Colonisation de la viande par Escherichia coli O157 H7 : caractérisation moléculaire, cellulaire et tissulaire des interactions

Caroline Chagnot

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Caroline CHAGNOT

Colonisation de la viande par *Escherichia coli* O157:H7 : Caractérisation moléculaire, cellulaire et tissulaire des interactions

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Liste des abréviations

AafA : aggregative adherent fimbrilin A
Acm : collagen adhesin de *Enterococcus faecium*
Afa : afimbrial adhesin
AIP : Appel Incitatif Programmé
ATP : Adenosine triphosphate
BFFCs : Beaded Filaments-Forming Collagen
CADF : *Campylobacter* adhesin to fibronectin
CBD : collagen binding domain
CbpA : collagen binding protein A
CbsA : collagen-binding S-layer protein A
CEPIA : Caractérisation et Elaboration des Produit issus de l'Agriculture
ClfA : Clumping factor A
Cna : collagen adhesin
CWB proteins : CW-binding proteins
EC : enveloppe cellulaire
DAEC : *E. coli* à adhésion diffuse
DraE : Dr haemagglutinin
EAEC : *E. coli* entéroaggrégatifs
EbpS : elastin-binding protein of *S. aureus*
ECM : matrice extracellulaire
EcmbP : ECM-binding protein
EDA : extra domain A
EDB : extra domain B
EIEC : *E. coli* entéroinvasifs
EhaA : enterohemorhagic *Escherichia coli* autotransporter A
EhaB : enterohemorrhagic *E. coli* autotransporteur B
EHEC : *E. coli* entérohémorragiques
EPEC : *E. coli* entéropathogènes
ETEC : *E. coli* entérotoxinogènes
ExPEC : *E. coli* extra-intestinales
FACIT : fibril-associated collagen with interrupted triple helixes
FBD : fibronectin binding domaine
FbpA : fibronectin-bindind protein A
FbpB : protéine de liaison fibronectine B
Fbp54: fibronectin-binding protein de 54 kDa
FEDER : Fonds Européen de Développement Régional
FFC : fibril-Forming Collagen
FnBP : fibronectin-binding protein
FoG : fibrinogen-binding protein de streptocoques G
GBD : gelatin binding domaine
HBD : heparin binding domaine
Hlp3 : HMW3-like protein
i-fibronectine : fibronectines insolubles

IMP : integral membrane protein
InPEC : *E. coli* intestinale pathogéniques
INRA : Institut National de la Recherche Agronomique
IPR : Interpro motif numbers
LigA : *Leptospira* immunoglobulin-like protein A
LipL32 : *Leptospira* lipoprotein de 32 kDa
Lp : lipoproteine
Lpf : long polar fimbriae
LPS : lipopolysaccharide
Lsa24 : *Leptospira* surface adhesin de 24 kDa
Lsa63 : *Leptospira* surface adhesine of 63 kDa
MACIT : membrane-associated collagens with interrupted triple helixes
MAP : MHC class II analogous protein
MB : Membrane basale
MC : membrane cytoplasmique
ME : membrane externe
MI : Matrice interstitielle
MICA : Microbiologie et Chaîne Alimentaire
MP : membrane plasmique
MPCs : Multiplexin collagens
MSCRAMM : microbial surface components recognizing adhesive matrix molecules
Mtp : *Mycobacterium tuberculosis* pili
NFC : Network-Forming Collagen
OMP : out membrane protein
OmpL47 : protéine de la membrane externe des Leptospira de 47 kDa
PARF : peptide associated with rheumatic fever
Pava : pneumococcal adhesion and virulence protein A
PC : paroi cellulaire
pH : potentiel hydrogène
pHu : pH ultime
PlpA : pneumoniae-like protein A
PP : periplasme
PTT : purpura thrombotique thrombocytopénique
RevA : reverse strand encoding protein
RrgA : RlrA-réglemented gene A
RspA : Rhusiopathiae surface protein A
SDRG : sérine -aspartate protéines repeatcontaining G
Stx : Shigatoxines
s-fibronectine : fibronectine soluble
SHU : syndrome hémolytique et urémique
SSURE : streptococcal surface repeat
Tp0136 : protéine codée par locus tp0136 chez *Treponema pallidum*
UafB : uroadherance factor B
UPEC : *E. coli* uropathogènes
YadA : Yersinia adhesin A

INTRODUCTION

A travers le monde, de nombreuses toxi-infections alimentaires liées aux *Escherichia coli* entérohémorragiques (EHEC) ont été rapportées. Ces bactéries sont des pathogènes à Gram-négative qui produisent des toxines, appelées shigatoxines, responsables de graves maladies touchant principalement les jeunes enfants. Ces maladies se caractérisent par des diarrhées parfois sanglantes, susceptibles d'engendrer pour les cas les plus graves, des complications telles que le Syndrome Hémolytique et Urémique (SHU) ou le Purpura Thrombotique et Thrombocytopenique (TTP) (Karch *et al.*, 2005, Tarr, 2009) pouvant mener à de graves séquelles ou décès. La souche bactérienne principalement incriminée lors de toxi-infections à EHEC, est le sérotype O157:H7. Cependant, il existe plus de 300 sérotypes d'EHEC répertoriés, ces EHEC appelées non-O157:H7 représentent 20 à 70% des infections à EHEC chaque année. Les derniers rapports européens de l'EFSA (European Food Saftey Authority) et américains de l'USDA (United States Department of Agriculture) ont identifiés 6 sérotypes d'EHEC non-O157, les plus couramment isolés chez l'homme, O26, O45, O103, O111, O121 et O145 (EFSA *et al.*, 2013, USDA, 2012). En Europe, un sérogroupe est également étroitement surveillé, le sérotype O104 qui a été responsable de la principale intoxication à EHEC de 2011 (EFSA *et al.*, 2013). Lors de ces épidémies, les principaux aliments incriminés sont les produits carnés, en particulier les viandes de bœuf hachées (ou produits dérivés) mais aussi les produits laitiers, les végétaux ainsi que les eaux de boissons (Meng *et al.*, 1997). Le réservoir naturel de ces bactéries est le ruminant (principalement le bovin) et l'origine des contaminations est essentiellement fécale. Au cours de l'abattage et des étapes de découpe, les viandes se trouvent contaminées par des microorganismes dont certains induisent un risque d'intoxication alimentaire tels que les EHEC. Le respect de règles d'hygiènes strictes permet de réduire la contamination des carcasses cependant il ne garantit pas l'absence d'EHEC dans la viande.

Malgré la fréquence élevée des intoxications liées à la consommation de viandes contaminées par des EHEC et les nombreuses études microbiologiques dédiées à la contamination des produits carnés, les dialogues moléculaires de colonisation et d'interaction des bactéries à la viande restent méconnus. Or, la compréhension des mécanismes d'interaction et de colonisation à l'échelle moléculaire est un pré-requis à la maîtrise des écosystèmes bactériens des viandes et produits carnés. Certaines études ont montré que les EHEC sécrètent des protéines de surface capables d'adhérer et de coloniser certaines protéines de la matrice extracellulaire (ECM : extracellular matrix), appelées MSCRAMM (Microbial Surface Components Recognizing Adhesive Matrix Molecules) (Patti *et al.*, 1994).

Cependant, l'expression de ces protéines est dépendante des conditions environnementales (pH, température, milieux de culture, *etc*…) (Olson, 1993). Par conséquent, les évolutions physicochimiques du muscle au cours de sa transformation en viande, pourraient influer sur la contamination des bactéries. Jusqu'à présent, la plupart des études réalisées pour comprendre les interactions MSCRAMM/ECM n'ont pas pris en compte la variation des isoformes des protéines de l'ECM. Or, l'ECM est composée de protéines glycosylées formant un maillage fibrillaire majoritairement constitué de collagènes, fibronectines, laminines et élastine dont la proportion et les isoformes varient en fonction des tissus (Vakonakis *et al.*, 2007). Les isoformes protéiques constitutives de l'ECM du muscle squelettique sont susceptibles de moduler l'adhésion ou la colonisation des EHEC aux protéines de l'ECM musculaire.

Les muscles squelettiques, à l'origine de la viande, sont essentiellement constitués de cellules musculaires (ou fibres musculaires). Il existe 4 types pures de fibres musculaires se différenciant par leur métabolisme (oxydatif ou glycolytique) et leur vitesse de contraction (rapide ou lente). Leur contenu cytoplasmique est dépendant du type cellulaire. Chaque muscle est composé de différents types de fibres en proportions variables selon son origine anatomique et sa fonction. Ces variations de compositions cellulaires peuvent impliquer que le risque de contamination aux EHEC soit plus important pour certains muscles. De plus, l'évolution physicochimique *postmortem* du muscle est dépendante du type de fibres qui le compose, ce qui est également susceptible de moduler les interactions avec les bactéries. A l'heure actuelle, aucune étude ne s'est intéressée au tropisme bactérien vis-à-vis du type de fibre musculaire.

Ainsi l'objectif de ma thèse est de mieux comprendre les interactions qui s'établissent entre des bactéries, notamment pathogènes, et les viandes lors d'une contamination. Ces travaux de thèse ont été financés par la région Auvergne et par le Fond Européen de Développement Régional (FEDER) ainsi que par les départements de recherches INRA « Microbiologie et Chaîne Alimentaire » (MICA) et « Caractérisation et Elaboration des Produit Issus de l'Agriculture » (CEPIA) dans le cadre d'un Appel Incitatif Programmé (AIP). Le premier volet de ce travail de recherche consistait à caractériser les capacités d'adhésion et de colonisation de la souche *E. coli* O157:H7 EDL933 aux protéines purifiées de l'ECM musculaire et de les comparer aux 7 sérotypes majeurs d'EHEC. Pour cela, différentes conditions de cultures (milieux, pH et températures) ont été testées afin de mimer les conditions environnementales rencontrées par la bactérie dans le contexte d'une

contamination des viandes. Dans un second temps, il s'agissait de d'identifier les déterminants protéiques bactériens qui peuvent jouer un rôle dans l'adhésion de la souche EDL933 aux protéines de l'ECM musculaire en utilisant une approche de génétique fonctionnelle ciblant les principales MSCRAMMs connues dans la littérature.

Le second volet de mon travail de thèse a consisté à caractériser la colonisation bactérienne dans la viande. La rigueur expérimentale exigeait de contrôler finement les facteurs biologiques (type génétique, sexe, âge), l'élevage, et de maitriser la cinétique de prélèvement *postmortem* de muscles dont la taille devait être compatible avec les méthodes d'imagerie cellulaire. Pour cela, deux muscles modèles de rat ont été choisis en fonction de leurs différences métaboliques et contractiles, le Soleus et l'Extensor Digitorum Longus (EDL). Une caractérisation du type de fibre sur du matériel biologique exempt de tout apport chimique extérieur (fixateur, colorant, etc…) fut souhaitable pour établir le lien entre le type de fibres et adhésion bactérienne. La capacité d'autofluorescence des molécules intracellulaires a été exploitée pour tenter de discriminer les différents types de fibres musculaires par microspectroscopie de fluorescence UV couplée au rayonnement synchrotron. Dans un second temps, le tropisme bactérien en fonction du type de fibre (sur coupe de muscle) ainsi qu'en fonction du type de muscle (adhésion en surface de muscle entier) a été étudié à différent temps *postmortem*.

SYNTHESE BIBLIOGRAPHIQUE

-CHAPITRE 1-
Escherichia coli entérohémorragiques (EHEC)

1. Intoxications alimentaires bactériennes

En 2013, l'autorité européenne de sécurité des aliments (EFSA) et le centre européen de prévention et de contrôle des maladies (ECDC) ont publié les dernières données sur l'apparition de zoonoses et d'épidémies d'origine alimentaire en Europe répertoriant l'ensemble des informations communiquées par les 27 Etats membres de l'Union en 2011 (rapport EFSA 2013). Un total de 5648 épidémies d'origine alimentaire ont été signalés dans l'UE en 2011. Cela représente 69553 cas humains, 7125 hospitalisations et 93 décès enregistrés. Le lien entre les cas humains et l'aliment impliqué n'a été prouvé que pour 701 épidémies. Le plus grand nombre d'épidémies déclarées a été causé par *Salmonella* (26,6%), suivit par des toxines bactériennes (comprenant les shigatoxines) (12,9%), *Campylobacter* (10,6%) et les virus (9,3%). Cependant, en termes de nombre de cas, la campylobactériose est la zoonose la plus fréquemment rapporté, avec une augmentation du nombre de cas humains confirmé au cours des cinq dernières années atteignant 220209 cas. Dans l'UE, la viande de poulet est considérée comme le vecteur principal des contaminations par *Campylobacter*. Malgré une diminution depuis 2007, le nombre de cas de salmonellose chez l'homme, représente toujours 95 548 cas en 2011, ce qui la place en seconde position après la S*almonella*. Les principales denrées alimentaires contaminées par *Salmonella* sont des viandes et produits dérivés (responsables de 9485 contaminations humaines). En terme d'incidence, les *Escherichia coli* productrices de shigatoxines (STEC) arrivent en troisième position, mais avec le taux de mortalité le plus élevé des trois. De 2010 à 2011, l'augmentation de 159,4 % des contaminations par *E. coli* est consécutive à la grande épidémie de STEC O104 :H4 survenue en Allemagne principalement. La souche épidémique de STEC O104:H4 a été particulièrement virulente impliquant un grand nombre de cas graves et de décès. Cependant, le sérogroupe le plus fréquemment rencontré lors d'une intoxication alimentaire aux STEC est O157. Ce sérogroupe est principalement retrouvé dans le tractus digestif des bovins mais également retrouvé chez d'autres espèces comme le porc, le poulet et le mouton. Le nombre important d'intoxications alimentaires humaines et le taux élevé de mortalité lié àla consommation d'aliments contaminés (des viandes en particulier) par les

STEC, justifie la mise en œuvre de recherches sur le sujet. Une meilleure compréhension des mécanismes impliqués dans la contamination des aliments par *E. coli* permettra à plus long terme de réduire le niveau de contamination alimentaire. Nous nous sommes donc particulièrement intéressés à la souche *E. coli* O157:H7, la STEC plus couramment incriminée dans la contamination des viandes.

2. Historique : *Escherichia coli*

Dès 1885, le genre *Escherichia* a été isolé et initialement décrit par le pédiatre Theodor Escherich comme hôte commensal dans l'intestin des enfants. Cette espèce bactérienne fut baptisée *Bacillus* (ou *Bacterium*) *coli* commune, puis renommée en 1919 sous le nom d'*Escherichia coli* par Castellani et Chalmers (Grimont, 1987). Le genre *Escherichia* appartient à la famille des *Enterobacteriaceae* qui regroupe des bacilles à coloration Gram négatif, non sporulés, anaérobies facultatifs. Par ailleurs, bien que plusieurs espèces d'*Escherichia* aient été définies comme *E. blattae* (Burgess *et al.*, 1973), *E. fergusonii* (Farmer *et al.*, 1985), *E. hermanii (Brenner et al., 1982a)* et *E. vulneris* (Brenner *et al.*, 1982b), des analyses phylogénétiques du gène *ompA* suggèrent que seulement *E. fergusonii* est un vrai membre du genre *Escherichia*, avec les autres espèces qui divergent phylogénétiquement plus d'*E. coli* que des espèces des genres *Salmonelle* ou *Citrobacter* (Lawrence *et al.*, 1991). Une autre espèce, originellement désignée *Hafnia alvei* (Albert *et al.*, 1991), partage des caractéristiques génétiques et phénotypiques avec le genre *Escherichia* et a été renommée *E. albertii* (Huys *et al.*, 2003). Des analyses phylogénétiques ultérieures montrent que cette espèce est étroitement liée au sérotype 13 de l'espèce *Shigella boydii* (Hyma *et al.*, 2005). De façon générale, les membres du genre *Shigella* sont phylogénétiquement indistinguables du genre *Escherichia*. Des comparaisons génomiques entre des espèces de *Shigella* et d'*E. coli* ont montré qu'elles partageaient plus de 75% de similarité de séquence ce qui justifierait l'appartenance des *Shigella* à l'espèce *E. coli* (Brenner *et al.*, 1972). Néanmoins, le pouvoir pathogène des autres espèces d'*Escherichia* reste faible comparé à celui d'*E. coli,* où plusieurs types de souches sont à l'origine de pathologies humaines. A noter que le genre *Escherichia* est actuellement subdivisé en 8 groupes avec trois espèces nommés *E. coli*, *E. fergusonii* et *E. albertii*, et cinq clades numérotés de I à V (Walk *et al.*, 2009).

3. Caractéristiques microbiologiques des *E. coli* pathogènes

3.1. Généralités

Les *Escherichia coli* sont des bactéries commensales de la flore intestinale des animaux à sang chaud comme les mammifères et les oiseaux. *E. coli* est une espèce commensale du tractus digestif, elle est présente dès les premières heures qui suivent la naissance et se maintient dans le côlon tout au long de la vie de l'animal. Elle représente près de 80% de la flore aérobie facultative intestinale. Cependant, certaines souches d'*E. coli* sont responsables d'infections humaines, il en existe deux types : les infections extra-intestinales par les *E. coli* pathogènes extra-intestinales (ExPEC) et les infections intestinales par les *E. coli* pathogènes intestinales (InPEC). Les ExPEC peuvent être à l'origine d'infections du tractus urinaire (UPEC : uropathogenic *E. coli*), de méningites et de septicémies (NMEC : neonatal meningitidis *E. coli*), ou de nécroses (NTEC : necrotoxigenic *E. coli*). Les InPEC sont classées en fonction des symptômes observés et de leurs interactions avec les cellules épithéliales (Nataro *et al.*, 1998). On distingue six pathotypes : (i) les *E. coli* entéropathogènes (EPEC), (ii) les *E. coli* entérotoxinogènes (ETEC), (iii) les *E. coli* entéroaggrégatifs (EAEC), (iv) les *E. coli* entéroinvasifs (EIEC) dont les *Shigella*, (v) les *E. coli* à adhésion diffuse (DAEC), ainsi que les (vi) *E. coli* entérohémorragiques (EHEC) (Tableau 1). Récemment, plus de 3000 cas de gastroentérite ont été enregistrés durant l'épidémie d'*E. coli* producteurs de Shiga-toxines (STEC : Shiga-toxine-producing *E. coli*) en Allemagne (Frank *et al.*, 2011). Le sérotype associé à cette épidémie (O104:H4) possède les gènes de virulence typiques des EAEC, ainsi qu'un prophage codant pour la Shiga-toxine 2 (Stx2) et un plasmide codant pour la β-lactamase. Les souches isolées de cette épidémie ont été classées dans un nouveau pathotype, les EAHEC (Enteroaggregative haemoragic *E. coli*), afin de les distinguer des souches d'EHEC habituelles. L'analyse phylogénétique basée sur des gènes spécifiques de virulence a montré que les EHEC auraient probablement acquis la plupart de leurs facteurs de virulence par transfert horizontal de matériel génétique (Walk *et al.*, 2009). Les souches d'EHEC dérivent des souches d'EPEC qui auraient acquis les gènes codant des Shiga-toxines responsables d'hémorragies.

Tandis que tous les EHEC sont des STEC (*E. coli* produisant des Shiga-toxines), tous les STEC ne sont pas responsables d'infection et n'ont pas été isolées de cas cliniques, elles ne peuvent donc pas être considérées systématiquement comme des EHEC (Levine, 1987). Les infections causées par les EHEC constituent un problème majeur de santé publique en raison

de l'extrême sévérité des manifestations cliniques telles que le Syndrome Hémolytique et Urémique ou le Purpura Thrombotique Thrombocytopénique qui peuvent mener à la mort du patient ou occasionner de graves séquelles (Zhou *et al.*, 2010).

Pathotypes	Symptômes associés	Caractéristiques
Enterotoxigenic *E. coli* (ETEC)	Diarrhée aqueuse aiguë	Production d'entérotoxines Adhésion aux entérocytes via pili
Enteroinvasive *E. coli* (EIEC)	Diarrhée aqueuse, syndrome dysentérique	Invasion des cellules épithéliales Plasmide de virulence (140 MDa)
Enteroaggregative *E. coli* (EAEC)	Diarrhée persistante	Adhésion en «briques empilées» Facteur de virulence hétérogène Adhésine et Toxine
diffusely adherent *E. coli* (DAEC)	Diarrhée aqueuse aiguë ou persistante	Adhésion diffuse sur cellules *in vitro* Adhésine afimbriae
Enteropathogenic *E. coli* (EPEC)	Diarrhée aqueuse aiguë ou persistante	Adhésion localisée sur les entérocytes et cellules *in vitro* Destruction des microvillosités : lésion d'attachement-effacement, présence du LEE
Enterohemorrhagic *E. coli* (EHEC)	Diarrhée aqueuse aiguë Colite hémorragique Syndrome Hémolytique et Uremique Purpura Thrombotique et Trombocytopénique	Production de shigatoxine lésion d'attachement-effacement, présence du LEE plasmide de virulence (60 MDa)

Tableau 1 : Affections associées aux pathotypes d'*E. coli* responsables de diarrhées chez l'homme. (Blattner *et al.*, 1997, Kaper *et al.*, 2004, Milon *et al.*, 1999, Trabulsi *et al.*, 2002)

3.2. Classification des *E. coli* producteurs de shigatoxines (STEC)

Afin de classer les STEC, deux nomenclatures sont utilisées. La première tient compte du séropathotype (Karmali *et al.*, 2003) et l'autre du sérotype de la souche (Johnson *et al.*, 2006).

Lorsque les STEC sont classés en fonction du séropathotype, c'est leur incidence et leur association avec le Syndrome Hémolytique et Uremique qui sont étudiées (Tableau 2). Le séropathotype A comprend les sérotypes les plus virulentes et les plus fréquemment associées aux épidémies de SHU, tels que les souches de sérotype O157:H7, tandis que le séropathotype E comprend des sérotypes qui n'ont jamais été associées à des pathologies humaines. La classification phylogénétique des espèces a pour objectif de rendre compte des degrés de parenté entre les différentes espèces analysées. Cette classification s'effectue àpartir des données génétiques et permet de comprendre l'évolution des souches. 72 souches d'*E. coli* d'origines diverses ont été collectées (ECOR : *Escherichia coli* collection of reference) et analysées. La variabilité génétique de 11 enzymes ainsi que leurs profils électrophorétiques ont permis de classer les différentes souches en 6 séropathotypes majeurs : A, B1, B2, C, D et E (Ochman *et al.*, 1984). Par la suite, Boyd et Hartl (1998) ont montré une dispersion des *E. coli* parmi les différents groupes phylogénétiques des souches de la collection ECOR et de la collection DEC (diarrheagenic *E. coli*) (comprenant 15 souches). Les souches ExPEC (extra-intestinales) appartiennent majoritairement aux groupes B2 et D. Les souches entéropathogènes sont massivement présentes dans les groupes A et B1, et en particulier les souches des pathovars EHEC, ETEC et EIEC. Les souches des pathovars EPEC et EAEC ont des pathologies associées plus modérées que les 3 précédentes et sont présentes dans l'ensemble des groupes (Escobar-Paramo *et al.*, 2004). Les souches de STEC/EHEC sont présentes majoritairement dans les groupes A et B1. Les souches d'EHEC de sérotype O157:H7 sont regroupées dans le groupe D (Girardeau *et al.*, 2005).

La classification antigénique est également utilisée pour classer les STEC. Elle est basée sur une combinaison du sérotype O:H:K des antigènes du lipopolysaccharide (LPS) (O), flagellaire (H) et capsulaire (K) des souches. Cependant, la combinaison O:H est la plus couramment utilisée pour décrire les STEC. Plus d'une centaine de sérotypes ont été identifiés chez les STEC isolées à partir d'échantillons humains (Johnson *et al.*, 2006).

Le sérotype le plus fréquent et le plus virulent est O157:H7. Cependant, des souches provenant d'autres sérogroupes tels que O26 ; O145 ; O103 ; O111 ; O91 ; O55 ; O121 ont été isolés de cas cliniques (Tableau 2)
(Rapport AFSSA 1996-2008 http://www.anses.fr/Documents/MIC2010sa0031.pdf).

Séropathotype	Incidence relative	Implication dans des épidémies	Association avec les SHU ou les colites hémorragiques	Sérotypes
A	Elevée	Fréquente	Oui	O157:H7, O157:NM*
B	Modérée	Non fréquente	Oui	O26:H11, O103:H2, O111:H8/NM, O121:H19, O145:NM
C	Faible	Rare	Oui	O91:H21, O104:H21, O113:H21 et autres sérotypes.
D	Faible	Rare	Non	Multiples sérotypes
E	Non-humain	n.a.	n.a.	Multiples sérotypes

Tableau 2 : Classification des STEC en fonction de la fréquence d'isolement lors d'épidémies et de la sévérité de la pathologie engendrée.
(d'après Karmali *et al.*, 2003)

3.3. Environnement

Le réservoir principal des STEC est le tube digestif des bovins (Keen *et al.*, 2010). Les STEC sont adaptés à tous les microhabitats du tractus gastro-intestinal des bovins. Cependant, des STEC, principalement de sérotype O157:H7, ont également été retrouvés chez le porc, le poulet, le mouton ainsi que d'autres espèces. Contrairement à l'Homme, le portage des STEC par les bovins est asymptomatique. La prévalence des STEC varie en fonction de plusieurs critères : l'animal, le mode d'élevage, les saisons ou encore le pays (Hussein, 2007, Jenkins *et al.*, 2002, Zhao *et al.*, 1995). Par exemple, aux Etats-Unis, 11.4% des bovins sont porteurs d'EHEC O157:H7 dans les fèces, au Brésil, 82% de bovins laitiers sont porteurs de STEC (tous sérotypes) dans les fèces, alors qu'en France seulement 7.9% des bovins sont porteurs de STEC (tous sérotypes) (Rapport de l'AFSSA: Bilan des connaissances relatives aux STEC).

3.4. Infection alimentaire à EHEC

3.4.1. Origine des infections humaines par les STEC

Différentes causes peuvent mener à une contamination par les STEC. Tout d'abord, la viande contaminée est la première cause d'infections à EHEC. Si les règles d'hygiène lors de la découpe sont respectées, le risque de contamination reste faible car le contact avec le contenu du tube digestif, porteur d'EHEC, est restreint. En revanche, si ces règles ne sont pas respectées, le risque de contamination de la viande augmente s'il y a contact direct avec le contenu du tube digestif ou des cuirs des bovins, également porteurs d'EHEC. D'autres aliments tels que les produits laitiers (laits et fromages au lait cru) ont également été incriminés lors d'infections à EHEC suite à des contacts avec des cuirs de bovins lors de la traite. Les fèces de bovin peuvent également être un vecteur de la contamination à EHEC sur des fruits et des légumes. De la même façon, l'Homme peut être contaminé par contact direct avec l'animal. Enfin, des contaminations interhumaines ont été rapportées lors d'épidémies, en particulier dans les hôpitaux et dans les crèches.

3.4.2. Symptômes

La dose infectieuse des EHEC est faible en comparaison d'autres pathogènes alimentaires, elle est de moins de 50 bactéries (Rapport de l'AFSSA). Cette faible dose infectieuse des EHEC est due en partie aux mécanismes de résistance à l'acidité très performants des EHEC qui leur permettent de résister efficacement à l'acidité gastrique. Les symptômes ainsi que l'issue de l'infection varient en fonction de l'âge de l'individu et de l'état de son système immunitaire. Les symptômes de l'infection apparaissent séquentiellement dans les 15 jours suivant l'ingestion de l'aliment contaminé. Les premiers symptômes apparaissent sous forme de diarrhées aqueuses modérées entre 1 et 9 jours après l'ingestion (Brunton *et al.*, 1994). Dans 90% des cas, les diarrhées deviennent sanglantes et s'accompagnent de sévères douleurs abdominales spasmodiques ainsi que d'une déshydratation modérée. Cette colite hémorragique persiste entre 2 et 10 jours, pouvant mener à des vomissements. Dans 10% des cas, des complications apparaissent une semaine environ après le début des symptômes, pouvant affecter le pronostic vital de l'individu. C'est la production de Stx dans l'intestin qui est à l'origine de ces complications, elles se traduisent par des microangiopathies thrombotiques de deux types : le SHU et le PTT (Nivet *et al.*, 1995,

Gillis, 1996). Le SHU se caractérise par une anémie microangiopathique hémolytique, une thrombopénie ainsi qu'une insuffisance rénale aiguë. Il a également été observé des troubles du système nerveux central tels que léthargie, convulsions, et encéphalopathie (Tesh *et al.*, 1993). Le SHU touche essentiellement les enfants de moins de 5 ans et est la première cause d'insuffisance rénale aiguë en France. Le PTT présente les mêmes caractéristiques physiopathologiques que le SHU mais il diffère du SHU sur le fait que les patients présentent une fièvre ainsi que des symptômes neurologiques plus marqués. Cette forme touche plus particulièrement les personnes âgées.

3.4.3. Diagnostic et traitements

Le diagnostic des infections à EHEC repose sur leur isolement et leur identification dans les selles, sur la mise en évidence de la présence de Stx libres ou des gènes *stx1* et *stx2* (par PCR) et/ou sur l'augmentation du titre sérique des anticorps spécifiques dirigés contre le LPS et la flagelline.

A ce jour, il n'existe aucun traitement contre les infections à EHEC. Les patients sont principalement traités contre les symptômes de la maladie : diurèse, hémodialyse, transfert de plasma frais (Andreoli *et al.*, 2002). Ces traitements permettent de diminuer la mortalité de la maladie de 40 à 4% pour les patients atteints du SHU et de 90 à 10% pour les patients atteints de PTT (Gillis, 1996). Cependant, il est important de noter que les séquelles sont fréquentes et durables, notamment au niveau rénal (30%). Habituellement les infections bactériennes sont soignées par antibiothérapie. Cependant dans le cas d'une infection à EHEC, ce traitement n'est pas préconisé. Plusieurs études ont en effet montré que l'antibiothérapie est inefficace sur les infections à EHEC, elle serait même un facteur de risque pour l'apparition du SHU. Les gènes codant la toxine (Stx) sont portés par des phages de type λ en phase lysogénique. Dans le cas d'un traitement antibiotique, l'expression et la synthèse de Stx sont favorisées par l'induction de cassures de l'ADN bactérien, activant le cycle lytique du phage.

Certains traitements alternatifs sont actuellement à l'étude :

- Des traitements aux antibiotiques bactériostatiques comme l'Imipenem, un dérivé semi synthétique de la thiénamycine qui diminue la cytotoxicité des EHEC de sérotypes O26, O111 et O157 sur les cellules Vero en reduisant la libération des Stx par les EHEC (Lee *et al.*, 2009) ou encore la fosfomycine qui lutte contre les infections à

EHEC aux stades précoces en diminuant l'expression des gènes codant la Stx (Ichinohe *et al.*, 2009).

- L'utilisation d'anticorps anti-Stx testés chez la souris et le porcelet gnotobiotique. Chez la souris, l'administration d'anticorps anti-Stx diminue la mortalité lors d'une infection expérimentale par les EHEC (Yamagami *et al.*, 2001). Par ailleurs, des bactéries recombinantes produisant des analogues structuraux du récepteur de la toxine, ont été administrées à un modèle murin. L'administration de ces bactéries supprime toute létalité due à *E. coli* O157:H7 (Paton *et al.*, 2000). Chez le porcelet gnotobiotique, ces anticorps anti-Stx diminue la probabilité d'apparition de symptômes neurologiques ainsi que la mortalité (Sheoran *et al.*, 2005).
- L'administration par voie orale de composés peptidiques (Nishikawa *et al.*, 2006) ou glucidiques (Takeda, 1998) permet de séquestrer la toxine dans la lumière intestinale en mimant le récepteur de la toxine.
- Plus récemment, lors de l'épidémie de 2011 en Allemagne, des chercheurs ont administrés à des patients atteints du SHU un anticorps dirigé contre la fraction C5 du complément (Eculizumab) qui inhibe l'activation du complément par la Stx, ce complément étant à l'origine des symptômes les plus graves de l'infection à EHEC. Des résultats prometteurs ont été observés pour les trois patients traités avec cette molécule. Ils ont rapidement guéris de leurs lésions cérébrales et rénales (Lapeyraque *et al.*, 2011). Cependant, l'administration d'anticorps reste très coûteuse.

D'autres études sont encore nécessaires pour proposer des stratégies alternatives efficaces et peu coûteuses afin de lutter efficacement contre les infections à EHEC.

3.5. Epidémies à EHEC

3.5.1. Première épidémie à EHEC

Les EHEC ont été décrits pour la première fois en 1982, lors de deux épidémies qui ont touché le continent nord-américain. Le sérotype à l'origine de ces épidémies était O157:H7. L'aliment incriminé était de la viande hachée peu cuite, retrouvée principalement dans les hamburgers, 47 personnes ont été infectées. La souche de référence EDL933 a été isolée de patients contaminés lors de l'une de ces épidémies.

3.5.2 Epidémies alimentaires liées aux EHEC

A travers le monde, différents centres de contrôle et de prévention en matière de santé publique ont été créés. Les trois principaux sont l'EFSA et l'ECDC pour l'Europe et le CDC (Centers for Disease Control and Prevention) pour les Etats-Unis. Ils récencent les cas et les causes des infections à travers l'Europe et les Etats-Unis et ont également pour rôle de réaliser des bilans d'évolution et d'émergence des maladies infectieuses à travers les années. On peut citer comme exemple, une des plus grandes épidémies à EHEC, en 1996, au Japon où la consommation de pousses de radis crues avait été incriminée. 9451 personnes ont été infectés, dont 101 présentaient un SHU et 12 sont décédées. La souche O157:H7 *Sakai* a été isolée lors de cette épidémie. Toutefois, la viande de bœuf est considérée comme la source principale des intoxications aux EHEC. Parmi les plus graves, on peut citer les Etats-Unis en 1993 où de la viande hachée était contaminée par la souche O157:H7, 501 personnes ont été infectées, 45 ont présenté un SHU et 3 sont décédées. De nouvelles épidémies aux EHEC sont recensées chaque année, de nombreuses souches sont incriminées. Néanmoins, le sérotype le plus virulent et le plus fréquemment associé aux épidémies de SHU est EHEC O157:H7 (Tableau 3).

	2011		2010	
serogroupe	nombre de cas	%total	nombre de cas	%total
O157	2185	41.2	1502	41.1
O104	1064	20.1	0	0
O26	287	5.4	258	7.1
O103	141	2.7	91	2.5
O91	116	2.2	57	1.6
O145	76	1.4	61	1.7
O63	0	0	42	1.1
O128	53	1	30	0.8
O111	52	1	41	1.1
O146	48	0.9	28	0.8
nd	795	15	1230	33.7
Autre	484	9.1	315	8.6
Total	5,301		3,655	

Tableau 3 : Nombre de cas d'infections aux EHEC confirmés entre 2010 et 2011 en Europe en fonction des principaux sérogroupes. (nd :non déterminé) (d'après le rapport de l'EFSA et de l'ECDC 2013)

Les dernières épidémies liées à la viande contaminée par *E. coli* O157:H7, datent de 2011 :

- Aux Etats-Unis, suite à la consommation de saucisson, 14 personnes ont été touchées mais aucune n'a développé de SHU ou de PTT.
- En France, des steaks hachés contaminés ont provoqué 10 cas de SHU, essentiellement chez de jeunes enfants.

En Europe, l'année 2011, àvu le nombre de cas d'infection aux STEC augmenter significativement par rapport aux années précédentes, passant de 3271 cas en 2007 à 9435 en 2011 (Tableau 4). Cette forte augmentation est due àl'épidémie causée par la souche STEC entéroaggrégative O104: H4 (EAEC) (Rasko *et al.*, 2011; Scheutz *et al.*, 2011) qui a eu lieu en Allemagne entre mai et juillet 2011. L'aliment incriminé était des graines de fenugrec en provenance d'Egypte (Rapports EFSA, 2011 et 2013). 3816 cas humains ont été rapportés, 845 ont développé un SHU et 54 sont décédés. Contrairement aux principales STEC (dont la souche *E. coli* O157:H7) qui touchent majoritairement les enfants, la majorité des cas de SHU liés à O154:H4 s'est produite chez des adultes, surtout des femmes (Figure 1). Peu d'enfants ont été atteints par la souche O104:H4, certainement parce que les graines germées ont majoritairement été consommées par des femmes et non des enfants.

Année	2011	2010	2009	2008	2007
nombre de cas humains confirmés	9435	3656	3583	3159	3271

Tableau 4 : Augmentation du nombre de cas humains d'infections aux STEC confirmés entre 2007 et 2011 en Europe (d'après le rapport de l'EFSA et de l'ECDC 2013).

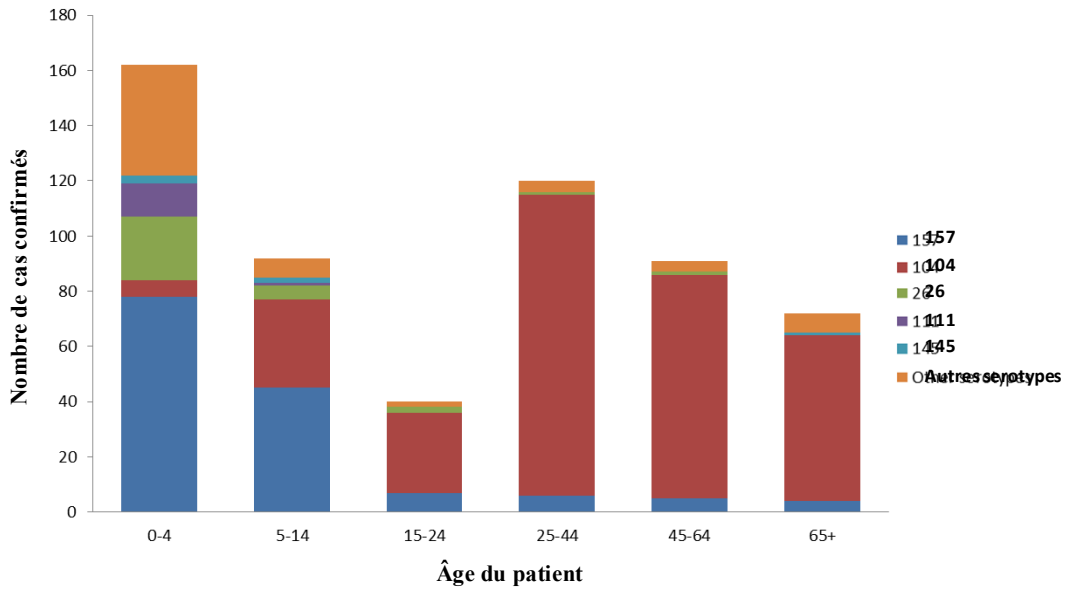

Figure 1 : Nombre de cas europeen, en 2011, de SHU en fonction de l'âge du patient et du sérotype.
(D'après le rapport de l'EFSA 2013)

Les caractéristiques microbiologiques de cette souche de *E. coli* O104:H4, la classent parmi les *E. coli* entéroaggrégatifs (EAEC) (Rasko *et al.*, 2011; Scheutz *et al.*, 2011). Son génome est identique à 93% à celui de la souche d'*E. coli* entéroaggrégative O104:H4 (souche 55989) isolée en Afrique à partir d'un patient atteint du HIV qui présentait des diarrhées chroniques. La souche O104:H4 responsable de l'épidémie allemande présente un profil de multi-résistance aux antibiotiques (ampicilline, céfotaxime, ceftazidime, streptomycine, sulfaméthoxazole, triméthoprime, cotrimoxazole, tétracycline et acide nalidixique) (Rasko *et al.*, 2011). La souche O104 :H4 possède les gènes codant les facteurs d'adhésion des EAEC, responsables notamment de la persistance des EAEC dans l'intestin. De plus, la souche O104:H4 possède le gène *stx2* (Rasko *et al.*, 2011), qu'elle aurait acquis par transfert horizontal avec une souche de STEC. La combinaison de la persistance des EAEC avec la production de Stx est probablement la cause de l'incidence élevée de l'apparition du SHU lors de cette épidémie due à une plus longue exposition à la toxine. Les EAEC n'ont jamais été retrouvés chez les bovins. Le réservoir connu des EAEC est principalement l'Homme. L'origine probable de la contamination serait humaine.

-CHAPITRE 2-

MUSCLE ET MATRICE EXTRACELLULAIRE

La viande est le produit de la transformation du muscle squelettique après la mort d'un animal de boucherie. Cette transformation s'accompagne de nombreux changements structuraux et physiologiques. Au cours de l'abattage et de la découpe, les viandes sont naturellement contaminées par des microorganismes dont le développement peut limiter la durée de conservation (bactéries d'altérations) et induire un risque d'intoxication alimentaire (bactéries pathogènes). Lors de contaminations aux EHEC O157:H7 par exemple, la viande de bœuf est l'aliment le plus souvent incriminé. Malgré la fréquence des associations viandes-bactéries et les nombreuses études microbiologiques (dénombrement, biodiversité, procédés technologiques) dédiées à la contamination des produits carnés, les mécanismes moléculaires de colonisation et d'interaction des bactéries à l'échelle des fibres musculaires de la viande sont totalement méconnus. Or, la compréhension de ces mécanismes est un pré-requis à la maîtrise de la contamination des viandes. La connaissance de la structure et de l'organisation du muscle et de son enveloppe conjonctive est nécessaire à la caractérisation et à la compréhension des interactions entre les bactéries et les muscles.

1. Muscle strié

Le muscle strié est un organe capable de se contracter permettant aux organismes de se mouvoir et de prendre des positions adaptées, sous l'influence d'une stimulation. Les muscles des vertébrés sont classés en trois types selon leurs fonctions et leurs localisations :

- **Les muscles squelettiques ou muscles striés** qui représentent de 30 à 40% du poids du corps d'un animal vivant. Ils assurent les mouvements du corps grâce à leurs fixations sur les os.
- **Les muscles lisses**, qui sont responsables du mouvement involontaire des organes (artères, veines, utérus…)
- **Le muscle cardiaque**, un muscle strié en fonctionnement permanent assurant la circulation du sang et l'apport continu de nutriments et d'oxygène aux tissus.

Le muscle squelettique est quantitativement le tissu le plus important de l'organisme. Il est majoritairement composé d'eau (entre 73 et 75%) et contient 19 à 25% de protéines, dont 60% sont des protéines myofibrillaires. Le muscle contient également 1 à 6% de lipides intramusculaires. Les muscles sont différents en fonction de leur forme, de leur taille mais aussi de leur composition tissulaire. Le tissu nerveux (fibres motrices afférentes et fibres sensitives efférentes) assure le contrôle de la contraction et de la relaxation musculaire. Les vaisseaux sanguins fournissent les nutriments et l'oxygène. Enfin le tissu conjonctif maintient la structure musculaire.

Le muscle strié (Figure 2) résulte de la juxtaposition de cellules multinucléés de forme allongée : les fibres musculaires. Elles constituent 75 à 90% du volume musculaire et représentent l'élément de base du muscle. Elles sont entourées par l'endomysium, une mince gaine de tissu conjonctif ou matrice extracellulaire (ECM), riche en protéines fibrillaires (collagène, fibronectine, laminine, elastine). Les fibres musculaires sont rassemblées en faisceaux primaires enveloppés par une gaine conjonctive fine, le périmysium. Les faisceaux primaires sont à leur tour regroupés en faisceaux secondaires enveloppés dans une gaine de périmysium plus épaisse. Enfin l'épymisium enveloppe l'ensemble du muscle. Aux extrémités du muscle cette trame conjonctive se prolonge par les tendons, assurant la liaison du muscle avec le squelette.

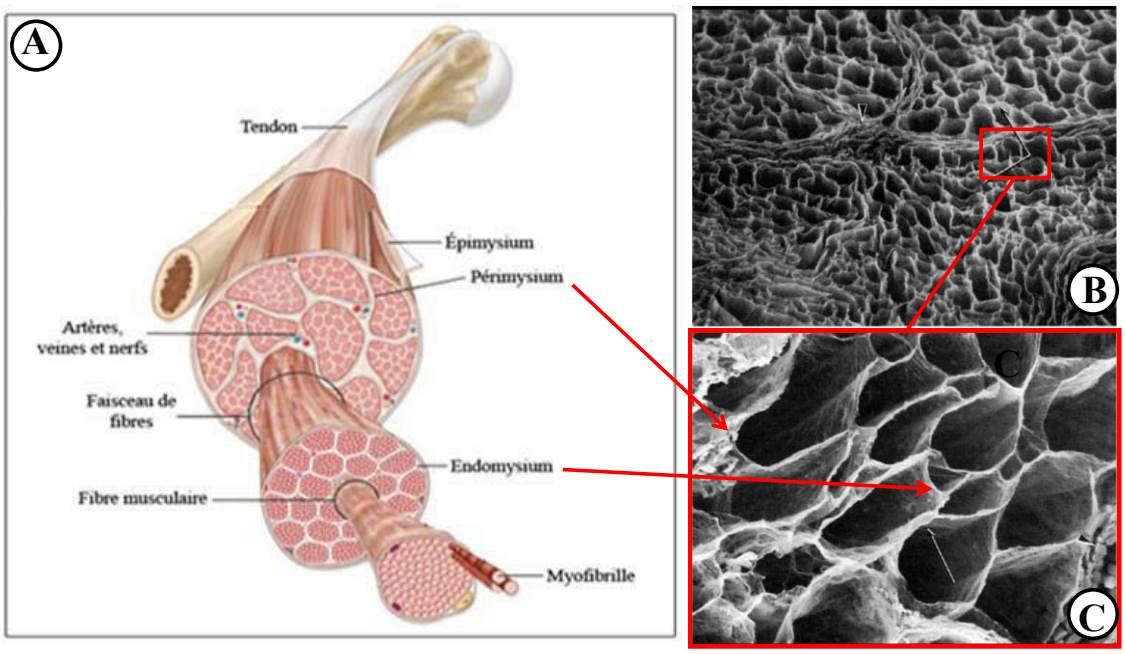

Figure 2 : Organisation structurale d'un muscle squelettique. (A) Structure d'un muscle. (B et C) Observation, en microscopie électronique à balayage, de l'endomysium et du périmysium après digestion des fibres au NaOH, (Trotter *et al.*, 1992)

2. Fibres musculaires

La fibre musculaire est l'une des cellules les plus longues de l'organisme pouvant mesurer jusqu'à 34 cm de longueur et de section, plus ou moins circulaire, de 10 à 100µm de diamètre. Ces fibres sont des syncytiums issus de cellules fusionnées pouvant contenir plusieurs centaines de noyaux localisés en position périphérique. L'espace intracellulaire ou sarcoplasme est majoritairement occupé par les myofibrilles (éléments contractiles).

Il existe essentiellement 4 types de fibres musculaires différentes chez le mammifère. La proportion de chacun de ces types de fibres est extrêmement variable dans le muscle squelettique et dépend de nombreux facteurs biologiques (espèce, race, âge…) et physiologiques (fonction du muscle, activité physique…). A quelques exceptions près, un muscle strié est généralement constitué de trois à quatre types de fibres, le type prédominant donne les caractéristiques essentielles du muscle. La disponibilité de plusieurs types de fibres avec des caractéristiques fonctionnelles différentes permettent au muscle strié une optimisation du travail aux tâches diverses qu'il devra accomplir.

La fibre musculaire est composée d'un assemblage de petites unités fonctionnelles parallèles au grand axe de la fibre : les myofibrilles de diamètre d'un micron. Elle est principalement formée de deux types de myofilaments : les myofilaments fins et les myofilaments épais (Figure 3).

Les myofilaments fins ont une structure complexe comprenant : deux chaines d'actine en hélice, trois protéines régulatrices (la troponine I, C et T) chacune ayant un rôle spécifique au niveau de la contraction musculaire et de la tropomyosine disposée le long des chaines d'actine.

Les myofilaments épais sont principalement composés d'un assemblage de myosine comportant des têtes globulaires bilobées et une tige. La myosine est constituée de deux chaines lourdes de myosine MyHC (Myosin Heavy chain) au niveau des têtes globulaires et de quatre chaines légères MLC (Myosin light chain). C'est au niveau des têtes qu'est située l'activité ATPasique qui catalyse la dégradation de l'ATP en ADP, libérant l'énergie chimique indispensable à la contraction musculaire.

Il existe 4 différentes isoformes de chaines lourdes de myosine dans le muscle de mammifère. Chaque type cellulaire « pure » est composé d'une isoforme de MyHC (isoforme

1 pour les fibres de type I, isoforme 2a pour les fibres IIA etc…). Le type n'est pas fixé rréversiblement. En effet, le type cellulaire peut évoluer au cours de l'âge ou avec un changement d'activité physique. Dans ce cas, l'évolution passe par des fibres hybrides, intermédiaires, qui présentent les 2 isoformes de MyHC des types purs qui l'encadrent (I, I-IIA, IIA, IIA-IIX, IIX, IIX-IIB et IIB; Schiaffino et al. 1989).

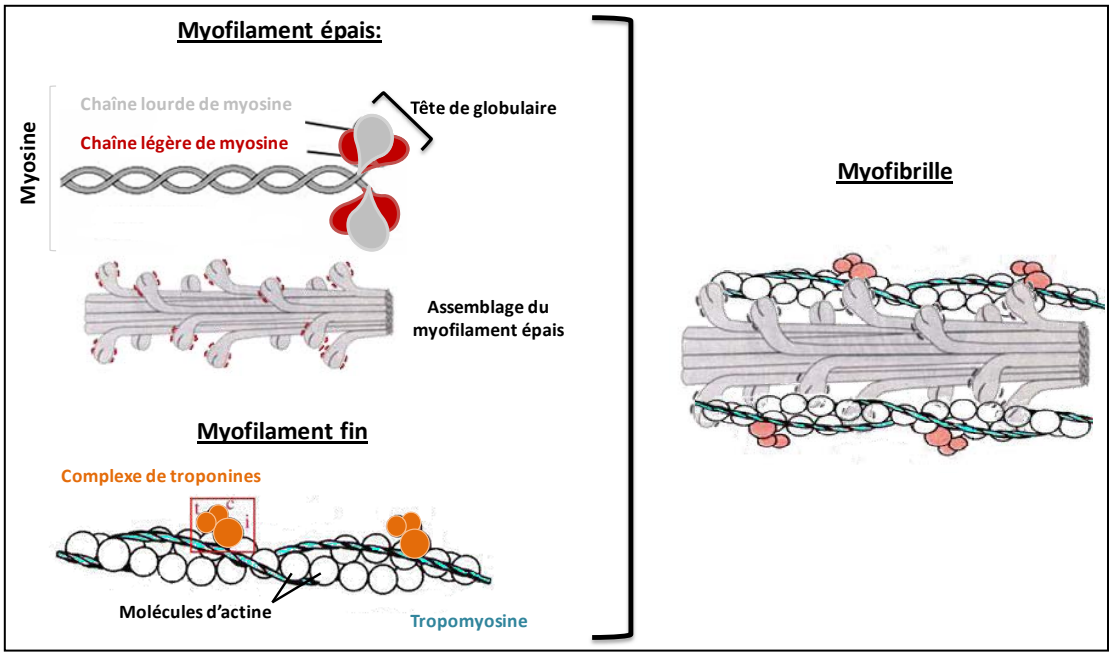

Figure 3 : Représentation de la structure des myofilaments et de leur organisation dans la myofibrille

Les fibres musculaires ne sont pas des structures statiques et leur nature dynamique rend difficile leur classement en catégories différentes (Bottinelli *et al.*, 2000). De nombreuses nomenclatures ont été établies pour rendre compte de la diversité des types de fibres musculaires. La classification des fibres musculaires a longtemps reposée sur un critère fonctionnel (la vitesse de contraction) et/ou sur un critère métabolique (le type de métabolisme énergétique régénérateur de l'ATP).

La classification des fibres selon la vitesse de contraction (type contractil) dépend de leur composition en chaines lourdes de myosine qui est la seule protéine myofibrilliaire présentant une activité enzymatique. Cette classification est basée sur la révélation histochimique de l'activité de l'ATPase myofibrilliaire après différents pH de pré-incubation (Guth *et al.*, 1969, Brooke *et al.*, 1970a). L'activité ATPasique associée aux fibres à vitesse de contraction lente (fibres de type I) est plus acido-résistante que celle associée aux fibres à vitesse de contraction rapide (fibre de II, acido sensible). Des variations de la sensibilité de

l'ATPase à différents pH acides de pré-incubation et des différences histochimiques ont permis de subdiviser les fibres de type II en sous types IIA, IIB et IIX, cependant elle ne permet pas de discriminer les types hybrides tels que IIAB, IIAX et IIBX et IIC constitués par des mélanges de myosine (Brooke *et al.*, 1970b, Hamalainen *et al.*, 1993). C'est une population hétérogène (mélange de myosine I et IIA) transitoire présente dans les muscles immatures.

La classification des fibres par leur type métabolique est en relation avec les voies métaboliques utilisées pour régénérer l'ATP nécessaire à la contraction. L'étude de l'importance respective de la voie aérobie (oxydative) et la voie anaérobie (glycolytique) permet de classer les fibres en oxydatives, glycolytiques ou oxydoglycolytiques (Nachlas *et al.*, 1957). D'une façon générale, les fibres à métabolisme oxydatif sont riches en myoglobine (Essen-Gustavsson *et al.*, 1990), composé qui pigmente le muscle en rouge et assure le transport de l'oxygène dans les tissus. Ces fibres sont aussi appelées « fibres rouges ». Elles disposent de beaucoup de mitochondries, ce qui permet de régénérer l'ATP par la voie oxydative avec un fort rendement (37 ATP/ résidu glycosyl) et leur octroie une bonne résistance à la fatigue. Les fibres à métabolisme glycolytique ont peu de mitochondries et régénèrent principalement l'ATP par la voie de fermentation lactique. De ce fait, la myoglobine est quasiment absente et les fibres apparaissent blanches (fibres blanches). Le faible rendement de leur voie métabolique (3 ATP/ résidu glycosyl) explique leur faible résistance à la fatigue. Les fibres oxydo-glycolytiques utilisent les deux voies métaboliques et sont intermédiaires aux deux précédentes. Les caractéristiques fonctionnelles et métaboliques des différents types de fibres sont détaillées dans le tableau 5.

Pour améliorer la robustesse et la précision de la caractérisation des fibres musculaires, Guth et Samaha (1970) ont combiné la réponse de l'activité ATPasique et celle de l'activité métabolique généralement caractérisée par l'activité histoenzymologique de la succynyl déshydrogénase, une enzyme de la chaine respiratoire des mitochondries. Un résumé est présenté dans le tableau 5. Les fibres de type I présentent une vitesse de contraction lente et un métabolisme oxydatif impliquant une concentration très importante de mitochondries en périphérie cellulaire. Les fibres IIA, IIX et IIB sont des fibres à contraction rapide. En moyenne, les fibres IIA ont un métabolisme oxydo-glycolytique tandis que les fibres IIX et IIB ont un métabolisme glycolytique.

Plus récemment, il a été mis en évidence que le type métabolique et contractil d'une fibre musculaire était également étroitement lié à sa composition en isoforme de chaines lourdes de myosines (Schiaffino et al. 1989). La disponibilité d'anticorps monoclonaux dirigés contre ces isoformes de chaines lourdes de myosine permet aujourd'hui d'identifier les types cellulaires par immunohistochimie (Schiaffino *et al.*, 1989, Meunier *et al.*, 2010). Cette méthode est la seule permettant de discriminer avec précision les types hybrides, des types pures. De façon générale, chez les mammifères et en particulier les rongeurs, la transition est continue entre les fibres I à IIB avec un passage par les formes intermédiaires IIX, IIB et leurs hybrides.

Caractères	Type de fibres			
	I	IIA	IIX	IIB
Physiologie				
Vitesse de contraction	Lente	Rapide	Rapide	Rapide
Résistance à la fatigue	+++	+++	++	+
Morphologie				
Couleur	Rouge	Rouge	Blanche	Blanche
Myoglobine	+++	+++	++	+
Densité capillaire	+++	++		+
Nombre de mitochondries	+++	+++	++	+
Richesse en collagène	+++	++		++
métabolisme				
Type	Oxydatif	Oxydatif	Oxydo-glycolitique	Glycolitique
Réserves glycogène	+	++	+++	++++
Réserves Lipides	+++	+++	++	+
Activités enzymatiques				
ATPase myofibrillaire	+	+++	+++	+++

Tableau 5 : Caractéristiques des différents types de fibres musculaires (Hamalainen *et al.*, 1993, Lefaucheur, 1989)

3. Matrice extracellulaire

La matrice extracellulaire (ECM) est constituée d'un complexe protéique, dont la composition et l'organisation structurelle influence de nombreux processus biologiques tels que l'adhérence, la migration, la prolifération ou la différenciation des cellules eucaryotes (Patti *et al.*, 1994). En tant que constituant ubiquitaire des tissus animaux, l'ECM peut également servir de substrat à la fixation de microorganismes, dans le cas d'une infection par exemple. Le rôle principal de l'ECM est le soutien et le lien entre les cellules des tissus. Deux

grandes classes de macromolécules constituent l'ECM: (i) les protéines fibrillaires et (ii) les protéoglycanes. La composition protéique de l'ECM est vaste et complexe, avec de multiples domaines distincts, mais hautement conservés parmi les différents taxons. Les bactéries sécrètent des protéines qui peuvent être exprimées à la surface de la cellule interagissant spécifiquement avec les composants de l'ECM. Ces protéines sont qualifiées de MSCRAMM (Microbial Surface Components Recognizing Adhesive Matrix Molecules). L'étude des intéractions des MSCRAMM à l'ECM nécessite une parfaite connaissance des composants de l'ECM.

3.1. Organisation supramoléculaire des composants majeurs de l'ECM

Alors que la composition et la concentration protéique de l'ECM peuvent varier en fonction du tissu étudié, son organisation reste similaire. Les protéines sont immergées dans un gel constitué de protéoglycanes afin de former une matrice complexe, un réseau fonctionnel structuré grâce à des interactions spécifiques entre les composants (Vakonakis *et al.*, 2007) (Figure 4). Cette matrice est le résultat d'une organisation supramoléculaire complexe qui n'est toujours pas entièrement résolue et comprise.

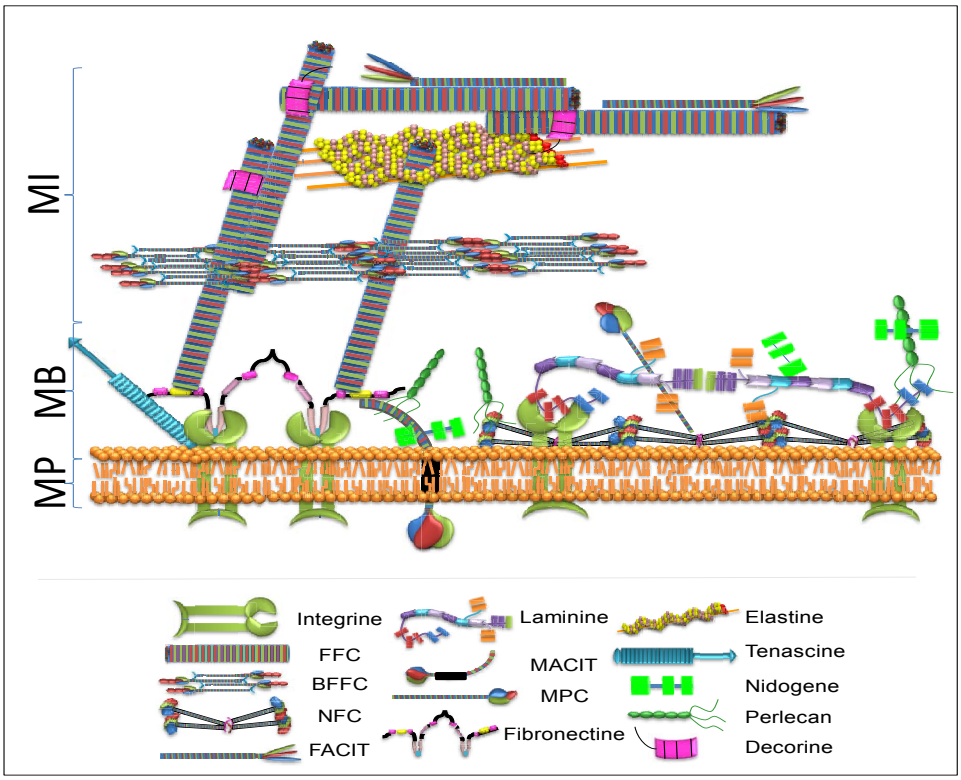

Figure 4 : Représentation de l'ECM au sein d'un tissu.
La matrice extracellulaire entoure les fibres musculaires et est constituée de protéines glycosylées qui forment un maillage fibreux dense. L'ECM est constituée principalement de collagène, élastine, fibronectine, et de laminine. MACIT (Membrane-Associated Collagen with Interrupted triple helices) ou collagènes transmembranaires, FFC (Fibril-Forming Collagen) ou collagènes formant des fibrilles, FACIT (Fibril Associated Collagen with Interupted Triple helices) ou collagènes retrouvés à la surface des collagènes fibrillaires, BFFC (Beaded Filaments-Forming Collagen) ou collagènes à l'origine de filaments perlés, NFC (Network-Forming Collagen) ou collagènes en réseaux, MPC (Multiplexin Collagens). MI: Matrice Interstitielle ; MB: Membrane Basale; MP:Membrane Plasmique (d'après Chagnot *et al* 2012).

Les collagènes forment la structure de base permettant la fixation d'autres composants de l'ECM (Orgel *et al.*, 2011). Les différents types de collagènes en fibrilles ou FFC (Fibril-Forming Collagen) (Tableau 6) s'assemblent pour former des fibres. Les collagènes en filaments perlés ou BFFCs (Beaded Filaments-Forming Collagen) et les collagènes associés au FFC ou FACIT (Fibril-Associated Collagen with Interrupted Triple helices) se fixent à la surface des FFC (Figure 5). Les MPCs (Multiplexin Collagens) se fixent sur la fibronectine et la laminine (Hurskainen *et al.*, 2010). Les collagènes membranaires ou MACITs (Membrane-Associated Collagens with Interrupted Triple helices) relient la cellule et l'ECM par l'intermédiaire de leur domaine N-terminal intracellulaire, d'un domaine transmembranaire ancré à la membrane plasmique et d'un domaine ectodomaine collagénique, permettant de fixer la fibronectine, le nidogène et les perlecans. En plus de permettre la fixation de la

fibronectine et de la laminine, ces différents types de collagènes peuvent interagir avec les récepteurs présents à la surface cellulaire, tels que les intégrines (Leitinger, 2011).

La fibronectine forme un pont moléculaire entre le réseau de collagènes et les autres composants de l'ECM (Figure 4). Les modules de fibronectine contiennent des sites de liaison aux intégrines ainsi qu'à d'autres composants, comme à la fibuline, la thrombospondine, la fibrine ou la tenascine (Schultz *et al.*, 2009). La tenascine peut également interagir avec des protéoglycanes, le perlecan et les intégrines (Orend *et al.*, 2003). Les intégrines sont des protéines transmembranaires qui interviennent dans la fixation des cellules à l'ECM, elles ont un rôle dans la transduction des signaux entre l'ECM et la cellule (Geiger *et al.*, 2011). En plus des collagènes et de la fibronectine, les intégrines ont la capacité de se lier à la laminine. Bien que les intégrines représentent la plus grande famille de récepteurs de surface, d'autres récepteurs peuvent également être impliqués dans la liaison des composants de l'ECM (Heino *et al.*, 2009).

La laminine interagit avec de nombreux protéoglycanes et types de collagènes, notamment les NFC (Network-Forming Collagen) (Durbeej, 2010) (Figure 4). Le nidogène interagit avec la fibuline et de nombreuses autres protéines de l'ECM, y compris l'élastine (de Vega *et al.*, 2009). L'élastine, fournissant l'élasticité à l'ECM, détient des sites d'interaction avec les protéoglycanes, la fibuline et la fibrilline (Hayes *et al.*, 2011). L'assemblage de ces molécules forme des microfibrilles qui s'associent avec la tropoélastine pour former les fibres élastiques, elles même associées à d'autres molécules de l'ECM telles que les collagènes (Figure 4). Cela souligne encore que la structure supramoléculaire de l'ECM est un amalgame de molécules biologiques qui diffèrent par leur identité et leur abondance relative (Bruckner, 2010).

Composants de l'ECM		Type	Structure	Organisation supramoléculaire	Localisation dans le tissu
Collagène	FFC	I, II, III,V,XI,XXIV,XXVII	Heterotrimère ou Homodimère	Collagène Elastine Fibronectine decorine	MI
	FACIT[a]	IX, XII, XIV, XVI, XIX, XX, XXI, XXII		Collagène	MI
	MPC	XV, XVIII		Fibronectine Laminine	MB
	NFC	IV, VIII, X		Laminine perlecan	MI
	BFFC	VI, VII, XXVI, XXVIII		Collagène	MB
	MACIT	XIII, XVII, XXIII, XXV		Fibronectine Nidogène Perlecan	MB MP
Fibronectine		Soluble Insoluble	Homodimère covalent	Integrine Integrine collagène	PLASMA MI MB
Laminine		111, 121 211 212/222, 213, 221, 3A11, 3A21, 3A32, 3A33, 3B32, 411, 421, 423, 511, 521, 522, 523	Heterotrimère	Integrine Collagène Proteoglycans Nidogène Perlecan Tenascine Microfibrile	MB
Elastine		n/a	Multimère	Proteoglycans Collagene Microfibrile	MI

Tableau 6 : Caractéristiques des composants de l'ECM. (d'après Chagnot *et al* 2012)
FACIT (Fibril Associated Collagen with Interupted Triple helices) ou collagènes retrouvés à la surface des collagènes fibrillaires, BFFC (Beaded Filaments-Forming Collagen) ou collagènes à l'origine de filaments perlés, NFC (Network-Forming Collagen) ou collagènes en réseaux, MPC (Multiplexin Collagens). MI: Matrice Interstitielle ; MB: Membrane Basale; MP:Membrane Plasmique (d'après chagnot *et al* 2012).

3.2. Localisation de l'ECM dans les tissus

Outre cette organisation supramoléculaire générique, il existe une certaine spécificité de l'ECM en fonction des tissus étudiés. Par exemple, en fonction de son état physiologique, l'organisation de l'ECM varie énormément d'un tissu à l'autre et même au sein d'un même type de tissu. Alors que dans les tissus conjonctifs, les composants de l'ECM sont synthétisés classiquement par les fibroblastes, ces molécules peuvent être sécrétées par des cellules spécifiques d'un tissu donné. Par exemple, les fibres musculaires sécrètent certaines protéines de l'ECM au cours de la myogenèse. Le collagène IV est exprimé par les cellules épithéliales ou encore ce sont les cellules de Shawn qui synthétisent la fibronectine dans les tissus nerveux. L'ECM a deux formes de base dans le tissu: (i) la membrane basale, également appelée lame basale et (ii) la matrice interstitielle (Figure 4). Dans certains tissus conjonctifs tels que le sang ou la lymphe, la matrice est fluide et appelée plasma.

La membrane basale est présente à la base de l'épithélium et du tissu conjonctif (Yurchenco, 2011). Les laminines sont les composantes majeures de la membrane basale et ne sont pas présentes dans la membrane interstitielle (Figure 4). La membrane basale forme un réseau complexe et structuré qui contient entre autres des protéoglycanes (essentiellement perlecan et nidogéne) et des collagènes, en particulier le collagène IV non fibrillaire (Tableau 6). Le Collagène IV forme un réseau qui constitue la membrane basale et le collagène VII sert d'ancrage cellulaire (Amano *et al.*, 2001). La diversité fonctionnelle de la membrane basale découle de la diversité moléculaire des isoformes de collagène, de laminine, ainsi que des constituants mineurs de l'ECM.

La composition de la matrice extracellulaire dépend grandement du tissu considéré, par exemple, il s'agit essentiellement de collagène dans le tissu conjonctif ou de fibronectine et de vitronectine dans le tissu sanguin. L'ECM la plus développée se trouve dans les tissus conjonctifs. Dans les tissus conjonctifs denses, comme le cartilage et les tendons, l'espace entre les cellules est presque entièrement rempli de collagènes fibrillaires, ce qui représente 60 à 85 % du poids sec de ces tissus. Dans le cartilage articulaire, le collagène II est le composant principal, mais les protéoglycanes et la décorine, contribuent également à la composition de la membrane interstitielle (Fox et al., 2009).

3.3 Principaux composants de l'ECM

3.3.1. Collagènes

À ce jour, 28 types de collagènes (de I à XXVIII) ont été décrits (Ricard-Blum, 2011) (Tableau 6). Les collagènes I à V sont les types les plus couramment rencontrés. Ils sont toujours formés de trois chaines α spécifiques au type de collagène afin de former un trimère en triple hélice (Figure 5). Les domaines en triple hélice sont alternés avec des domaines non hélicoïdaux qui diffèrent selon le type de collagène. Le degré d'hydroxylation et la glycosylation des résidus hydroxylysine dépend du type de collagène et du tissu. Les collagènes peuvent former diverses structures supramoléculaires (Figure 5), à savoir des fibres (FFC), des réseaux (NFC), les filaments perlés (BFFC), MACITs, FACIT ou multiplexin (MPC) (Thorsteinsdottir *et al.*, 2011).

Il existe une certaine spécificité de l'ECM en fonction des tissus étudiés, les types protéiques et leurs proportions varient.

Dans le tissu musculaire, les collagènes sont les principaux composants de l'ECM, les types I, III , IV , V , VI , XI , XII , XIV , XV et XVIII sont représentés (Voermans *et al.*, 2008, Gillies *et al.*, 2011). Cependant la proportion de chaque type n'est pas homogène, les principaux collagènes composant l'endomysium, le périmysium et l'épimysium sont les types fibrillaires I et III. Plusieurs études ont suggéré que dans le périmysium le collagène I prédomine, alors que pour l'endomysium et l'epimysium, c'est le collagène de type III (Light *et al.*, 1984). Cependant, ces études sont en grande partie qualitative en raison de la difficulté à isoler les diverses régions de l'ECM. Le collagène de type V, également fibrillaire, est associé avec les types I et III dans le perimysium et endomysium (Fitch *et al.*, 1984). Les types XII et XIV, collagène FACIT, dont la fonction exacte n'est pas connue, sont principalement localisés dans le perimysium (Listrat *et al.*, 2000). La membrane basale des cellules musculaires se compose principalement de collagènes en réseaux de type IV, mais également des types VI , XV et XVIII (Gillies *et al.*, 2011). Les collagènes MPC de types XV et XVIII sont également présents et se lient à d'autres glycoprotéines membranaires.

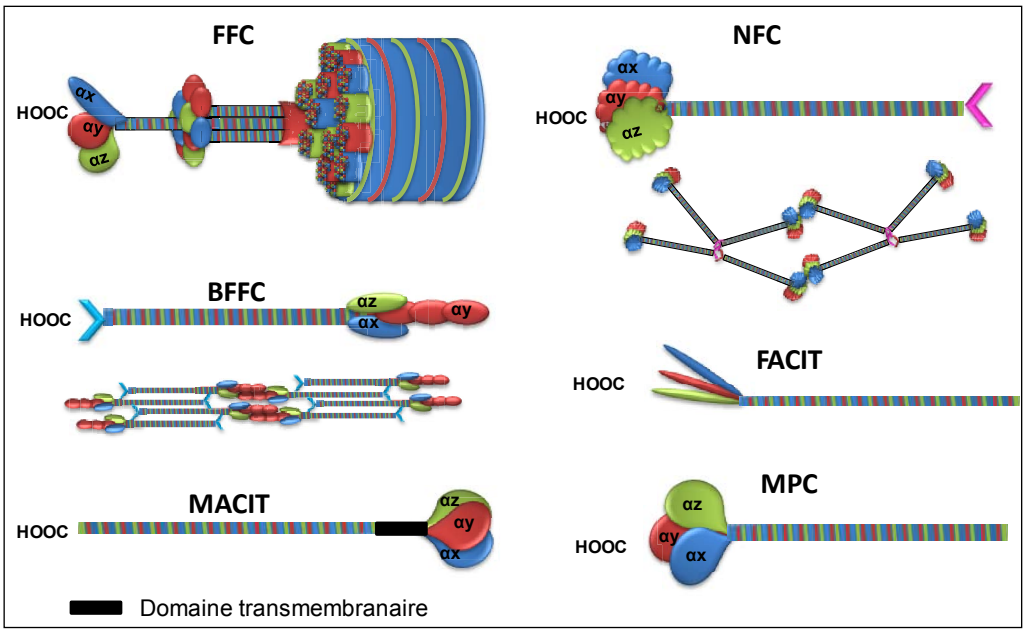

Figure 5 : Structure et organisation des différents types de collagènes.
MACIT (membrane-associated collagen with interrupted triple helices), FFC (fibril-Forming Collagen), FACIT (Fibril Associated Collagen with Interupted Triple helices), BFFC (Beaded Filaments-Forming Collagen), NFC (network-forming collagen), MPC (Multiplexin collagens) (d'après Chagnot *et al* 2012).

3.3.2. Fibronectine

La fibronectine est constituée de dimères liés de manière covalente par deux liaisons disulfures au niveau des extrémités carboxyliques (Pankov *et al.*, 2002) (Figure 6). Les fibronectines ont une architecture modulaire composée d'une combinaison de trois types différents de domaines homologues, à savoir les types I (FnI; IPR000083), II (FnII; IPR000562) et III (FnIII; IPR003961). Ces modules répétitifs sont principalement composés de feuillets β antiparallèles. Le domaine FnI est la région la plus conservée de la protéine, elle est retrouvée dans les différents taxons. Les modules FnI1-5 constituent le domaine liant l'héparine (HBD) et les modules FnI6-9 correspondent au domaine de liaison de la gélatine au collagène (GBD) (Erat et al., 2009). Le domaine FnII est impliqué dans la liaison au collagène. Contrairement aux deux autres domaines, FnIII ne peut pas former de ponts disulfures inter ou intramoléculaires mais constitue la plus grande partie de la fibronectine. La présence ou l'absence des domaines supplémentaires A (EDA) et B (EDB) partage en deux types les fibronectines: (i) fibronectine insoluble (i-fibronectine), également appelée fibronectine cellulaire, contenant une proportion variable d'EDA et EDB et (ii) la forme soluble (s-fibronectine), également appelée fibronectine plasmatique, sans domaine de type

III, uniquement présente dans l'ECM de certains tissus comme le sang ou la lymphe (Pankov *et al.*, 2002).

Dans le tissu musculaire, la fibronectine insoluble est la seule forme retrouvée dans l'ECM et est reliée à la membrane par des intégrines α7β1 (Voermans *et al.*, 2008). Toutefois, le muscle étant un organe fortement vascularisé, la forme soluble de la fibronectine est présente au niveau des capillaires sanguins.

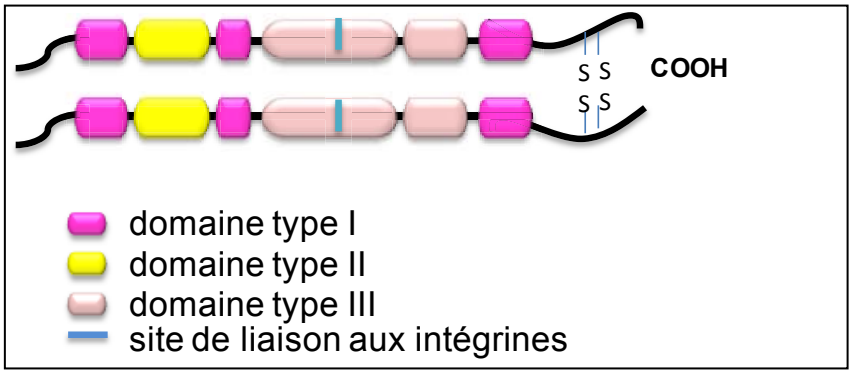

Figure 6 : Structure moléculaire de la fibronectine insoluble. (Chagnot *et al.* 2012)

3.3.3. Laminines

Les laminines sont des hétérotrimères composés de trois sous-unités distinctes, appelées α, β et γ, liées entre elles par des ponts disulfures (Durbeej, 2010) (Figure 7). Les variations de séquence des sous-unités, ainsi que l'épissage alternatif, expliquent qu'il existe aujourd'hui cinq chaines α, trois β et trois γ. La nomenclature des laminines est basée sur le numéro des trois sous-unités qui les compose, par exemple, la laminine-111 a une composition α1β1γ1. À ce jour, 18 isoformes de laminine distinctes ont été identifiées (Tableau 6). C'est une grande structure supramoléculaire (400 à 900 kDa) en forme de croix ou en forme de Y. La lamine s'attache à des molécules d'adhésion cellulaire spécifiques afin de former des jonctions entre les membranes cellulaires et les autres composants de l'ECM. Les laminines possèdent de nombreux domaines de liaison aux autres protéines, elles sont par conséquent des éléments clés dans l'organisation de l'ECM.

Dans le muscle squelettique, il a été établit que la forme α2 est majoritaire et plus particulièrement la laminine-211 (composé des chaines α2, β1 et γ1), autrefois appelé

mérosine, ainsi que la forme -221 (composé des chaines α2, β2 et γ1) (Voermans *et al.*, 2008). Les deux formes se lient entre elles et interagissent avec d'autres molécules telles que le nidogène ou les fibulines. La forme α4 est également retrouvé au niveau des zones de contact entre les nerfs et le muscle (Gawlik *et al.*, 2011).

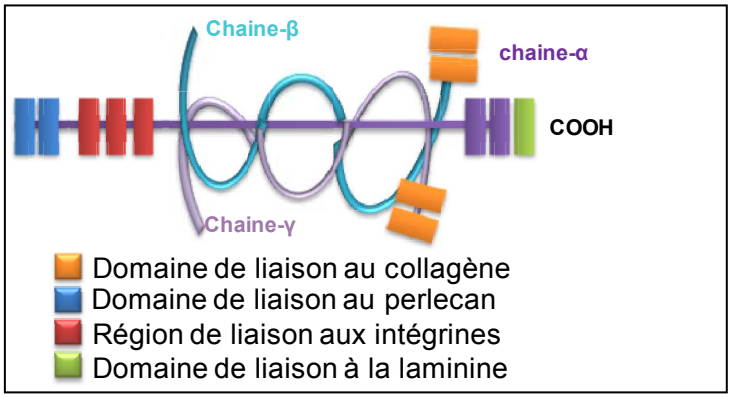

Figure 7 : Structure moléculaire de la laminine. (D'après Chagnot *et al.* 2012)

3.3.4. Elastine

L'élastine est un polymère résultant de l'assemblage de monomères de tropoélastine (Muiznieks *et al.*, 2010). La tropoélastine se compose en une alternance de domaines hydrophobes et de domaines de liaisons. Les domaines hydrophobes sont essentiellement composés d'acides aminés G, V, P et A, disposés en motifs responsables de l'organisation structurale de la protéine et sont directement impliqués dans un processus d'auto-association appelé coacervation. Les domaines hydrophobes jouent un rôle crucial dans la propriété élastique de la protéine. Les domaines de liaison sont principalement composés de lysine, ce qui permet la liaison covalente des monomères de tropoélastine sous l'action d'une lysyl-oxydase (Figure 8). Comme dans beaucoup de tissus, l'élastine est également présente dans le tissu musculaire, lui conférant de l'élasticité.

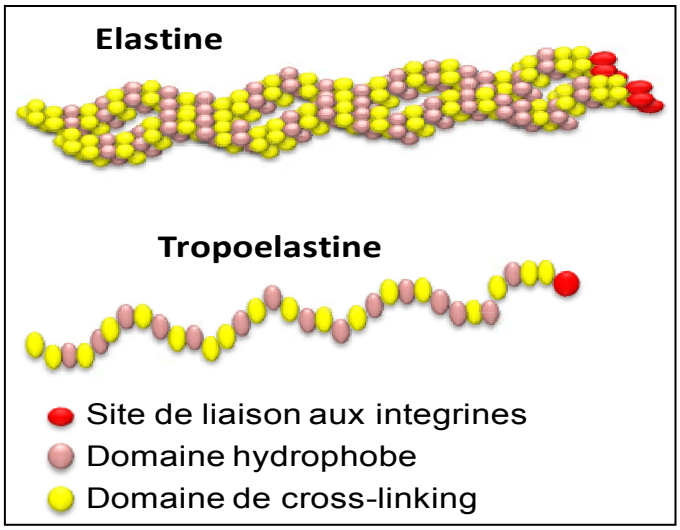

Figure 8 : Structure moléculaire de l'élastine et du monomère de tropoélastine.
(D'après Chagnot *et al*. 2012)

3.3.5. Protéoglycanes

En plus des principaux composants de l'ECM précédemment présentés, d'autres protéines jouent un rôle primordial dans le maintien de l'ECM, ce sont les protéoglycanes. Ils sont classés en trois grandes familles (Schaefer *et al.*, 2010) (i) SLRP (Small Leucine-Rich Proteoglycans), (ii) protéoglycanes modulaires et (iii) protéoglycanes associés à la membrane cellulaire. Les glycosaminoglycanes (GAG) sont composés d'un ensemble de chaines de protéoglycanes. Les GAG se partagent en deux groupes les sulfatés et les non sulfatés. L'acide hyaluronique est le seul GAG qui n'est pas complexé avec une autre protéine. On observe, au sein d'une même molécule de GAG, que la présence et la fréquence des chaînes sulfatées peuvent varier ainsi que le nombre et la longueur des GAG autour de la protéine centrale, ce qui s'explique par la grande hétérogénéité des chaines de protéoglycanes qui les composent. Les protéoglycanes forment des ponts protéiques reliant les différents composants de l'ECM entre eux afin de former une substance gélatineuse fortement hydratée. Cette substance maintient le volume de l'ECM et assure une résistante à la compression. Dans l'ECM musculaire, comme dans d'autres tissus, la présence de la fibuline, de perlecan, de tenascin, nidogène, et de décorine a pu être observée (Voermans *et al.*, 2008).

4. Evolution *postmortem* du muscle

4.1. Transformation du muscle en viande

Les réactions biochimiques mises en jeu lors de la transformation des muscles en viandes entraînent des modifications physico-chimiques du muscle. A l'abattage, le muscle est privé de l'oxygène et des nutriments qui jusque là étaient apportés par le sang. Pour maintenir son homéostasie, la cellule musculaire consomme alors ses réserves énergétiques par la voie anaérobie de la glycogénolyse dont le produit final est le lactate. L'ensemble des réactions *postmortem* dans la cellule conduit à une accumulation de protons et de lactate en proportions sensiblement identiques (Bendall, 1973). Le pH, voisin de 7,2 *in vivo* diminue jusqu'à une valeur finale et stable, appelée pH ultime (pH_u). La valeur du pH ultime dépend de l'espèce animale, du muscle et des conditions de pré-abattage.

Quand les réserves énergétiques sont épuisées, les gradients membranaires disparaissent et les concentrations ioniques s'équilibrent La formation de métabolites et la libération dans le cytoplasme d'ions inorganiques initialement concentrés dans des organites cellulaires et le réticulum sarcoplasmique entraine une augmentation sensible de l'osmolarité des cellules (Ouali, 1991). Pendant cette période, le muscle qui entre en rigidité cadavérique subit également des modifications structurales. Les myofibrilles et les cellules subissent une contraction latérale avec une augmentation concomitante des espaces extramyofibrillaires et des espaces extracellulaires (Heffron *et al.*, 1974, Lahucky *et al.*, 1997, Offer *et al.*, 1988). L'eau est expulsée dans les espaces extracellulaires puis exsudée.

4.2. Maturation

Après la rigor, le muscle qui a perdu toute propriété de contraction et d'extensibilité est progressivemlent dégradé lors de processus physico-chimiques et enzymatiques complexes. Sur le plan morphologique, les membranes se détachent des myofibrilles périphériques (Taylor *et al.*, 1995, Taylor *et al.*, 1998) et s'altèrent (Currie *et al.*, 1983, Astruc, 2014). Les myofibrilles présentent des ruptures caractéristiques le long des stries Z (Davey *et al.*, 1970, Dutson *et al.*, 1974, Abbott *et al.*, 1977, Taylor *et al.*, 1995, Taylor *et al.*, 1998) (Figure 9). Les fibres blanches (types IIX et IIB) semblent plus altérées que les fibres rouges (I et IIA) dans les mêmes conditions expérimentales (Abbott *et al.*, 1977).

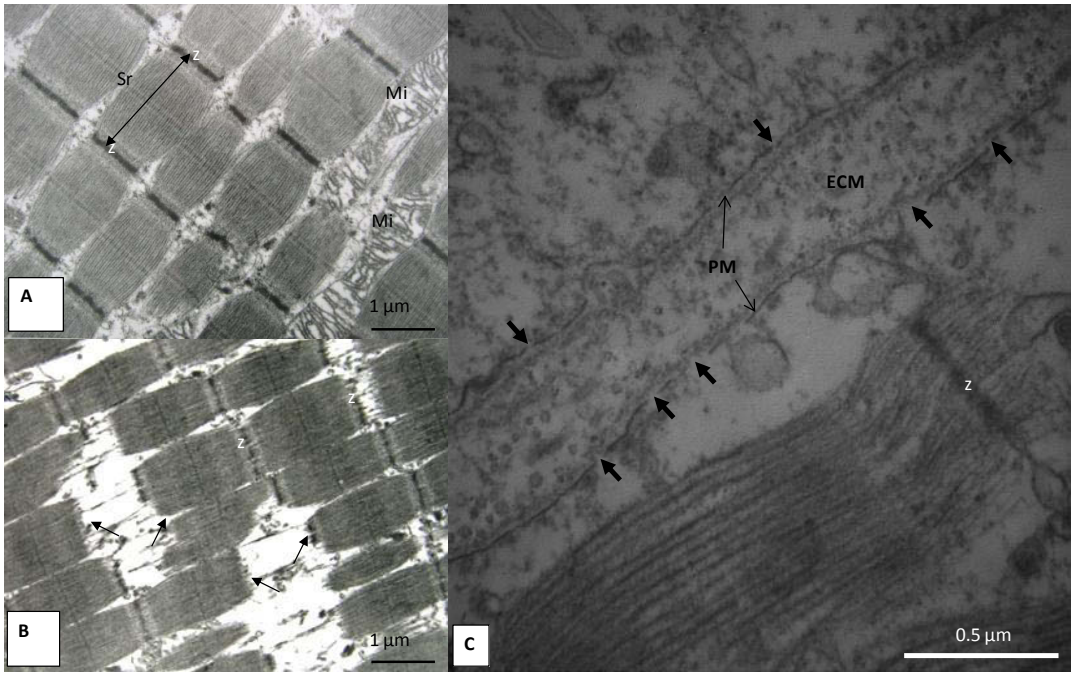

Figure 9 : Changements ultrastructuraux *postmortem des muscles*. Observation du muscle squelettique en miscroscopie electronique à transmission. Muscle semitendineux de bovin à 1jour (A) et 14jours (B) *postmortem*. (C) Coupe longitudinale de muscle Longissimus Dorsis de souris à 24h *postmortem.* Cassure au niveau des stries Z des myofibrilles resultant de la protéolyse *postmortem (*flèche sur B). Altération de la membrane plasmique (flèches sur C). PM : membrane plasmique ; Z=stries Z ; ECM : espace extracellulaire (Astruc, 2014).

Sur le plan biochimique, les produits de dégradation de l'ATP apparaissent comme l'AMP, transformée en IMP par desamination qui elle-même est lentement dégradée en hypoxantine et ribose. Le glycogène résiduel est finalement dégradé en glucose (Sharp, 1957).

L'action protéolytique des différentes protéases endogènes telles que cathepsines, calpaines, caspases et protéasome conduit à une dégradation partielle des proteines musculaires. Les protéines sarcoplasmiques sont assez peu affectées par la maturation (Davey *et al.*, 1966) alors que les proteines myofibrillaires sont fortement altérées et présentent une augmentation de leur solubilité au cours de la maturation. La protéolyse se traduit par la libération de peptides issus de la dégradation de proteines myofibrillaires et du cytosquelette (troponine T, desmine, connectine, titine actine, myosine (Greaser, 1986, Greaser *et al.*, 1995). Les organites cellulaires tels que le réticulum sarcoplasmique, les mitochondries et les lysosomes ne sont pas épargnés et montrent des signes de dégradation importants avec la durée *postmortem* (Greaser, 1986).

Le tissu conjonctif subit également quelques modifications lors de la phase de maturation des viandes (Bailey *et al.*, 1989, Nishimura, 2010). Les modifications morphologiques du tissu conjonctif intramusculaire sont sensibles dès 12h *postmortem* chez le poulet (Liu *et al.*, 1995) alors qu'elles ne deviennent évidentes qu'après 2 semaines *postmortem* chez le bovin (Nishimura *et al.*, 1995). Cette dégradation *postmortem* faciliterait la solubilisation du collagène au cours de la cuisson, avec comme conséquence une amélioration de la tendreté des viandes après cuisson. Des hypothèses récentes Nishimura, 2010 suggèrent que la dégradation *postmortem* des protéoglycanes qui stabilisent la matrice extracellulaire conduirait à un délitement des fibres de collagène, ce qui contribuerait à l'attendrissement des viandes.

Toutes ces modifications conduisent à une modification sensible de la composition du tissu musculaire, en particulier dans le compartiment intracellulaire ou les évolutions semblent les plus marquées.

-CHAPITRE 3–
INTERACTIONS MUSCLE ET BACTERIES

La contamination des viandes et les mécanismes moléculaires impliqués dans l'adhésion bactérienne restent mal connus. De nombreuses études montrent que les composants de la matrice extracellulaire musculaire peuvent être de bons substrats pour l'adhésion bactérienne. De plus, la transformation du muscle en viande s'accompagne de nombreuses variations biochimiques (acidification) et mécaniques (contraction cellulaire), pouvant augmenter le risque de développements bactériens dans les viandes. Afin de mieux contrôler ces contaminations, il semble indispensable de mieux comprendre les mécanismes moléculaires impliqués dans l'adhésion bactérienne à la viande. Les bactéries sécrètent des protéines de surface qui peuvent interagir spécifiquement avec les composants de l'ECM. Ces protéines sont qualifiées de MSCRAMM (Microbial Surface Components Recognizing Adhesive Matrix Molecules). Leur étude nécessite non seulement de bien connaitre les composants de l'ECM mais également la biologie des cellules. Alors que l'interaction spécifique entre les bactéries et l'ECM se produit à un niveau moléculaire grâce à l'interaction entre deux protéines, la structure et l'organisation des composants de l'ECM sont aussi à prendre en considération au niveau moléculaire, cellulaire et tissulaire. Ces aspects ne doivent pas être négligés afin de comprendre les mécanismes d'interaction ECM/bactérie dans un contexte hôte infecté par exemple.

1. MSCRAMM

Les protéines MSCRAMM se localisent à l'interface de la cellule bactérienne et de son environnement, c'est-à-dire la surface de la cellule. Cela implique nécessairement la translocation des protéines, via des systèmes de sécrétion, à travers une membrane biologique (monoderme) ou deux (diderme) en fonction de l'enveloppe cellulaire (Desvaux *et al.*, 2006, Desvaux *et al.*, 2009, Gupta, 2011, Sutcliffe, 2010) (Figure 10). Ces protéines sécrétées possèdent des domaines spécifiques à l'acheminement correct de la protéine vers la membrane, par exemple le Peptide signal (N-terminal) nécessaire à la translocation des protéines par la voie Sec ou le domaine LPXTG (C–terminal) essentiel à l'ancrage à la paroi

cellulaire bactérienne. L'exposition des protéines de surface diffère en fonction de l'architecture de l'enveloppe cellulaire bactérienne, monoderme ou diderme (Desvaux *et al.*, 2006, Sutcliffe, 2010). Chez les bactéries, la caractérisation moléculaire des domaines protéiques d'interaction avec l'ECM est uniquement décrite pour les principaux composants de l'ECM, à savoir le collagène, la fibronectine, la laminine et l'élastine.

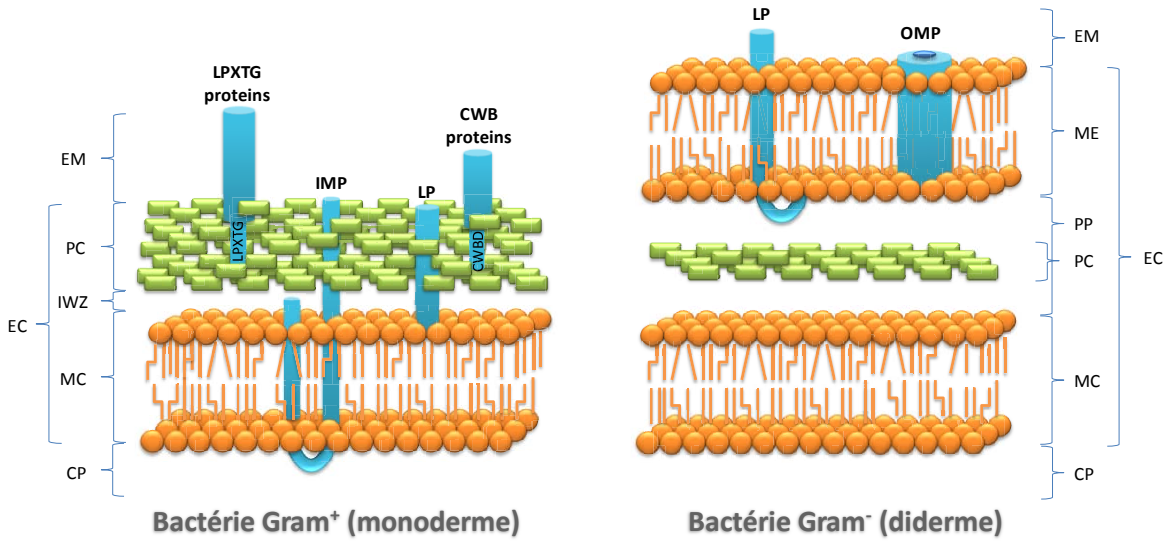

Figure 10 : Représentation des protéines membranaires exposées à la surface des cellules bactériennes Gram positives ou Gram négatives.
Dans les bactéries monodermes, ces protéines sont soit intrinsèques à la membrane cytoplasmique (MC) ou situées sur la paroi cellulaire (PC) lorsqu'elle est présente (Desvaux et al, 2006). Dans la membrane cytoplasmique, les protéines font partie intégrante de la membrane cytoplasmique, ce sont les IMP (integral membrane protein), ou fixées sur la partie externe de la membrane cytoplasmique comme les lipoprotéines (LP) Dans la paroi cellulaire, les protéines peuvent être discriminées en deux groupes les protéines ancrées de manières covalentes, à savoir les protéines LPXTG et les protéines associées de manière non covalente comme les CW-binding proteins (CWB proteins).
Dans les bactéries didermes, les protéines exposées à la surface cellulaire ne sont ni intrinsèques à la membrane cytoplasmique ni ancrées à la paroi cellulaire, qui est présente dans le périplasme (PP), mais intrinsèque à la membrane externe (ME). EIles sont soit intégrées à la membrane externe comme les OMP (Out Membrane Protein) soit ancrées à la membrane externe comme les lipoprotéines (LP)(Kovacs-Simon *et al.*, 2011).
CP: cytoplasme; IWZ : paroi interne; EM: milieu extracellulaire.

1.1. Protéines bactériennes et domaines de liaisons à l'ECM

Un grand nombre d'études a mis en évidence des interactions entre les protéines de l'ECM et les bactéries, toutefois de nombreuses interactions restent encore mal comprises. Afin de mettre en évidence les connaissances actuelles sur ces interactions, nous nous sommes intéressés non seulement aux protéines bactériennes mais surtout aux domaines de liaisons connus et référencés dans les bases de données par des codes IPR (InterPro). La mise en évidence de ces domaines permet de mieux comprendre les liaisons entre ECM et protéines

de liaison bactériennes mais également d'émettre des hypothèses sur le rôle que peuvent jouer certaines autres protéines bactériennes dans l'adhésion à l'ECM.

1.1.1. Protéines de liaison aux collagènes

Les domaines de liaison au collagène, appelés CBD, ont d'abord été identifiés et caractérisés à partir de l'adhésine Cna (collagen adhesin) chez un *Staphylococcus aureus* (Zong *et al.*, 2005). Les domaines CBD (IPR008456), d'environ 160 acides aminés de long sont composés de deux hélices α et de deux feuillets β antiparallèles. Ils s'associent avec les domaines de type B des collagènes (IPR008454 et IPR008970), qui ne sont pas considérés comme des domaines d'adhésion au collagène mais des domaines nécessaires dans la mise en place d'une conformation fonctionnelle de la protéine, exposant ainsi le domaine de liaison au collagène en surface (Deivanayagam *et al.*, 2000). Le domaine CBD appartient à la superfamille des domaines structuraux de l'adhésion bactérienne. Selon IPR v36.0, la grande majorité des domaines CBD est retrouvée principalement dans les protéines bactériennes des Firmicutes mais également chez les *Actinobacter* et quelques bactéries didermes. Généralement étudié à travers la proteine Cna, le domaine CBD permet une forte liaison au collagène de type I, mais il peut y avoir des différences d'adhésion au collagène d'une protéine à une autre. Par exemple, l'Acm (adhésine de collagène d'*Enterococcus faecium*) se lie au collagène I et a plus faible échelle au type IV (Nallapareddy *et al.*, 2003). La proteine Cne adhère au collagène de types I, II et III, mais pas au collagène IV (van Wieringen *et al.*, 2010). La proteine CbpA (Collagène protéine de liaison A) se fixe de façon similaire aux collagènes I et IV-XI et dans une moindre mesure aux collagènes V et IX (Pietrocola *et al.*, 2007).

En plus des domaines CBD, d'autres domaines impliqués dans l'adhésion aux collagènes ont été décrits, mais aucun modèle ou profil n'est encore disponible dans les bases de données. Chez les *Streptococcus,* la protéine M interagit fortement avec le collagène IV grâce à son motif N-terminal PARF (Peptide Associated with Rheumatic Fever)(Reissmann *et al.*, 2012). La région N-terminale de la protéine CbsA (Collagen-binding s-layer protein A) se lie aux collagènes I et IV (Antikainen *et al.*, 2002). Chez les bactéries didermes, la conformation structurale particulière de l'autotransporteur trimerique YadA (*Yersinia* adhesin A) lui confère la capacité de se lier aux collagènes I, II, III, IV, V et XI. L'activité de liaison

de la protéine réside dans la portion centrale et C-terminale du domaine apical impliquant le motif répété NSVAIG-S (Tahir *et al.*, 2000). Pour plusieurs autres protéines d'adhésion au collagène, le domaine responsable de l'adhésion reste encore indéterminé, comme par exemple pour les protéines p26 (protéine de 26 kDa) (Howard *et al.*, 2000), FoG (fibrinogen-binding protein de streptocoques G) (Nitsche *et al.*, 2006), Lsa63 (leptospiral surface adhesine de 63 kDa) (Vieira *et al.*, 2010) ou Ehab (enterohemorrhagic *Escherichia coli* autotransporter B) (Wells *et al.*, 2009).

1.1.2. Protéines de liaison aux fibronectines

Huit domaines bactériens impliqués dans l'adhésion à la fibronectine ont été identifiés et référencés dans les bases de données. Ces domaines liant la fibronectine (FBD) ont été classés du type 1 à 8.

Le domaine de liaison à la fibronectine FBD1 (IPR011252) a été identifié pour la première fois chez *S. aureus* ClfA (Clumping factor A) une protéine d'adhésion aux chaines γ des fibronectines (Deivanayagam *et al.*, 2002). Il a également été montré dans la protéine SdrG (Serine-aspartate proteines repeat containing G), que ce domaine lie son ligand par un mécanisme dynamique (Ponnuraj *et al.*, 2003). Le domaine FBD1 va généralement de pair avec le domaine de laison à la fibronectine FBD2 (IPR011266), comme dans les deux principales adhésines de S. aureus, FnBPA (Fibronectin-Binding Protein A) et FnBPB (Fibronectin-Binding Protein B). Le domaine FBD1 est retrouvé essentiellement au sein des Firmicutes et des *Actinobactéries* et FBD2 exclusivement chez les Firmicutes majoritairement des Staphylocoques et Streptocoques.

Le domaine de liaison à la fibronectine FBD3 (IPR008616) a été initialement caractérisée dans la protéine FbpA (Fibronectin-bindind protein A) du *Streptococcus gordonii* (Christie *et al.*, 2002), et des formes orthologues comme les protéines Fbp54 (Fibronectin-binding protein de 54 kDa) et PavA (Pneumococcal adhesion and virulence protein A). Or, le domaine FBD3 est également impliqué dans la liaison au fibrinogène, en interagissant avec les domaines HBD de la fibronectine. Fait intéressant, la capacité de lier la fibronectine soluble varie d'une protéine d'adhésion à la fibronectine (FnBP) à une autre, par exemple contrairement à la protéine Fbp54, la protéine Pava ne fixe pas la forme soluble (Henderson *et al.*, 2011). FBD3 est le domaine FBD le plus conservé, il est retrouvé chez 1160 espèces

bactériennes différentes. Il est retrouvé principalement au niveau du phylum Firmicutes y compris dans la classe *Clostridium* (Desvaux *et al*., 2005), mais il est également présent dans les phylums *Proteobacteria* (classes et des protéobactéries), les Cyanobactéries ou les *Spirochaetes*.

Pour le domaine de liaison à la fibronectine FBD4, le motif répété FIVAR (retrouvé dans diverses architectures) (IPR011490), formé àpartir d'un ensemble d'hélices α, permet une interaction spécifique avec la fibronectine. Par exemple, la protéine EcmbP (ECM-binding protein) interagit avec le module FNIII de la fibronectine (Christner *et al.*, 2010). Le domaine FBD4 est majoritairement retrouvé dans le phylum des Firmicutes et de façon ponctuel dans les phylums des *Actinobacteria Thermotogae* et *Bacteroidetes*.

Le domaine de liaison à la fibronectine FBD5 (IPR004237) est certainement le domaine le plus étudié (Henderson *et al.*, 2011). Il se trouve exclusivement chez les Firmicutes, essentiellement chez les genres *Staphylococcus* et *Streptococcus*. Chaque répétition du domaine FBD5 peut lier un module FNI de la fibronectine au niveau du domaine liant l'héparine, provoquant un changement de conformation de la protéine (Bingham *et al.*, 2008). De plus, ce domaine est retrouvé dans certain cas avec les domaines FBD1 et FBD2, comme dans les protéines FnBPA et FnBPB.

Le domaine de liaison à la fibronectine FBD6 (IPR010801) a été identifié dans une protéine FAP (Fibronectin Attachement Protein) chez *Mycobacterium*, et se limite à ce genre bactérien. Ce domaine interagit avec les domaines liant l'héparine de la fibronectine.

Le domaine de liaison à la fibronectine FBD7 ou SSURE (Streptococcal Surface Repeat) (IPR021021), a d'abord été découvert dans la protéine SP0082 du *Streptococcus pneumoniae (Bumbaca et al., 2004)*. Ce domaine est exclusivement présent chez les Firmicutes, essentiellement du genre *Streptococcus*.

Le domaine de liaison à la fibronectine FBD8 (IPR010841) a été initialement identifié chez *Listeria monocytogenes* Lmo0721 au niveau de la protéine FbpB (Fibronectine inding protein B) (Gilot *et al.*, 2002). Le domaine FBD8 est principalement présent dans le phylum Firmicutes et dans quelques espèces des phylums *Proteobacteria, Bacteroidetes* et *Chloroflexi*. Les informations structurelles en termes de repliement des protéines et de reconnaissance spécifique du site de liaison à la fibronectine ne sont toujours pas décrites pour les domaines FBD7 et FBD8.

Etonnamment, plusieurs protéines possédant un domaine de liaison à la fibronectine contiennent également un domaine de liaison aux collagènes, par exemple la protéine RspA (Rhusiopathiae surface protein A) abrite à la fois un domaine FBD1 et un domaine CBD (Shimoji *et al.*, 2003). Fait à noter, l'implication du domaine (IPR005298) des protéines MAP (MHC class II analogous protein) dans la liaison à la fibronectine reste à vérifier. Outre les domaines de types FBD, d'autres domaines impliqués dans la liaison fibronectine ont été décrits, mais aucun modèle ou profil n'est encore disponible dans les bases de données (Henderson *et al.*, 2011), par exemple le motif EWYYQ de l'antigène 85B, le motif FRLS de la proteine CadF (*Campylobacter* adhesin to Fibronectin), les motifs riches en glutamine de la proteine Hlp3 (HMW3-like protein) et de la protéine PlpA (Pneumoniae-like protein A) ou le domaine terminal Ig-like (IPR003343) des protéines LigA (*Leptospira* immunoglobulin-like protein A) et LigB. Dans certaines protéines Yada, la présence d'un motif N-terminal (appelé le domaine d'absorption) dans la région apicale de la protéine interagit avec la fibronectine (Heise *et al.*, 2006).

D'autres régions non caractérisées sont également connues pour interagir avec la fibronectine (Henderson *et al.*, 2011) : la région C-terminale de la protéine LipL32 (*leptospira* lipoprotein de 32 kDa), la région centrale des adhésines de type Cha (cell surface high molecular weight adhesin de *Granulicatella adiacens*) ou la région N-terminale de la protéine RevA (Reverse strand encoding protein). Dans certaines autres protéines de liaison à la fibronectine, les caractéristiques structurales et fonctionnelles ne sont pas encore décrites, comme pour UafB (uroadherance factor B) (King *et al.*, 2011).

1.1.3. Protéines de liaisons aux laminines

Contrairement au collagène ou à la fibronectine, aucun domaine de liaison à la laminine n'est actuellement enregistré dans les bases de données. Néanmoins, plusieurs régions protéiques sont connues pour conférer une adhérence spécifique à la laminine. La protéine CbsA (collagen-binding S-layer protein A) peut se lier à la laminine grâce à sa région N-terminale (Antikainen *et al.*, 2002). En l'absence du domaine d'absorption, la protéine YadA (Yersinia adhesin A) à la capacité d'adhérer au collagène mais aussi à la laminine (Heise *et al.*, 2006). Bien que l'analyse structurale et fonctionnelle reste à faire, d'autres protéines adhérantes à la laminine ont été identifiées, comme Ehab (Enterohemorrhagic *E. coli* autotransporteur A) (Wells *et al.*, 2009), Tp0136 (protéine codée

par locus tp0136 chez *Treponema pallidum*) (Brinkman *et al.*, 2008), OmpL47 (protéine de la membrane externe des *Leptospira* de 47 kDa) (Pinne *et al.*, 2010) ou Lsa24 (*Leptospira* surface adhesin de 24 kDa), Lsa27 et Lsa63 (Vieira *et al.*, 2010).

1.1.4. Protéines de liaison à l'élastine

Comme pour la laminine, les domaines protéiques bactériens responsables de la liaison spécifique à l'élastine ont été peu étudiés, ce qui contraste avec la fibronectine. Dans la protéine membranaire EbpS (Elastin-binding protein of *S. aureus*), le motif TNSHQD est la séquence minimale nécessaire à la reconnaissance de la région N-terminale de l'élastine (Park *et al.*, 1999). Pour la protéine FnBPA, il a été démontré que les domaines FBD1 et 2 ont des propriétés d'adhérence pour l'élastine, en plus de la fibronectine (Keane *et al.*, 2007). Les Ig-like repeats de la protéine LigB, sont impliqués dans les interactions électrostatiques avec l'élastine (Lin *et al.*, 2009). Bien que les caractéristiques structurales et fonctionnelles ne soient pas encore disponibles, la protéine OmpL37 montre une spécificité marquée pour l'élastine (Pinne *et al.*, 2010).

1.1.5. Structures supramoléculaires de surface

Chez les bactéries didermes, plusieurs systèmes de sécrétion protéique sont impliqués dans l'assemblage des pili. Ces structures supramoléculaires peuvent avoir des propriétés d'adhésion aux composants de l'ECM (Desvaux *et al.*, 2009). Outre les pili, le rôle des flagelles H6 et H7 dans l'adhérence spécifique au collagène, la laminine et la fibronectine ont été mise en évidence (Erdem *et al.*, 2007). Chez les bactéries didermes, le pili de type 4 ou HCP se lie spécifiquement à la laminine et à la fibronectine mais pas au collagène (Ledesma *et al.*, 2010). Les P-fimbriae sont capables de se lier au domaine de la fibronectine (Westerlund *et al.*, 1991) tout comme les fimbriae de type 1. Cependant cette capacité de liaison aux domaines HBD (heparin binding domain) et GBD (gelatin binding domain) de la fibronectine varie en fonction des souches bactériennes et résulte de l'hétérogénéité de séquence de la fimbriline FimH (Sokurenko *et al.*, 1994). Le motif de liaison de FimA, a été identifié (Murakami *et al.*, 1996). Pour la protéine AafA (Aggregative adherent fimbrilin A), il a été démontrée que le domaine de liaison se situe au sein du domaine glycosylé (Farfan *et al.*, 2008)). Les pili Lpf (Long polar fimbriae)

adhérent aux plaques de PEYER et aux cellules intestinales en interagissant avec la fibronectine, le collagène IV et la laminine(Farfan *et al.*, 2011). DraE (Dr haemagglutinin) de la famille des Afa (afimbrial adhesin) se lie au collagène IV (Korotkova *et al.*, 2006). Les Curli permettent des interactions spécifiques avec la laminine et la fibronectine (Zogaj *et al.*, 2003).

Chez les bactéries monodermes, les pili Ebp (endocardit- and biofilm- associed pilus) contribuent à l'adhésion au collagène et au fibrinogène (Nallapareddy *et al.*, 2011). Les Mtp (*Mycobacterium tuberculosis* pili) se lient à la laminine (Alteri *et al.*, 2007). La principale sous-unité adhésive du protofilament, RrgA (RlrA-réglemented gene A), permet la fixation du collagène I, de la laminine, de la fibronectine et du fibrinogène (Hilleringmann *et al.*, 2008).

1.2. MSCRAMM chez *E. coli* O157:H7

Chez les bactéries à Gram négatif comme les EHEC, les MSCRAMMs sont : soit ancrées à la membrane externe par un tonneau β, essentiellement des protéines sécrétées par la voie de sécrétion de type V (T5SS) comme les autotransporteurs, soit sécrétées et assemblées sous forme de structures supramoléculaires par différentes voies, comme les pili (fimbriae, curli, injectisome) et les flagelles.

1.2.1. Autotransporteurs

Les autotransporteurs sont des protéines modulaires synthétisées sous la forme de pré-pro-protéines dans le cytoplasme et relarguées sous forme de pro-protéines dans l'espace périplasmique. La translocation de cette pro-protéine a lieu via le système Sec au travers de la membrane interne et via le system Bam (β-barrel assembly machinery) au niveau de la membrane externe, ce qui entraîne son exposition à la surface de la cellule bactérienne (Leyton *et al.*, 2012). Leur structure primaire est modulaire, elle se compose de trois domaines principaux: (i) le peptide signal N-terminal pour le routage de la protéine vers la membrane interne, (ii) le domaine passager qui se présente en hélice β suivi d'une région C-terminale, appelée autochaperone, et (iii) le domaine C-terminal, l'unité de translocation (IPR005546), formée de deux segments: une petite hélice α appelée région de liaison et un

segment formé de 14 brins antiparallèles enchâssées dans la membrane externe, appel tonneau β (Figure11).

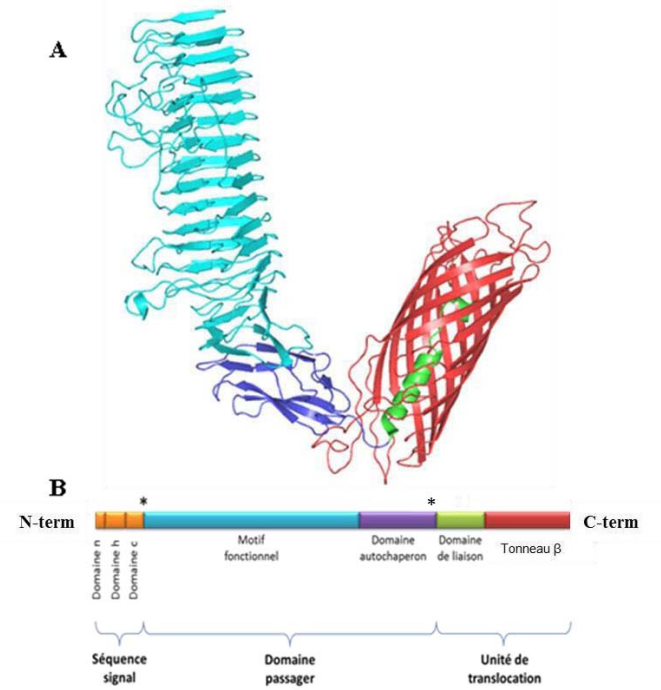

Figure 11 : Représentation (A) de la structure tridimensionnelle et (B) de la Structure primaire d'un autotransporteur.
Les deux astérisques symbolisent les points du clivage N-term et C-term. Le clivage du domaine passager se limite à certains autotransporteurs comme AIDA-I et l'Ag43 (Desvaux *et al.*, 2004).

Chez *E. coli* O157:H7, 9 autotransporteurs sont codés par le génome et certains ont été caractérisés. Suite à l'interrogation de la base de données Pfam différents gènes *eha* (EHEC autotransporter) ont été identifiés. EhaA (EHEC autotransporter A) est impliqué dans l'adhésion à des cellules épithéliales bovines. EhaB permet la liaison à la laminine et au collagène I de l'ECM (Wells *et al.*, 2009). EhaG favorise l'agrégation cellulaire, la formation de biofilms et adhère à plusieurs protéines de l'ECM notamment le collagène de type IV et l'élastine (Totsika *et al.,* 2012). EhaG entraîne une adhésion spécifique des bactéries aux cellules épithéliales colorectales. EhaJ a la capacité de se lier à une grande variété de composants de l'ECM comme les collagènes, la laminine, la fibronectine, l'élastine puis à un certain nombre de protéoglycanes et de glycosaminoglycanes (Easton *et al.*, 2011). A noter que l'implication de ces autotransporteurs dans l'adhésion à des composants de l'ECM ne semble pas prévalente puisque la délétion des gènes n'entraîne ni une absence ni une diminution de l'adhésion. Ceci résulterait de l'expression prépondérante d'autres facteurs

d'adhésion. (Klemm *et al.*, 2006) ont décrit une nouvelle famille d'autotransporteurs, il s'agit des SAATs (Self-associating autotransporters). Les SAATs sont impliqués dans l'adhésion à des cellules épithéliales, dans la formation du biofilm et dans l'autoagrégation bactérienne. A ce jour, cette famille compte trois protéines: le facteur d'autoagrégation appelé antigène 43 (Danese *et al.*, 2000), l'adhésine impliquée dans l'adhérence diffuse (AIDA-I) (Benz *et al.*, 1989), et l'adhésine/invasine TibA (Côté, 2011).

1.2.2. Structures supramoléculaires de surface

Plusieurs types de pili sont présents chez les EHEC dont l'aspect, la structure et les mécanismes d'assemblage peuvent être très différents**.** Ils sont impliqués dans un grand nombre de fonctions comme l'adhésion, l'agrégation cellulaire, la formation de biofilms mais aussi la motilité, la sécrétion ou le transfert horizontal de matériel génétique (Mandlik *et al.,* 2008).

Chez *E. coli* O157:H7 EDL933, 17 pili sont codés par le génome. Parmi les pili de type fimbriae sécrétés et assemblés par la voie du chaperone-escorteur (CU : chaperone-usher ou T7SS) (Desvaux *et al.,* 2009), les fimbriae de type F9, dont la piline majeure est codée par le gène *Z22000,* ont la capacité de se lier à l'élastine, à la fibronectine et aux différents collagènes de l'ECM (Low *et al.,* 2006). Les fimbriae de type I codés par le gène *fimA* ainsi que les Lpf (long polar fimbriae) codés par les gènes *lpf1* ou *lpf2* participent à la liaison de la bactérie aux protéines de l'ECM telles que la fibronectine, la laminine et le collagène IV (Farfan *et al.*, 2011). Les pili de type Curli codés par le gène *csgA* et mis en place par la voie ENP (Extracellular Nucleation Precipitation ou T8SS) (Desvaux *et al.,* 2009), interagissent avec la laminine et la fibronectine (Saldana *et al.*, 2009b). Les pili de type IV (gène *ppdD*) aussi appelés HCP (Haemorrhagic Coli Pilus) ont la capacité de se fixer à différentes protéines de l'ECM telles que la fibronectine, la laminine et les collagènes (Xicohtencatl-Cortes *et al.,* 2009). A noter que l'expression de tous ces pili est extrêmement dépendante des conditions environnementales.

Outre le pili, le flagelle est aussi une structure supramoléculaire de surface. Au-delà de son rôle dans la motilité bactérienne, il peut aussi agir comme adhésine comme cela a été montré chez certains EHEC où les flagelles H6 et H7 permettent l'adhésion spécifique au collagène, à la laminine et à la fibronectine (Erdem *et al.,* 2007). L'injectisome, appartenant comme le flagelle à la voie de sécrétion de Type III (T3SS), peut lui aussi être impliqué dans

l'adhésion bien que son implication dans la reconnaissance de composants de l'ECM n'ait pas encore été démontrée.

2. Modifications biochimiques et mécaniques du muscle pouvant influencer l'adhésion bactérienne

Malgré la capacité des bactéries à sécréter des protéines de surfaces capables d'interagir avec les protéines de l'ECM, la structuration de l'ECM ne facilite probablement pas la colonisation de la partie interne du muscle natif. L'ECM étant un maillage protéique dense et complexe, il représente une barrière mécanique face à la contamination bactérienne. C'est pourquoi d'autres mécanismes peuvent également être impliqués. En effet, lors de la transformation du muscle en viande, le muscle subit des transformations mécaniques et biochimiques qui peuvent jouer un rôle dans la contamination bactérienne intramusculaire.

2.1. Acidification du muscle

Ces modifications de pH du muscle au cours de sa transformation peuvent favoriser la colonisation des bactéries. En effet, de nombreuses études ont montré que la variation de pH a un effet sur l'expression des gènes ainsi que sur la structure protéique des bactéries. Le pH peut avoir différents effets comme celui de modifier la structure et donc l'activité d'une protéine ou encore de modifier son routage à travers la membrane. Les protéines membranaires sont extrêmement sensibles aux modifications environnementales en particulier, les protéines transmembranaires (Olson, 1993). Or les protéines de surface étant des facteurs d'adhésion à l'ECM, le pH pourrait jouer un rôle important dans la contamination des viandes. Parmi les facteurs d'adhésion connus pour être sensible au pH, le flagelle des *E. coli* peut être cité comme exemple. En effet, il a été montré qu'à pH acide, la motilité de la bactérie ainsi que l'expression des gènes codant le flagelle étaient fortement diminuées (Soutourina *et al.*, 2002). De plus une étude traitant des effets du pH sur l'expression de structures de surface chez *Salmonella* a montré que la croissance en pH environnemental extrême modifie la distribution des structures de surface de la cellule. Ainsi, à la surface cellulaire, le nombre de fimbriae varie à pH 9 seuls 3% sont exprimés contre 52% à pH 7 et 20% à pH 4 (McDermid *et al.*, 1996).

2.2. Modifications morphologiques *postmortem*

Après quelques heures d'anoxie, les fibres se contractent latéralement, et la taille des espaces extracellulaires augmente, le maillage de l'ECM se relâche (Offer *et al.*, 1988). Le maillage étant moins dense cela peut permettre aux bactéries de se frayer un chemin vers l'intérieur du muscle. L'évolution des propriétés mécaniques du muscle après l'abattage (perte d'élasticité et d'extensibilité) est étroitement liée aux modifications affectant le système protéique myofibrillaire responsable de la contraction musculaire. Les deux principales protéines fonctionnelles de ce système sont l'actine et la myosine. Elles ont la propriété de se lier et de former un complexe actomyosine. Ce complexe est indispensable à la contraction du muscle, tout comme l'ATP qui fournit l'énergie nécessaire. Néanmoins, en l'absence de stimulation nerveuse, la présence d'ATP est nécessaire au maintien de l'état relaxé du muscle, c'est-à-dire à l'inhibition du complexe actomyosine. Or, la disparition progressive de l'ATP s'accompagne de la formation de complexes actomyosine, le muscle se contracte et entre alors en *rigor mortis*, cette contraction est d'autant plus importante que le pH est acide. La *rigor mortis* provoque l'expulsion de l'eau intracellulaire vers l'espace extracellulaire. Globalement, la chute de pH et l'installation de la *rigor mortis* entraine une réduction de 40% du volume myofibrilliaire (Offer, 1984). De plus, les membranes plasmiques des cellules musculaires s'altèrent et se perforent (Astruc *et al.*, 2008, Rowe, 1989) entraînant la libération de substrats intracellulaires (sucre, protéines…) susceptibles de stimuler la croissance et donc les interactions bactéries-cellules musculaires. Ces phénomènes de dégradation membranaire des cellules musculaires pourraient favoriser la colonisation intracellulaire.

OBJECTIFS DE RECHERCHE

Les EHEC sont des pathogènes producteurs de Shiga-toxines responsables de toxi-infections alimentaires. De nombreuses épidémies sont régulièrement recensées, et la consommation de viandes est souvent incriminée. Dans le monde, le sérotype majoritairement impliqué dans les infections à EHEC est O157:H7, c'est également le plus virulent. Malgré l'existence de techniques de prévention et de détection des EHEC, des aliments contaminés se retrouvent régulièrement sur le marché. La premiere étape clé de la contamination est le dialogue moléculaire mis en jeu entre le pathogène et l'aliment qui reste encore aujourd'hui mal connu. Dans le but de limiter la contamination des viandes par les EHEC, il est nécessaire de mieux comprendre les mécanismes protéiques d'adhésion et de colonisation des EHEC avec la viande afin de proposer de nouvelles stratégies de prévention. Dans la littérature, des protéines bactériennes appelées MSCRAMM ont la capacité d'adhérer à l'ECM. Or l'ECM musculaire partage de grande similitude avec l'épimysium, la couche de tissu conjonctif la plus exposée du muscle. L'ECM pourrait jouer un rôle important dans l'adhésion des EHEC à la viande.

Les objectifs de ma thèse étaient :

I. D'étudier la capacité d'adhésion et de colonisation de la souche *E. coli* O157 :H7 aux protéines de l'ECM purifiées :
   - Identifier les protéines de l'ECM pouvant être impliquées dans l'adhésion.
   - Déterminer les conditions environnementales d'adhésions optimales (pH, température et conditions de croissances).
   - Comparer les capacités d'adhésion et de colonisation des STEC non-O157 majeurs et O157.
   - Identifier des déterminants bactériens impliqués dans l'adhésion aux protéines de l'ECM.

II. D'étudier le potentiel tropisme bactérien en fonction des caractéristiques métaboliques et contractiles du muscle :
   - Développer une méthode de discrimination des types cellulaires sur coupes vierges de tout marqueur.
   - Caractériser le tropisme bactérien en fonction du muscle et des types de fibres.

RESULTATS

Résultats partie I : Adhésion et colonisation des EHEC aux protéines purifiées de l'ECM

Alors que la pathogénicité des EHEC est particulièrement bien étudiée dans la littérature, les aspects moléculaires de la contamination des viandes sont très peu documentés. Or, la compréhension des mécanismes d'interaction et de colonisation des bactéries à l'échelle moléculaire est un pré-requis à la maîtrise des écosystèmes bactériens dans les aliments afin de limiter les risques de contamination à EHEC. La contamination bactérienne primaire a lieu lors de l'abattage où les bactéries peuvent être transférées de la peau à la carcasse (Aslam *et al.*, 2003). Après la mort de l'animal, le muscle subit des modifications biochimiques et structurales ce qui conduit à sa transformation en viande. Cette transformation s'accompagne de diminutions de pH et de température pouvant jouer un rôle dans l'adhésion bactérienne. La partie la plus exposée de la carcasse est l'épimysium, tissu conjonctif enveloppant les muscles, dont les composants majeurs sont les mêmes que l'ECM musculaire (ou endomysium) essentiellement des collagènes I, III et IV, de la fibronectine insoluble (i-fibronectine), de la laminine-α2 et de l'élastine (Voermans *et al.*, 2008). Certaines protéines bactériennes de surface sont connues pour leur capacité d'adhésion aux protéines de l'ECM, ce sont les protéines MSCRAMMs (Microbial Surface Components Recognizing Adhesive Matrix Molecules). Cependant l'expression de ces protéines dépend fortement des conditions environnementales de la bactérie (pH, température et milieux de culture) (Xicohtencatl-Cortes *et al.*, 2009, Brady *et al.*, 2011).

Cette première partie de mon travail de thèse a consisté à caractériser l'adhésion des EHEC aux protéines de l'ECM. Pour cela la capacité d'adhésion et de colonisation aux protéines purifiées de l'ECM musculaire de la souche *E. coli* O157:H7 a été particulièrement étudiée en raison de sa prévalence lors des toxi-infections alimentaires à EHEC (Article 1). Afin de mimer les conditions environnementales subies lors de la maturation de la viande, l'adhésion et la colonisation des bactéries aux protéines de l'ECM ont été testées dans différentes conditions de culture, de pH et de température représentatives de la chaîne de production. Ces résultats ont ensuite été comparés aux 7 sérogroupes majeurs d'EHEC non-O157 (O26, O45, O103, O111, O121 O145 et O104) (Article 2).

Après avoir démontré la capacité de la bactérie *E. coli* O157:H7 à adhérer et à coloniser les protéines de l'ECM musculaire, mes recherches se sont focalisées sur l'identification de déterminants moléculaires, en particulier protéiques, impliqués dans ces interactions. La colonisation de matrices biologiques, comme la viande, par les bactéries est étroitement liée à leur capacité à exprimer des adhésines capables d'interagir avec les composants de l'ECM. Ces déterminants protéiques sont appelés MSCRAMM, leur localisation en surface cellulaire implique nécessairement le transport de ces protéines par l'intermédiaire des systèmes de sécrétion et leurs translocations à travers les deux membranes biologiques des bactéries diderm–LPS (Desvaux *et al.*, 2006, Desvaux *et al.*, 2009). Chez les bactéries diderm-LPS comme les *E. coli,* les protéines MSCRAMM sont soit (i) ancrées à la membrane externe par un tonneau β et principalement sécrétées par le système de sécrétion de type V (T5SS), ou (ii) sécrétées et assemblées en structures supramoléculaires par différents systèmes de sécrétion (T3bSS, T2cSS, T3aSS, T4aSS, T4bSS, T7SS et T8SS) tels que les flagelles et les pili (Chagnot *et al.*, 2013b). L'expression de ces protéines dépend fortement des conditions environnementales. Or, l'adhésion de *E. coli* O157:H7 dépend également des conditions de culture, ce qui renforce l'idée que les interactions de la bactérie avec le muscle dépend de l'expression d'une ou plusieurs MSCRAMM(s). Cependant, les protéines bactériennes responsables de ces interactions dans la viande n'ont pas été caractérisées.

E. coli O157:H7 possède plusieurs protéines MSCRAMM dont le rôle dans l'adhésion aux tissus musculaires reste méconnu. Parmi les 17 pili codés par le génome de *E. coli* O157: H7, certains sont des MSCRAMMs connues pour leur rôle dans l'adhésion, l'agrégation cellulaire et/ou la formation du biofilm (par exemple : les fimbriae F9, les curli, les pili HCP, les flagelles H7 et/ou l'injectisome) (Mandlik *et al.*, 2008). En plus des pili et flagelles, 9 autotransporteurs sont codés par le génome de *E. coli* O157: H7, certains peuvent être impliqués dans les interactions aux protéines de l'ECM, le plus étudié étant l'antigène 43 (Ag43) qui participe à l'auto-agrégation et la formation de biofilm (Torres *et al.*, 2002). Dans tous les cas, le rôle de ces déterminants protéiques de surface dans l'interaction des bactéries à l'ECM du muscle n'est pas clairement établit dans la littérature. Cette partie de mon travail de thèse visait à identifier les déterminants protéiques pouvant jouer un rôle dans l'adhésion et la colonisation non-spécifique d*'E. coli* O157:H7 observées dans les Articles 1 et 2 en utilisant une approche de génétique fonctionnelle. Le rôle de 7 gènes d'intérêt a été étudié dans les conditions optimales d'adhésion et colonisation non-spécifiques déterminées dans l'Article 1.

ARTICLE n°1

Caroline CHAGNOT, Allison AGUS, Sandra RENIER, Frédéric PEYRIN, Régine TALON, Thierry ASTRUC and Mickaël DESVAUX.

Colonization of the Muscle Extracellular Matrix Components by *Escherichia coli* O157:H7: Evidences of the Influence of Growth Media, Temperature or pH on Initial Adhesion and of the Induction of Biofilm Formation by Collagens I and III

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In Vitro Colonization of the Muscle Extracellular Matrix Components by *Escherichia coli* O157:H7: The Influence of Growth Medium, Temperature and pH on Initial Adhesion and Induction of Biofilm Formation by Collagens I and III

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Abstract

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 are responsible for repeated food-poisoning cases often caused by contaminated burgers. EHEC infection is predominantly a pediatric illness, which can lead to life-threatening diseases. Ruminants are the main natural reservoir for EHEC and food contamination almost always originates from faecal contamination. In beef meat products, primary bacterial contamination occurs at the dehiding stage of slaughtering. The extracellular matrix (ECM) is the most exposed part of the skeletal muscles in beef carcasses. Investigating the adhesion to the main muscle fibrous ECM proteins, insoluble fibronectin, collagen I, III and IV, laminin-α2 and elastin, results demonstrated that the preceding growth conditions had a great influence on subsequent bacterial attachment. In the tested experimental conditions, maximal adhesion to fibril-forming collagens I or III occurred at 25°C and pH 7. Once initially adhered, exposure to lower temperatures, as applied to meat during cutting and storage, or acidification, as in the course of post-mortem physiological modifications of muscle, had no effect on detachment, except at pH_u. In addition, dense biofilm formation occurred on immobilized collagen I or III and was induced in growth medium supplemented with collagen I in solution. From this first comprehensive investigation of EHEC adhesion to ECM proteins with respect to muscle biology and meat processing, new research directions for the development of innovative practices to minimize the risk of meat contamination are further discussed.

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Introduction

Worldwide, the occurrence of food poisoning following the consumption of products contaminated with enterohemorrhagic *Escherichia coli* (EHEC) is recurrent [1]. EHEC outbreaks and sporadic cases incriminate some meat, milk or vegetable products as well as water-based drinks [2,3]. Human infections, though, often follow the consumption of beef meat, especially minced meat, and often involve strains of the serotype O157:H7. The EHEC reference strain O157:H7 EDL933 was originally isolated from contaminated burgers (beef patty) responsible for an outbreak of hemorrhagic colitis that occurred in the United States of America [4]. From this episode in 1982, the significance of EHEC as a serious public health problem was first recognized. EHEC infection manifests clinically with diarrhea and abdominal cramps before proceeding to hemorrhagic colitis characterized by bloody diarrhea [5,6]. Although it can occur at any age, EHEC infection is predominantly a pediatric illness [7]. It can cause into most acute forms of life-threatening complications, namely haemolytic uraemic syndrome (HUS) especially in young children less than 5 years old, or more rarely into thrombotic thrombocytopenic purpura (TTP) [8,9].

Ultimately, EHEC contamination of food products originates primarily from faecal contamination. Ruminants such as bovines are the main natural reservoir for EHEC [1]. In the case of beef, hygienic slaughtering practices reduce faecal contamination of carcasses (prevention of evisceration accidents, cross contamination and poor hygiene) but cannot guarantee the absence of *E. coli* O157:H7 from meat [10]. Primary bacterial contamination occurs often inevitably at the dehiding stage of processing where bacteria can be transferred from hides to beef carcasses. Post animal slaughther, the muscle converts to meat undergoing a succession of post-mortem physiological changes [11]. According to the European Community (EC) regulation specifying the hygiene

rules for foodstuffs (n°853/2004), animal slaughter and cutting of carcases into quarters can be carried out at room temperature. However, during further cutting, storage and/or transport, meat must reach and be maintained at 7°C. From then on the temperature of meat preparations and minced meat must not exceed 4°C and 2°C respectively. While molecular mechanisms of EHEC pathogenicity have been subjected to intense research [12,13], the molecular aspects of food contamination clearly lags behind. Such basic knowledge is of great importance to allow for further improvements to quantitative risk assessment in the management of *E. coli* O157:H7 in the beef processing industry [14].

Attachment of *E. coli* O157:H7 to beef meat has been reported [15,16,17,18] and identified between muscle fibres within the connective tissue [19,20]. However, the molecular interactions between the bacterial cells and the muscle extracellular matrix (ECM) components are more controversial. ECM is composed of two main classes of macromolecules, the fibrous proteins and the proteoglycans [21]. In skeletal muscle tissue, fibrous proteins are the predominant components of the ECM, essentially comprised of collagens I, III and IV, insoluble fibronectin (i-fibronectin), laminin-α2 and elastin [22,23,24,25]. Using a surface plasmon resonance biosensor, binding of collagen I and laminin to the cell surface of *E. coli* O157:H7 was reported [20,26,27]. The role of pili in mediating the attachment of EHEC cells to meat was suggested but not ascertained and other cell surface determinants related to the virulence may also be involved [15,26,28]. Importantly, the expression of virulence factors in EHEC is inducible and depends on growth conditions [29,30]. Surprisingly enough, a most recent investigation could not provide evidence of significant attachment of O157:H7 EHEC strains to any of the tested immobilized ECM proteins [31]. Altogether, this prompted us to reinvestigate the adhesion of EHEC O157:H7 to the main ECM fibrous proteins present in beef meat.

Materials and Methods

Bacterial strains and culture conditions

The non-toxigenic isogenic mutant of EHEC O157:H7 EDL933, deleted of the *stx1* and *stx2* genes [32,33], were used in this study. Bacteria were cultured in different nutrient media either chemically defined, i.e. DMEM (Dulbecco's modified eagle medium, Gibco), M9 [34] and MinCa (minimal casein) media [35], or complex undefined, i.e. LB (lysogeny broth) [36], TSB (tryptic soy broth, Becton-Dickinson), BHI (brain-heart infusion, Becton-Dickinson), LH (Leedle Hespell) [37], BCC (bovine caecum content) and BJIC (bovine jejunum-ileum content) sterile media [38]. Contents of different parts of the bovine digestive tract were collected as previously described to prepare LH, BCC and BJIC [38]. From −80°C stock culture previously grown in the respective medium, strains were plated on the relevant agar medium and incubated overnight at 39°C (bovine temperature) [38]. A preculture was set up from one bacterial colony grown in the respective nutrient broth medium at 39°C in an orbital shaker at low speed (70 rpm) till stationary phase.

Fluorescent bacterial strains were obtained following transformation with the vector pSARE-Red1 expressing the fluorescent protein mRuby [39]. Briefly, the gene encoding mRuby was amplified by PCR with high-fidelity TaKaRa LA Taq DNA polymerase from pmRuby using mRubF (TTATATCATGAACAGCCTGATCAAAGAAAACATGCGG) and mRubR (TTATACTCGAGGCATGCTTACCCTCCGCCCAGGC) primers. The amplicon was cloned into pIMK2 [40] following DNA digest with NcoI/XhoI restriction enzymes, ligation and electroporation into *E. coli* TOP10 (Invitrogen). From the resulting construct, the

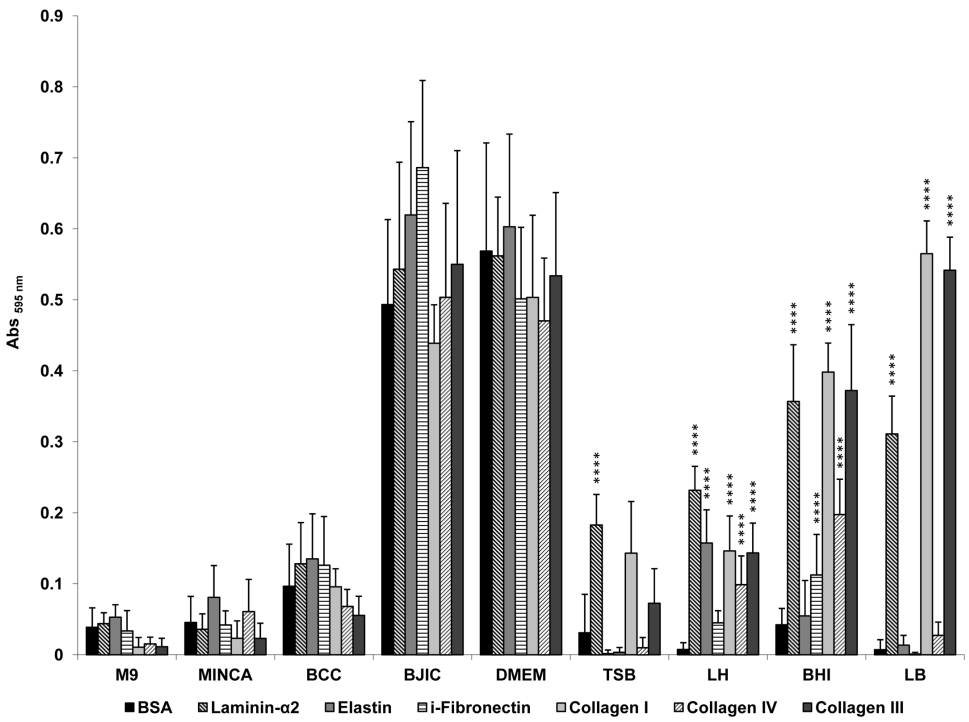

Figure 1. Adhesion to immobilized ECM proteins of *E. coli* O157:H7 EDL933 *stx*⁻ grown in different media. Specific bacterial adhesion assay to the main ECM fibrous proteins present in meat was performed at 25°C using BSA as a control and measured by the crystal violet staining method. Bacterial cells for the adhesion assay were first grown at 39°C.
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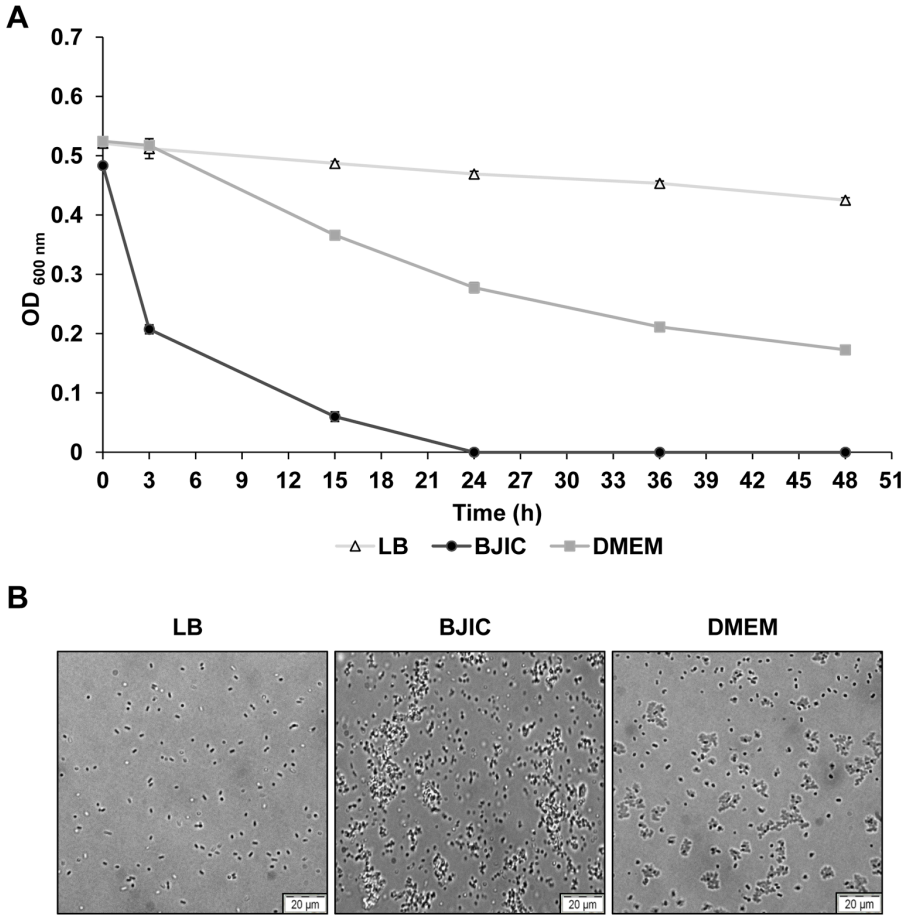

Figure 2. Autoaggregation of *E. coli* O157:H7 EDL933 *stx*$^{-}$. A: The autoaggregation assay was performed at 25°C on bacterial cells grown in DMEM or BJIC using LB as a control. B: The autoaggregation phenotype was visualized by phase-contrast microscopy following sampling at 24 h incubation time. Bacterial cells for the assay were first grown at 39°C.
doi:10.1371/journal.pone.0059386.g002

DNA fragment with promoter and CDS (coding DNA sequence) was restriction digested with SphI/ScaI and cloned into pAT18 [41] and resulted in pSARE-Red1. Transformants with pSARE-Red1 were selected on LB agar supplemented with 300 µg ml^{-1} of erythromycin.

Coating of microtiter plates with ECM proteins

Preparation of 96-wells polystyrene microtitre plates (Falcon) surface-coated with ECM proteins was based on a previously described protocol [42]. The ECM proteins consisted of collagen I (Millipore, 08-115), III (Millipore, CC078) and IV (Sigma, C7521), laminin-α2 (Millipore, CC085), elastin (Sigma, E1625) and insoluble fibronectin (i-fibronectin, Sigma, F2518). BSA (bovine serum albumin, Sigma, A3803) was used as a control for specific adhesion to ECM proteins. Basically, ECM proteins were solubilised in 0.1 M carbonate coating buffer (pH 9.6) and 250 µl was dispensed at a saturating concentration (50 µg ml^{-1}) to the well surface and incubated overnight at 4°C. The wells were washed with PBS (phosphate buffered saline, Sigma) containing 0.05% (v/v) Tween 20 (PBST, pH 7.3) at room temperature (rt) prior to blotting with 250 µl of 1% (w/v) BSA in PBST. After 2 h at 37°C, the wells were washed 3 times with PBST and used for bacterial adhesion or biofilm formation assays.

Bacterial adhesion assay

Precultures were diluted 1:100 and grown as described above. Relevant media were adjusted with NaOH (0.1 M) to reach a pH of 7 at the time of sampling. Sampling was performed during the exponential growth phase at an OD_{600nm} of 0.5, i.e. about 10^8 CFU ml^{-1}. Chloramphenicol was added and mixed gently at a final concentration of 90 µg ml^{-1} to prevent *de novo* protein synthesis and growth during the time of contact of bacterial cells with ECM proteins in the adhesion assay. Vigorous shaking, vortexing and centrifugation were avoided to preserve cell surface supramolecular structures potentially involved in adhesion. *E. coli* O157:H7 cell suspension (200 µl) was deposited in relevant protein-coated wells of the microtitre plate using wide-bore tips. Control wells were filled with sterile nutrient medium. Microtiter plates were incubated statically at relevant temperature for 2 h. After incubation, bacterial suspension was removed by pipetting. Wells were further first washed with TS (tryptone salt) to remove loosely attached cells. Adherent bacteria were fixed with 200 µl absolute ethanol for 20 min. Wells were then emptied by pipetting and dried for 30 min prior to 20 min staining with 200 µl of an aqueous-solution of crystal violet (0.1% w/v). Wells were emptied, washed a second time with TS to remove the excess of unbound crystal violet dye, and dried for 30 min. The bound dye was solubilized from stained cells using 200 µl of an aqueous solution of acetic acid (33% v/v) for 1 min under orbital shaking. Contents

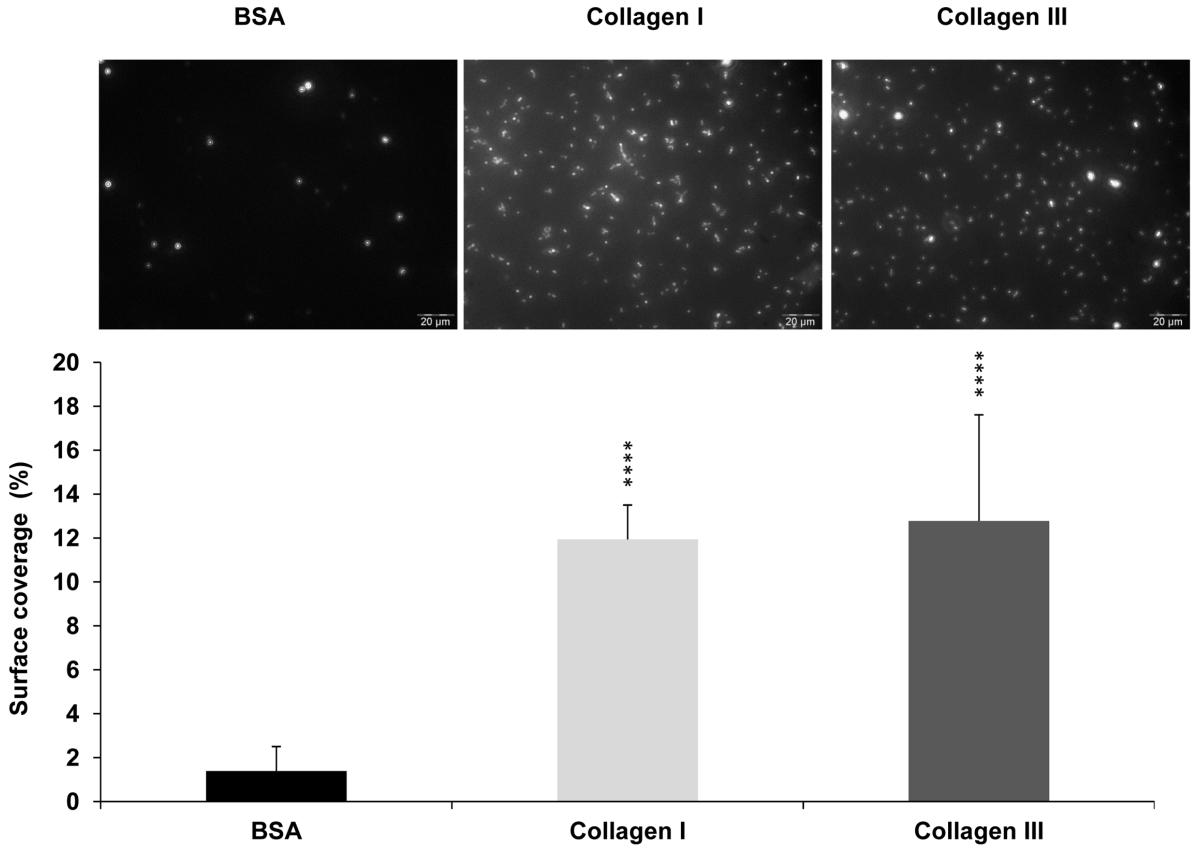

Figure 3. Adhesion to collagens I and III of *E. coli* O157:H7 EDL933 *stx*$^-$ strain grown in LB at 39°C. Epifluorescence microscopy data expressed as the percentage of area covered by fluorescent *E. coli* O157:H7 EDL933 *stx*$^-$ cells grown in LB in wells coated with collagen I or III with BSA used as control.
doi:10.1371/journal.pone.0059386.g003

of each well (150 µl) were transferred to a clean microtiter plate and absorbance was measured at 595 nm using a microtiter plate reader. The readings were corrected by substracting the average absorbance from control wells.

To observe the influence of lower temperatures on initial adhesion of bacteria at pH 7, the microtiter plates were incubated statically at relevant temperature (39, 25, 7 and 4°C) for 2 h. To test the influence of subsequent exposure to lower temperatures upon initial adhesion, bacterial suspension was removed after 2 h of incubation at 25°C. Then, 200 µl of sterile medium was added to each well and were further incubated for 2 h at the relevant temperature.

To investigate the influence of pH, cacodylate buffers (0.5 M final) were used to adjust the pH of the bacterial culture samples at relevant pH (7.0, 6.5, 6.0 and 5.5), resulting in a 1:3 dilution of the bacterial culture sample. After inoculation, the microtiter plates were incubated statically at 25°C for 2 h. To test the influence of subsequent exposure to acidic pH upon initial adhesion, bacterial suspension was removed after 2 h of incubation at 25°C and pH 7. Then, 200 µl of sterile LB, adjusted at the relevant pH with 0.5 M final cacodylate buffers, were added and the microplates were further incubated for 2 h at 25°C.

Autoaggregation assay

Precultures in LB, BJIC or DMEM were diluted 1:100 and grown as described above. Based on a previously described assay [43], the cell suspension was adjusted to the same $OD_{600\ nm}$, chloramphenicol was added at a final concentration of 90 µg ml^{-1} and each culture was placed in conical tubes. No vigorous shaking, pipetting or vortexing was ever applied to preserve cell surface determinants potentially involved in autoaggregation. The tubes were incubated statically and vertically at 25°C. Samples of 500 µl were taken from the top of the tube at different time points to measure the $OD_{600\ nm}$. To confirm the autoaggregation, observations in phase-contrast microscopy were performed after 24 h of incubation.

Biofilm formation assay

This assay was based on a previously described protocol [44]. For the starting inoculum, precultures in LB were diluted 1:100 and 200 µl of bacterial cell suspension were dispatched in protein-coated wells of the microtitre plate and further incubated at 25°C statically. Control wells were filled with sterile nutrient medium. To test the influence of collagens in solution on biofilm formation, collagen I or III was added to the cell suspension prior to dispatching in the wells of uncoated microtitre plates and BSA was used as a control. To follow the bacterial sessile development at various incubation times, wells were subjected to the same sequential procedure described for the bacterial adhesion assay, i.e. washing with TS, fixation with absolute ethanol, air drying, staining with crystal violet solution, washing with TS, air drying, dye recovering in acetic acid solution and reading of the absorbance at 595 nm. To back up the results of the crystal violet assay, fluorescence microscopic observations were performed with the *E. coli* O157:H7 EDL933 *stx*$^-$ pSARE-Red1.

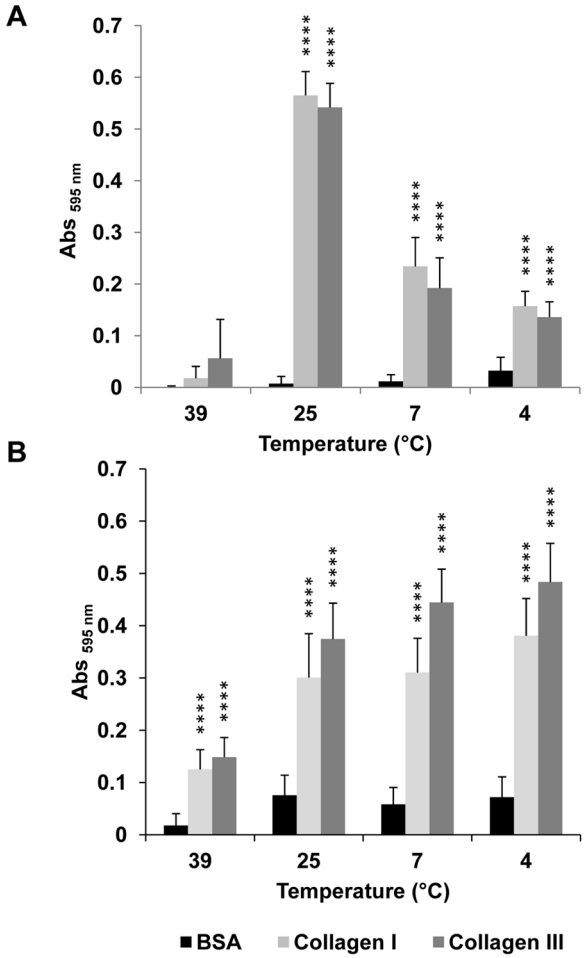

Figure 4. Effect of temperature on adhesion to immobilized collagen I or III of *E. coli* O157:H7 EDL933 *stx*$^-$. A: Bacterial adhesion was tested at decreasing temperatures encountered in meat along the production chain line, i.e. 39, 25, 7 and 4°C. Bacterial cells for the adhesion assay were first grown in LB at 39°C. B: The effect of temperature variation on bacteria initially adhered at 25°.
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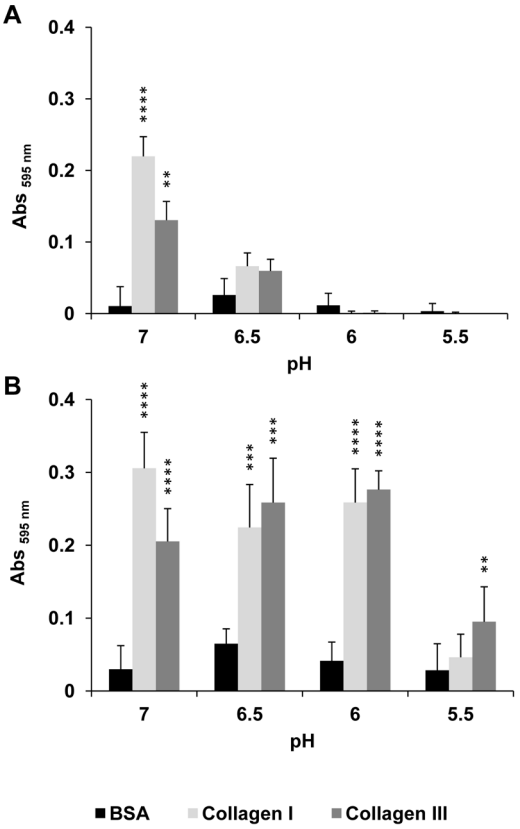

Figure 5. Effect of pH on adhesion to immobilized collagen I or III of *E. coli* O157:H7 EDL933 *stx*$^-$. A: Bacterial adhesion was tested at decreasing pH encountered post-mortem in skeletal muscle using cacodylate buffers to adjust the samples to the different pH. B: The effect of acidic pH on bacteria initially adhered at pH 7 was tested using sterile LB, which pH was adjusted with cacodylate buffers.
doi:10.1371/journal.pone.0059386.g005

Image acquisition and analysis from microscopic observations

Sample preparations were inserted on the stage plate to take one image for each well. This operation was repeated 12 times in order to acquire satisfactory statistical information. Field of view was chosen in the center of the well in order to avoid artifacts such as edge optical aberrations or biased bacterial spatial distribution. Fields of bacteria were imaged using a 60× magnification (40× objective with ×1.5 intermediate magnification).

Observations were performed in phase contrast transmitted light or fluorescence reflected light. The corresponding images were acquired using an inverted phase-contrast microscope (Olympus IMT-2) coupled to a cooled CCD camera (Olympus DP30BW) optimized for high sensitivity fluorescence work and driven by the CelÎA software v3.2 (Olympus France SAS, Rungis, France). Images were acquired with a 40x objective allowing phase contrast microscopy (Olympus LWD-CD-PLAN40FPL, 0.55, 160/1). The fluorescence light source was a mercury short arc lamp (HBO103W/2, OSRAM, Augsburg, Germany). Fluorescence acquisition was fitted with a "Cyanine 3" cube containing 540 nm exciter, 620 nm emitter, and 565 nm dichroic mirror (31002a filter cube, Chroma, Rockingham, VT, USA).

Images were processed with the public-domain image processing and analysis program ImageJ v1.43 (NIH-RSB, Bethesda, MD, USA) (http://rsb.info.nih.gov/nih-image/about.html). The pixels corresponding to bacteria were extracted by thresholding segmentation of the light gray levels. The relative area of bacteria was assessed by quantifying the number of these pixels in regard to the total number of pixels in the field of view.

Statistical analysis

Statistical analysis was performed from Excel using XLSTAT v2009.3.02. Data of assays result from at least five independent experiments, i.e. five biological replicates. On the figures, error bars thus represent the standard deviation from five independent experiments. For each experiment, a value was calculated from the average of repetitions performed in triplicate at fewest. The mean values from the biological replicates were compared to the mean values obtained with BSA used as a control. Data were statistically analyzed following Student's t-test with differences considered significant ($p<0.05$, *), very significant ($p<0.01$, **), highly significant ($p<0.001$, ***) or very highly significant ($p<0.0001$, ****).

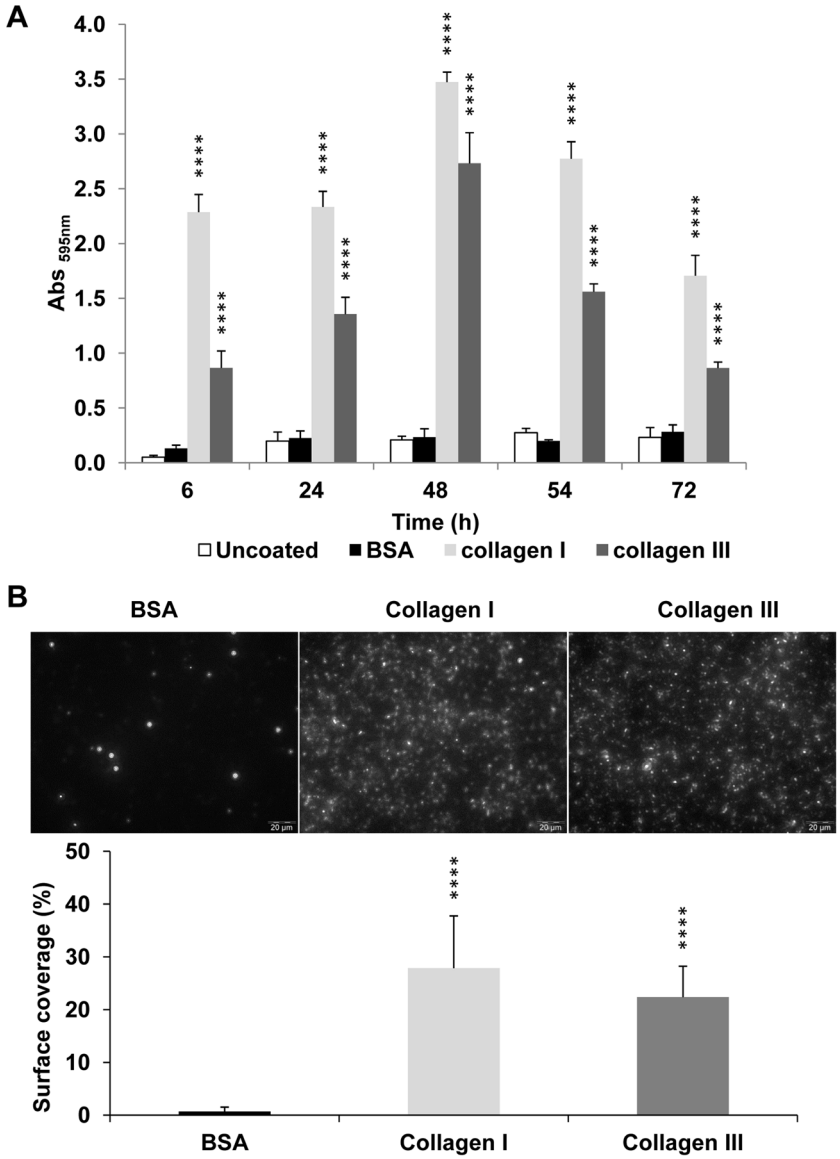

Figure 6. Colonization of immobilized collagen I and III by *E. coli* O157:H7 EDL933 *stx*$^-$. A: Kinetics of biofilm formation was performed in LB at 25°C using BSA as a control and measured by the crystal violet staining method. B: Percentage of surface coverage at 24 h from epifluorescence microscopy data of fluorescent *E. coli* O157:H7 EDL933 *stx*$^-$ pSARE-Red1 growing in LB at 25°C and colonizing surface with immobilized collagen I or III compared to immobilized BSA.
doi:10.1371/journal.pone.0059386.g006

Results

Growth media influence bacterial adhesion to the main muscle ECM components

Different growth media were used to evaluate bacterial adhesion to the predominant fibrous proteins of the muscle ECM. Besides chemically defined or complex basal nutrient media generally used for growth of EHEC strains in laboratory, more specific undefined media close to the conditions encountered in the digestive tract of ruminants, i.e. before faecal contamination of meat products, were also used, namely LH (Leedle Hespell) containing fluid rumen content (22), BJIC (bovine jejunum-ileum content) and BCC (bovine caecum content) [38].

It appeared that as for the control wells coated with BSA, *E. coli* O157:H7 EDL933 *stx*$^-$ grown in the chemically defined media M9 or MinCa could not adhere to the main muscle ECM components (Figure 1). In contrast, bacterial cells grown in BCC, BJIC or DMEM adhered similarly to the different ECM proteins tested as well as to BSA, indicating bacterial adhesion was non-specific. The levels of non-specific bacterial adhesion were especially high with BJIC and DMEM. As revealed by assays and miscroscopy observations (Figure 2), autoaggregation occurred in DMEM and BJIC but not in LB, where specific adhesion to some ECM components could be observed as described here below.

For the remaining media tested, specific adhesion of *E. coli* O157:H7 EDL933 *stx*$^-$ to ECM proteins could be observed (Figure 1). It clearly appeared that adhesion ability greatly depends on the growth media. Specific bacterial adhesion to laminin-α2 from cells grown in TSB, LH, BHI and LB could be observed as well as to elastin in LH or insoluble fibronectin (i-fibronectin) in BHI. Specific bacterial adhesion to both FFC (fibril-forming

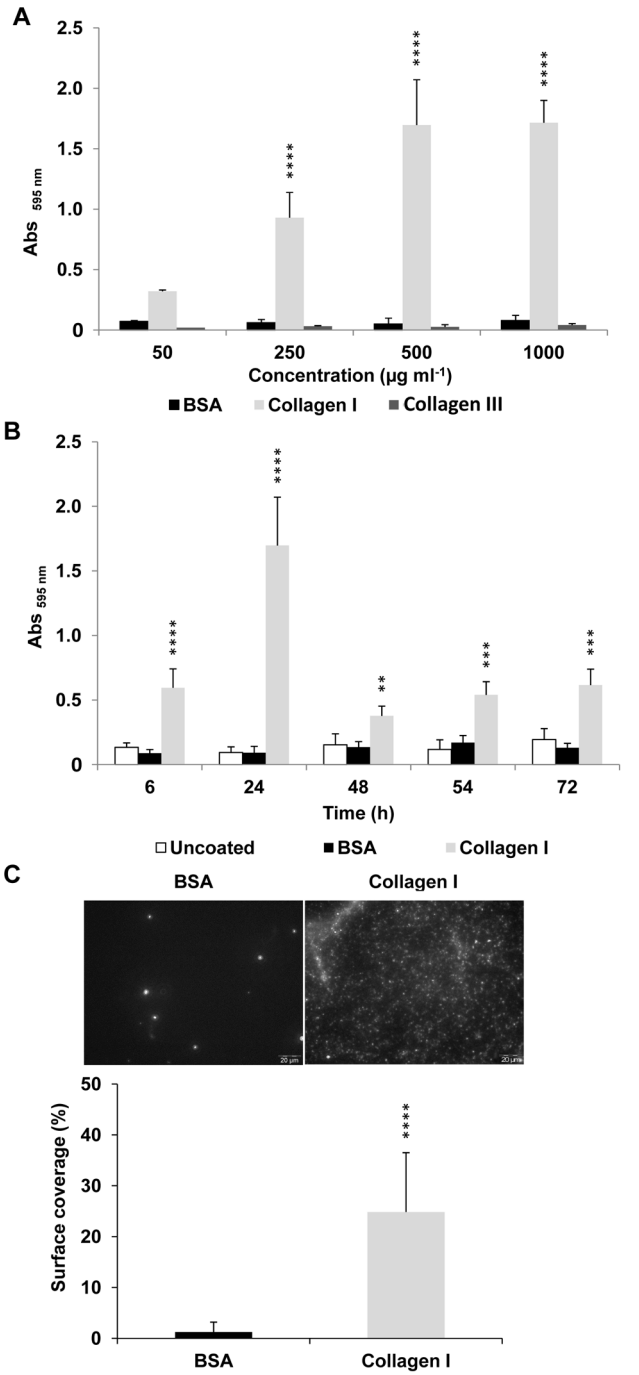

Figure 7. Surface colonization of *E. coli* O157:H7 EDL933 *stx*$^-$ with ECM proteins in solution. A: Surface colonization at 24 h of bacteria growing in LB at 25°C in presence of different concentration of collagen I or III in solution compared to BSA in solution and uncoated wells used as controls. B: Kinetics of biofilm formation in LB at 25°C with collagen I in solution (500 µg ml^{-1}) compared to BSA in solution and uncoated wells used as controls. C: Percentage of surface coverage from epifluorescence microscopy data of fluorescent *E. coli* O157:H7 EDL933 *stx*$^-$ pSARE-Red1 growing in LB at 25°C and colonizing surface with collagen I in solution (500 µg ml^{-1}) compared to BSA (500 µg ml^{-1}) in solution.

doi:10.1371/journal.pone.0059386.g007

collagen), i.e. collagens I and III, and NFC (network forming collagen), i.e. collagen IV, [25] occurred with bacterial cells grown in LH or BHI. The most prominent specific adhesion of *E. coli* O157:H7 EDL933 *stx*$^-$ was observed against fibrillar collagen of type I or III with bacterial cells grown in LB. The similar trend of bacterial adhesion to immobilized collagen I or III versus BSA were further backed up by fluorescent microscopy observations using *E. coli* O157:H7 EDL933 *stx*$^-$ pSARE-Red1 (Figure 3).

Temperature and pH influence bacterial adhesion to collagens I and III

Besides decrease of the meat temperature along the beef production chain (i.e. from 39°C, the temperature of the ruminant, work at room temperature until completion of slaughter, 7°C during transport, storage and/or further cutting, and 4°C during storage immediately after production), the decrease of pH (dropping from neutral to an ultimate value, i.e. pH_u, of about 5.5) is one of the most prominent post-mortem physicochemical modifications occurring in skeletal muscles. Focusing on specific adhesion of *E. coli* O157:H7 EDL933 *stx*$^-$ grown in LB at 39°C to collagen I or III, the influence of the temperature and pH was investigated.

No significant adhesion could be observed at 39°C but maximum specific bacterial adhesion occurred at 25°C. At the lower temperatures 7 or 4°C, specific bacterial adhesion to collagen I or III could still be observed but the amount of adherent biomass was lower (Figure 4A). On bacterial cells initially adhered at 25°C, though, subsequent exposure at 39°C maintained specific adhesion to these two FFCs and the amount of adhering cells to collagen I or III was not significantly affected at the lower temperatures of 7 and 4°C (Figure 4B).

While these previous data were obtained at pH 7, the pH of the samples was adjusted with cacodylate buffers to investigate the influence of lower pH on specific bacterial adhesion at 25°C. It appeared that maximal bacterial adhesion occurred at pH 7 and chiefly decreased at lower pH where no significant specific bacterial adhesion could be observed (Figure 5A). On bacterial cells initially adhered at pH 7, though, subsequent exposure at lower pH did not significantly affect the amount of adhering cells to collagen I or III, except when exposed at pH 5.5 (Figure 5B). These results demonstrate the importance of temperature and pH conditions at the time of initial adhesion of *E. coli* O157:H7 EDL933 *stx*$^-$ as well as pH_u to limit bacterial adhesion.

Collagens I and III promote biofilm formation

In addition to bacterial adhesion, the ability of *E. coli* O157:H7 EDL933 *stx*$^-$ to colonize immobilized collagen I or III was investigated. The presence of these coated ECM proteins chiefly increased the amount of sessile biomass over time when compared to uncoated surface or coated with BSA, especially immobilized collagen I (Figure 6A). In the course of sessile development on coated collagen, the sessile biomass increased up to 48 h of incubation before decreasing slowly as result of cell detachment upon washing following the crystal violet procedure. Observations in epifluorescence microscopy confirmed the increase of biofilm formation in the presence of immobilized collagen I or III (Figure 6B). There were very highly significant differences in biofilm formation on biotic surfaces made of immobilized BSA versus collagen I or III. The biofilm formation was clearly induced with coated collagen I or III.

To investigate the effect of ECM proteins in solution on bacterial surface colonization, different concentrations of collagen I or III were tested (Figure 7A). After 24 h growth, surface colonization was stimulated in a dose-dependent manner till

500 μg ml^{-1} of collagen I added to the culture medium but not by solubilized collagen III, which could not promote biofilm formation in this condition (Figure 7A). Following sessile development in the presence of 500 μg ml^{-1} of collagen I supplemented in the growth medium, the sessile biomass increased within the first 24 hours and decreased and stabilized from and after 48 h of incubation (Figure 7B). This decrease resulted from less adhering biomass. In the absence of collagen or BSA in solution, the amount of sessile biomass remained low and not significantly different from sessile development with coated BSA (Figures 7B and 6). Observations by epifluorescence microscopy confirmed that *E. coli* O157:H7 EDL933 *stx*$^{-}$ grown in LB supplemented with collagen I in solution, almost completely covered the surface but not with BSA in solution (Figure 7C).

Discussion

When initial faecal contamination of beef carcasses occurs at dehiding stage, bacterial adhesion can occur on exposed skeletal muscles. The ECM in skeletal muscle is structured into the epimysium on the most outer layer, then the perimysium around the muscle fascicle and finally the endomysium around the single muscle cells, i.e. the muscle fibers [45]. In close proximity of the muscle fibers, the ECM forms the basal lamina (BL), of which the main fibrous ECM proteins are laminin-α2 (laminin-211 and -221, formerly called laminin-2 and -4, or M- and S-merosin, respectively) and network-forming collagen (NFC) of type IV, whereas the interstitial matrix(IM) is essentially composed of fibril-forming collagens (FFCs) of Type I and III as well as elastin [46,47]. Insoluble-fibronectin (i-fibronectin) is present in these two forms of ECM (i.e. IM and BL). This knowledge of ECM organization at supramolecular, cellular, tissue and organ levels was here carefully considered for investigating adhesion of EHEC to the ECM [25]. In some previous reports on adhesion of EHEC to ECM muscle proteins, collagen III was overlooked and soluble fibronectin (s-fibronectin) or laminin-α1 (laminin-111, formerly called laminin-1) [26,27,31] were used although not relevant to skeletal muscle tissue.

This investigation pinpoints a crucial aspect quite overlooked in the literature [31,48], that is the great influence of growth media on subsequent EHEC adhesion to ECM proteins. This point, however, could be expected from the numerous investigations reporting differential expression of virulence factors, including adhesins, depending on environmental conditions [30,49,50,51,52,53,54,55,56,57,58,59]. Besides the usual laboratory nutrient growth media, more specific undefined media from intestinal contents of different parts of bovine gastro-intestinal tract (rumen, jejunum-ileum, caecum) were used for the first time to investigate bacterial adhesion in an attempt to mimic physiological conditions of the bovine intestine. In BJIC, non-specific bacterial adhesion to ECM proteins was observed and related with cell autoaggregation (similar result was observed for the chemically defined medium DMEM). This phenomenon could hinder specific bacterial adhesion to ECM proteins by some MSCRAMM (microbial surface components recognizing adhesive matrix molecules) proteins. In EHEC O157:H7 EDL933, at least one surface molecular determinant has been reported as potentially involved in such a phenotype. Indeed, its genome encodes an autotransporter characterized as a calcium-binding protein involved in autoaggregation and biofilm formation, namely Cah (calcium-binding antigen-43 homologue) [60,61,62]. Transcriptional gene reporter assays following fusion with the promoter of the *cah* gene indicated high expression level in DMEM over LB. However, genetic/protein expression and functional implication of *cah* in the autoaggregation phenotype has never been ascertained in EHEC O157:H7 EDL933, which would require further investigations in the light of our present findings.

While no specific ECM adhesion could be observed from bacterial cells in BCC, specific adhesion to laminin-α2, elastin, collagen I, IV and III occurred when grown in LH, a medium containing fluid ruminal content. This is especially relevant since this medium mimics an environment encountered by bacteria before shedding and subsequent faecal contamination of carcasses [63,64]. Among the lab growth media, the foremost specific ECM-protein adhesion was here observed with bacterial cells grown in LB and against collagen I or III. This result indicates that specific molecular factor(s) for adhesion to the main muscle FFCs are expressed in these experimental conditions. In all tested conditions, specific adhesion to the main fibrous proteins found in the basal lamina of skeletal muscle tissue, i.e. NFC IV and laminin-α2, is much weaker. Altogether, this finding somehow contrasts with previous reports in the literature where no adhesion to collagen I but adhesion to collagen IV and laminin-α1 was reported for *E. coli* O157:H7 EDL933 [31,48]. Besides environmental conditions (growth medium, temperature, pH or type of ECM proteins) [65,66], it should be stressed that, as detailed in the Material & Methods sections, great care was here taken for handling the bacterial samples and preserving as much as possible cell-surface molecular determinants potentially involved in adhesion to ECM proteins. This concern is not trivial and should be thoughtfully considered in future studies of specific bacterial adhesion to ECM proteins. In addition, our study provides strong basements for further investigation of expression and functional analysis of MSCRAMM proteins involved in adhesion of EHEC O157:H7 EDL933 to ECM [25].

Surprisingly enough, bacterial adhesion to immobilized ECM components has been observed only once in EHEC O157:H7 [67]. However, they were not all relevant to skeletal muscle tissue. Indeed, only soluble fibronectin (and different proteolytic fragments), laminin-α1 and collagen IV were considered. Two major types of molecular mechanisms could explain bacterial adhesion to ECM components [13], i) outer membrane proteins, namely autotransporters (e.g. EhaA, EhaB, EhaG), and ii) cell-surface appendages, especially pili (e.g. HCP, Lpf). Upon cloning and expression of the individual autotransporters in *E. coli* K12 derivatives, specific adhesion to different ECM components was demonstrated [48,68,69,70]. However, their implication has never been ascertained in an EHEC background because no adhesion was observed [48]. Using purified proteins, the affinity of pili for ECM components was investigated by plasmon resonance biosensor, flow cytometry or ELISA-based binding assay [26,67,71]. Of note, the binding of ECM components to EHEC was investigated using free forms and not immobilized forms of the ECM components, which is far different from a bacterial adhesion assay. Importantly, the different autotransporters and pili can each binds several different ECM components. In other words, adhesion of bacterial cells to ECM components is highly complex because it is multi-factorial and compensatory. While the use of LH, BJIC and BCC is highly biologically relevant, those media are also valued because of the difficulty to obtain the different contents of a bovine digestive tract. In the present investigation, the choice was then to use a common laboratory medium showing some similarities in the trend of bacterial adhesion to ECM components in LH. We also decided to focus on the most prominent ECM components enabling high specific bacterial adhesion, namely collagen I and III. Undoubtedly, much further in-depth investigations are necessary to elucidate the gene regulations and other molecular mechanisms responsible for the different patterns of

bacterial adhesion to the different ECM components as observed in the different growth media.

Besides growth media, bacterial adhesion capacity is greatly influenced by the temperature and pH. While this suggests the affinity of the MSCRAMM protein(s) towards ECM protein is modified by such physicochemical parameters, it also provides some new research directions for the development of innovative practices and/or prevention strategies at critical points of the slaughter of animals in order to minimize the risk of contamination by EHEC O157:H7. Indeed, it pinpoints that maximal bacterial adhesion to the main FFCs present in skeletal muscle, which are present on surface of carcasses, occurs at a standard room temperature of 25°C, which can be attained in meat during the time of slaughter and initial cutting into quarters. In addition, maximum specific adhesion to collagen I or III occurred at pH 7, which suggests the critical point for contamination occurs at the beginning of slaughter. Moreover, once bacteria adhered, it appeared that further modifications of temperature or pH had few effects on specific adhesion of EHEC to collagen I or III, except at pH_u.

In addition to specific adhesion, the presence of immobilized FFCs could promote the formation of dense biofilm. The induction of a dense biofilm was also observed in the presence of collagen I in solution but not with collagen III. It may be that collagen I in solution promotes aggregation, which in turn leads to flocculation and formation of a biofilm. While both collagens I and III are FFCs with the same supramolecular structure, they present differences in their composing subunits [25,72]. In *Streptococcus suis*, the influence of soluble versus immobilized fibrinogen was similarly tested and reported the induction of biofilm formation only in the presence of the soluble fibrinogen [73]; Still, the molecular basis for this difference in bacteria – protein interactions depending on the solubilized or immobilized form of the ECM proteins remain to be elucidated [74,75]. For EHEC, this constitutes a critical point for the control of bacterial contamination in the food plant environment considering residual collagen could be found solubilized in some fluids (meat juice, blood) resulting from meat processing (*e.g.* cutting, grounding) and/or immobilized (following soiling for instance) on sterilized workbenches or utensils, such as butcher knives.

Bearing in mind the main fibrous ECM proteins, especially collagen, are quite ubiquitous in animal tissues, bacterial cell attachment is certainly an adaptive strategy the bacterial species have evolved with in the course of interactions with eukaryotic cell [25,76]. While this study focused on bacteria-protein interactions, our future investigations on EHEC adhesion/colonization of muscle fibers and meat will complete information regarding the interactions at molecular, cellular, ultrastructural and organ levels. A better understanding of the molecular and cellular mechanisms involved in EHEC adhesion to meat is a prerequisite to limit the risk of food poisoning by EHEC, whose outcomes can be serious complications or even life-threatening pathologies especially for young children [7]. Eventually, results of this research could lead to more effective and innovative prevention strategies in the meat industry to further limit or avoid carcass contamination by bacterial pathogens. They also provide further insight in the physiopathology of EHEC with an integrated view from the reservoir, the contaminated food products to the subsequent ingestion resulting in host infection.

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Author Contributions

Conceived and designed the experiments: CC MD. Performed the experiments: CC AA SR FP. Analyzed the data: CC RT TA MD. Contributed reagents/materials/analysis tools: CC AA SR FP MD. Wrote the paper: CC RT TA MD.

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ARTICLE n°2

Caroline CHAGNOT, Nelly CACCIA, Estelle LOUKIADIS, Régine TALON, Thierry ASTRUC and Mickaël DESVAUX

Colonization of the muscle extracellular matrix proteins by O157:H7 and non-O157 enterohemorrhagic *Escherichia coli*

Pour publication dans Food Microbiology

Colonization of the muscle extracellular matrix proteins by O157:H7 and non-O157 enterohemorrhagic *Escherichia coli*

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ABSTRACT

Enterohemorrhagic *Escherichia coli* (EHEC) are anthropozoonotic agents that range third among food-borne pathogens respective to their incidence and dangerousness in the European Union. EHEC are Shiga-toxin producing *E. coli* (STEC) responsible for foodborne poisoning mainly incriminated to the consumption of contaminated beef meat. Among the hundreds of STEC serotypes identified, EHEC mainly belong to O157:H7 but non-O157 can represent 20 to 70 % of EHEC infections per year. Seven of those serotypes are especially of high-risk for human health, i.e. O26, O45, O103, O111, O121, O145 and O104. While meat can be contaminated all along the food processing chain, EHEC contamination essentially occurs at the dehiding stage of slaughtering. Investigating bacterial colonization to the skeletal-muscle extracellular matrix (ECM) proteins, revealed that environmental factors influenced specific and non-specific bacterial adhesion of O157 and non-O157 EHEC as well as biofilm formation. Importantly, mechanical treatment (i.e. shaking, centrifugation, pipetting and vortexing) inhibited and biased the results of bacterial adhesion assay. Besides stressing the importance of the protocol to investigate bacterial adhesion to ECM proteins, this study demonstrated that colonization abilities to ECM proteins varies among EHEC serotypes and could ultimately be taken into consideration to evaluate the risk of contamination for different types of food matrices.

Keywords: Enterohemorrhagic *Escherichia coli*, Shiga-toxin producing *E. coli*, Bacterial adhesion, Biofilm formation, Extracellular matrix protein, Meat contamination

INTRODUCTION

The enterohemorrhagic *Escherichia coli* (EHEC) are anthropozoonotic agents responsible for foodborne poisoning incriminating contaminated animal food products, vegetables and watery drinks (Bavaro, 2012). EHEC are one of the six pathotypes of intestinal pathogenic *E. coli* (InPEC) (Kaper *et al.*, 2004, Nataro *et al.*, 1998). Worldwide, EHEC infection is predominantly a pediatric disease frequently implicated in severe clinical illness, which is characterized by bloody diarrhea and, in most acute forms, can degenerate into hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP) (Tarr, 2009, Karch *et al.*, 2005). While the involvement of contaminated fruits and vegetables is increasing, EHEC infections are most often linked to the consumption of beef meat and are predominantly attributed to the *E. coli* O157:H7 (Berger et al., 2010). *E. coli* O157:H7 is only one of the over 300 distinct serotypes for Shiga toxin-producing *Escherichia coli* (STEC) isolated so far (Karmali et al., 2010), *i.e.* the previously called Verotoxin-producing *E. coli* (VTEC) (Griffin *et al.*, 1991, Schmidt *et al.*, 1996, Paton *et al.*, 1998). However, only a very limited number of serotypes appear to be associated with the majority of human diseases; all EHEC are pathogenic STEC but all STEC are not systematically InPEC (Naylor *et al.*, 2005, Karmali *et al.*, 2003). Respective to their incidence, EHEC range third in the European Union (EU) among food-borne pathogens after *Campylobacter* and *Salmonella* (EFSA *et al.*, 2013), in term of dangerousness, however, it is the worst of the three. While these serotypes vary in frequency with the country and year, non-O157 represents between 20 to 70 % of overall EHEC infections. The latest north American and EU reports, from the USDA (United States Department of Agriculture) and EFSA (European Food Saftey Authority) respectively, indicated the serotypes O26, O45, O103, O111, O121 and O145 are by far the most commonly isolated, the so-called "big six" (USDA, 2012, EFSA *et al.*, 2013). Since 2012, the USA (Unites States of America) legislation incorporates those non-O157 serotypes, which are considered as high-risk for human health and requiring high vigilance for food safety. In EU, the serotype O104 is watched closely since the last major outbreaks in 2011 (EFSA *et al.*, 2013).

In any cases, EHEC/STEC contamination of food products originates from faecal contamination, the ruminants being the main natural reservoir (Nguyen *et al.*, 2012, Chase-Topping *et al.*, 2008b). Normally, the respect of good hygienic slaughtering practices reduces the risk of contamination of carcasses but cannot guarantee the absence of EHEC/STEC from meat (Buncic *et al.*, 2013, Rhoades *et al.*, 2009). It has been shown that *E. coli* O157:H7 could adhere to the muscle tissue,

most certainly to the extracellular matrix (ECM) (Chen *et al.*, 2007, Cabedo *et al.*, 1997, Rivas *et al.*, 2006b, Li *et al.*, 1999). While binding to collagen I and laminin of the cell surface of *E. coli* O157:H7 was evidenced (Medina, 2001, Medina, 2002, Medina *et al.*, 1998), a later study could not report any significant attachment to any of the tested immobilized ECM proteins (Zulfakar et al., 2012). Using a non-toxigenic *E. coli* O157:H7 EDL933 Δ*stx* strain, i.e. *E. coli* O157:H7 CM-454 (Gobert et al., 2007), bacterial adhesion to muscle ECM proteins was re-investigated (Chagnot *et al.*, 2013a) by taking a great care to the types of ECM proteins to ensure the highest relevance to skeletal muscle tissue (Chagnot et al., 2012). It was evidenced that environmental factors such as the growth medium, temperature and pH had a strong influence on bacterial adhesion. Non-specific adhesion occurred when bacteria were grown in bovine small intestine content, with similar trend in DMEM (Dulbecco's Modified Eagle Medium), whereas specific adhesion to some ECM fibrillar proteins occurred when bacteria were grown in ruminal content, as well as in LB (lysogenic broth) where maximal specific adhesion to collagen I and III occurred at pH 7 and 25°C (Chagnot *et al.*, 2013a). Besides the use of different environmental conditions, it was hypothesized that the discrepancies with previous investigations for the attachment of the *E. coli* O157:H7 to ECM proteins (Zulfakar et al., 2013) could be linked to bacterial-surface protein determinants potentially involved in adhesion to ECM that were damaged by mechanical shaking-centrifugation-vortexing treatment. Altogether, this prompted us to test this hypothesis using *E. coli* O157:H7 wild type (Chagnot *et al.*, 2013a) as well as studying the specific and non-specific adhesion of the major non-O157 EHEC serotypes to some ECM proteins in relevant environmental conditions.

MATERIAL AND METHODS

Bacterial strains and culture conditions

EHEC strains investigated in this study are listed in Table 1. One strain of each of the major O157 and non-O157 serotypes recognized at risk by the USDA and EFSA was considered. Bacterial strains were cultured in different relevant nutrient media as previously determined (Chagnot *et al.*, 2013a), that is either chemically defined, *i.e.* DMEM (Dulbecco's modified eagle medium, Gibco) or complex undefined, i.e. LB (lysogeny broth) (Bertani, 2004). From -80°C stock culture previously grown in the respective medium, strains were plated on the relevant agar medium and incubated overnight at 39°C (bovine temperature) (Bertin et al., 2011). A preculture was set up from one bacterial colony grown in the respective nutrient broth medium at 39°C in an orbital shaker at low speed (70 rpm) till stationary phase.

Coating of microtiter plates with ECM proteins

96-wells polystyrene microtitre plates (Falcon) were surface-coated with ECM proteins as previously described (Chagnot *et al.*, 2013a). The ECM proteins consisted of collagen I (Millipore, 08-115), collagen III (Millipore, CC078) and a reconstituted ECM, i.e. MaxGel ECM (Sigma, E0282), including collagens, laminin, fibronectin, and elastin. BSA (bovine serum albumin (Sigma, A3803) was used as a control for specific adhesion to muscle ECM proteins. Basically, ECM proteins were solubilised in 0.1 M carbonate coating buffer (pH 9.6), 250 µl were dispatched at a saturating concentration of well surface (50 µg ml^{-1}) and incubated overnight at 4°C. The wells were washed 3 times with Tryptone Salt (TS) at room temperature for bacterial adhesion or biofilm formation assays.

Bacterial adhesion assay

Preculture was diluted 1:100 and grown as described above (39°C and low orbital shaking). The two media LB and DMEM were adjusted with NaOH (0.1 M) to reach a pH of 7 at the time of sampling, i.e. during the exponential growth phase at an OD_{600nm} of 0.5 (about 10^8 CFU ml^{-1}). Chloramphenicol was added and mixed gently at a final concentration of 90 µg ml^{-1} to prevent *de novo* protein synthesis and stop further bacterial growth. As previously mentioned (Chagnot *et al.*, 2013a) and to preserve cell surface supramolecular structures, no vigorous shaking, vortexing or centrifugation was ever applied. Cut tips were used to deposite cell suspension (200 µl) in relevant

protein-coated wells of the microtitre plates. As a control, some wells were filled with sterile growth medium. Microtiter plates were incubated statically at 25°C for 3 h. After incubation, bacterial suspension was removed by pipetting. To remove loosely attached cells, wells were washed with TS. Adherent bacteria were fixed with 200 µl absolute ethanol during 20 min. Wells were then emptied by pipetting and dried for 30 min prior to staining with 200 µl of an aqueous-solution of crystal violet (0.1 % w/v) during 15 min. Then, wells were washed a second time with TS to remove the excess of unbound crystal violet dye, and dried for 30 min. For 1 min, under orbital shaking, the bound dye was solubilized from stained cells using 200 µl of an aqueous solution of acetic acid (33 % v/v). Finally, contents of each well (150 µl) were transferred to a clean microtiter plate and a microtiter plate reader was used to measure absorbance at 595 nm. The readings were corrected by substracting the average absorbance from control wells.

To investigate the effect of mechanical treatment on bacterial adhesion, the alternative protocol described above with minimal mechanical treatment (MMT) was compared with the protocol usually described in the literature, which involved shaking-centrifugation-vortexing treatment (SCVT). The bacterial cells were grown under vigorous orbital shaking (150 rpm). Prior to the deposition of the bacterial suspension in the microtiter plate, the cells were centrifugated (3210 *g*, 5 min, 4°C) and washed twice in sterile growth medium. Cells were resuspended homogeneously by pipeting (using uncut tips) and vortexing. After the last resuspension, the cells were deposited in the wells of the microtiter plates (using uncut tips). The following steps for adhesion, washing, staining and absorbance reading were similar to those described above.

Biofilm formation assay

This assay was based on a previously described protocol (Chagnot *et al.*, 2013a). Preculture was diluted 1:100, 200 µl of bacterial cell suspension were dispatched in protein-coated wells of the microtitre plate and further incubated at 25°C statically. Wells filled with sterile growth medium were used as control. To estimate the attached biomass after 24 h of sessile development, wells were subjected to the same sequential procedure as described for the bacterial adhesion assay, namely, washing with TS, fixation with absolute ethanol, air drying, staining with an aqueous crystal violet solution, washing with TS, air drying, dye recovery with an aqueous acetic acid solution and reading of the absorbance at 595 nm.

Statistical analysis

Statistical analysis was performed from Excel using XLSTAT v2009.3.02. Results are presented as mean ± standard deviation (SD) and correspond to the mean values of at least three independent experiments, *i.e.* 3 biological replicates. For each independent experiment, the values result from the average of repetitions performed in triplicate at fewest. The mean values from the biological replicates were compared to the mean values obtained with BSA used as a control of specificity. Data were statistically analyzed following Student's t-test with differences considered significant ($p<0.05$, *), very significant ($p<0.01$, **), highly significant ($p<0.001$, ***) or very highly significant ($p<0.0001$, ****).

RESULTS

Mechanical treatment greatly impacts bacterial adhesion ability of *E. coli* O157:H7 EDL933 to immobilized ECM proteins

To test the hypothesis that SCVT (shaking-centrifugation-vortexing treatment) could affect the results of bacterial adhesion to ECM proteins, it was compared with the MMT (minimal mechanical treatment) protocol previously described (Chagnot *et al.*, 2013a). Using the reference strain *E. coli* O157:H7 EDL933, two conditions were tested, *i.e.* using bacterial cells grown (i) in LB, which induce bacterial specific adhesion to collagen I and III, and (ii) in DMEM inducing non-specific adhesion to ECM proteins. Specific bacterial adhesion of *E. coli* O157:H7 EDL933 to the tested collagens occurred when MMT was applied (Figure 1A). However, the specific bacterial adhesion was very highly significantly decreased upon SCVT. With DMEM, bacterial cells adhered similarly to collagens I and III and to BSA, indicating bacterial adhesion was non-specific (Figure 1B). Again, bacterial adhesion was very much lower upon SCVT than with the MMT protocol. This study clearly evidenced that mechanical treatment can influence and biase the results of bacterial adhesion assay when investigating the interaction of *E. coli* O157:H7 EDL933 with ECM proteins.

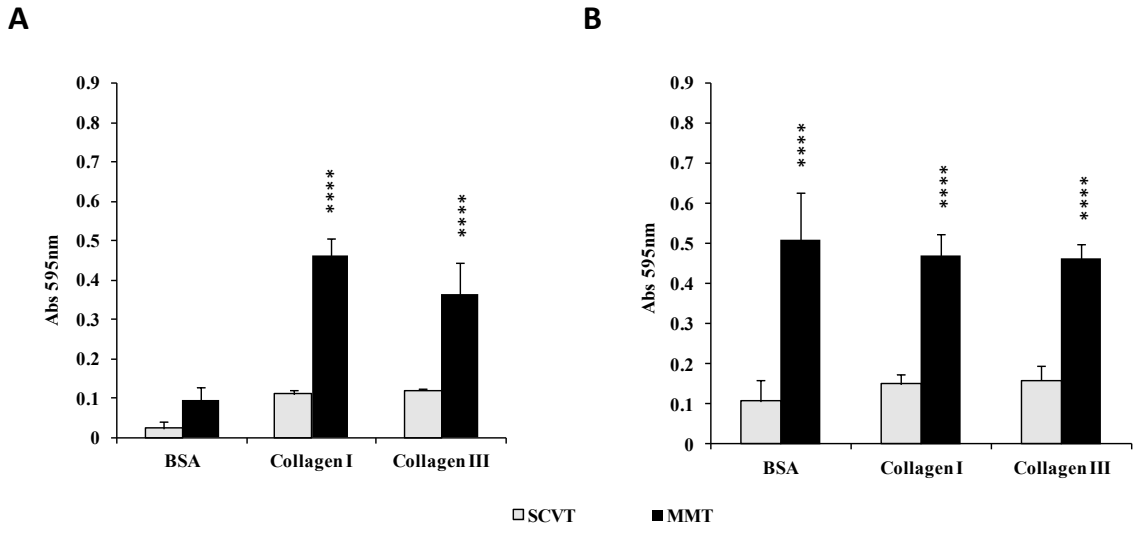

Figure 1: Effect of mechanical treatment on the adhesion of *E. coli* O157:H7 EDL933 *wt* to immobilized ECM proteins. Specific and non-specific bacterial adhesion to relevant collagens I and III were tested in LB (A) and in DMEM (B) respectively to compare the effect of SCVT (shaking-vortexing-centrifugation treatment) and MMT (minimal mechanical treatment) protocols. BSA was used a negative control for specific adhesion to ECM proteins. Bacterial adhesion assay was performed using the crystal violet staining method.

Bacterial adhesion of the major non-O157 EHEC to immobilized ECM proteins.

The attachment ability of the 7 major non-O157 EHEC serotypes (Table 1) was tested using previously reported conditions inducing the most exacerbates specific and non-specific adhesion of *E. coli* O157:H7 EDL933 against collagen I and III or reconstituted ECM respectively (Chagnot *et al.*, 2013a), *i.e.* at 25°C pH 7 using LB or DMEM as growth medium and applying the MMT (minimal mechanical treatment) protocol.

While *E. coli* O157:H7 EDL933 exhibited specific adhesion ability towards collagen I or III in LB, EHEC O26:H11 ED180 could not adhere to those ECM proteins in tested conditions (Figure 2A). In contrast, EHEC O45:H2 12047, O103:H2 CH087 and O111:H2 ED191 and O104:H4 CB13348 adhered similarly to collagens I and III as well as to BSA, indicating bacterial adhesion was non-specific. However, the levels of non-specific bacterial adhesion for EHEC O103:H2 CH087 and O111:H2 ED191 were much lower than those observed for O45:H2 12047 and O104:H4 CB13348. Besides *E. coli* O157:H7 EDL933, it appears that EHEC O145:H28 PH27 adhered specifically to both collagens I and III whereas EHEC O121:H19 32316 adhered specifically to collagen I only.

Bacterial adhesion was further tested in DMEM (Figure 2B). It first appeared that O103:H2 CH087 and O121:H19 32316 did not adhere in the tested conditions. As for *E. coli* O157:H7 EDL933, all the remaining non-O157 EHEC tested strains adhered similarly to a reconstituted ECM and BSA, indicating non-specific bacterial adhesion occurred. However, the levels of non-specific bacterial adhesion were higher for EHEC O45:H2 12047 and especially for O104:H4 CB13348. In the conditions tested and together with EHEC O111:H2 ED191, those strains exhibited non-specific adhesion ability both in LB and DMEM (Figure 2).

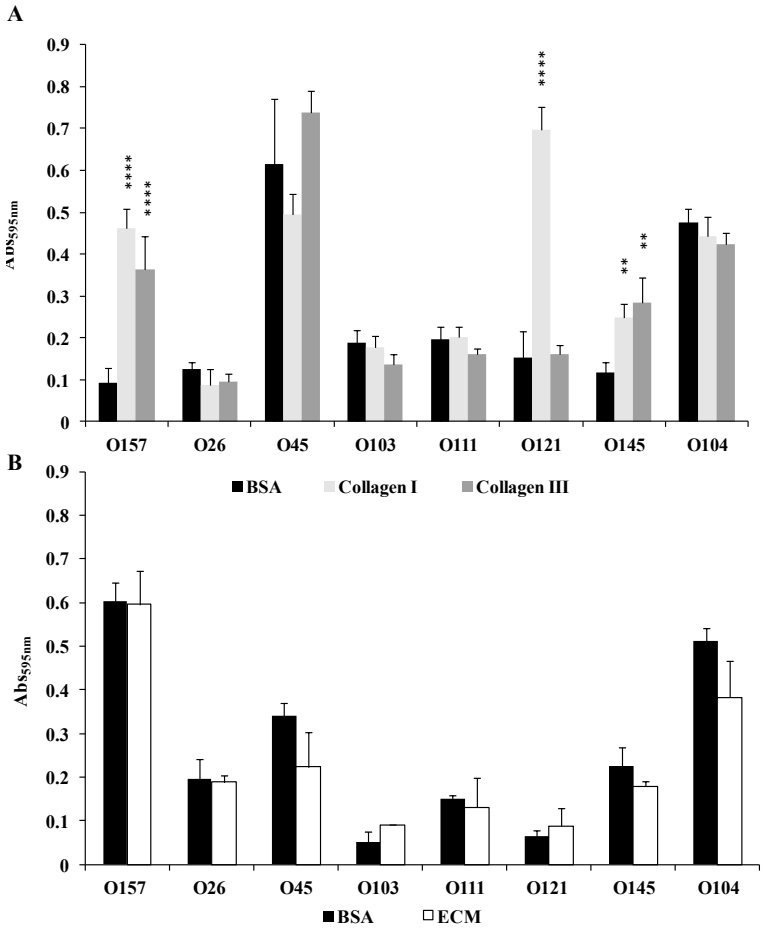

Figure 2: Adhesion of the major non-O157 EHEC to immobilized ECM proteins. Specific and non-specific bacterial adhesion to collagens I and III or a reconstituted ECM were tested in LB (A) and in DMEM (B) respectively applying the MMT (minimal mechanical treatment) protocol. BSA was used a negative control for specific adhesion to ECM proteins. Bacterial adhesion assay was performed using the crystal violet staining method. Tested bacterial strains are listed in Table 1.

Biofilm formation of the major non-O157 EHEC on immobilized ECM proteins.

Besides bacterial adhesion, biofilm formation of the 7 major non-O157 EHEC serotypes (Table 1) was investigated at 24 h using previously reported conditions promoting sessile development of *E. coli* O157:H7 EDL933 (Chagnot *et al.*, 2013a), *i.e.* at 25°C using LB with immobilized collagen I and III or DMEM with reconstituted ECM.

The presence of immobilized collagen I or III chiefly increased the amount of EHEC O157:H7 EDL933 sessile biomass at 24 h in LB compared to a surface coated with BSA (Figure 3A), EHEC O145:H28 PH27 formed biofilm specifically on collagen I or III. whereas O111:H2 ED191 and O104:H4 CB13348 formed biofilm similarly on collagen I or III as well as BSA, indicating sessile development was not specific to ECM proteins. As expected, O45: H2 12047 and O103:H2 CH087 could colonized specifically collagens I and III and O121:H19 32316 formed non-specific biofilm

from previous adhesion results. However, surprisingly unexpected from previous adhesion results, O26: H11 ED180 formed specific biofilm to ECM proteins tested.

Investigating biofilm formation in DMEM (Figure 3B), EHEC O45:H2 12047 and O104:H4 CB13348 formed a biofilm as well as EHEC O157:H7 EDL933 but with lower level of biomass adhered. The biofilm was formed at 24 h similarly on BSA and reconstituted ECM indicating that colonization was not specific. Unexpectedly and while they could adhere non-specifically to reconstituted ECM and BSA, the remaining non-O157 EHEC tested strains did not form a biofilm in the tested conditions.

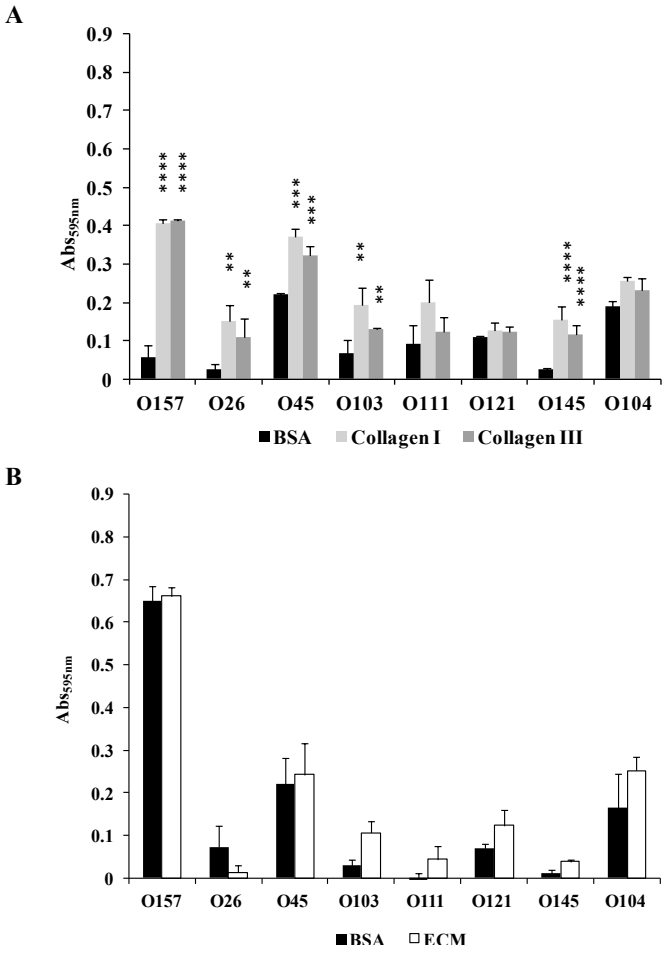

Figure 3: **Colonization of immobilized ECM proteins by *E. coli* O157:H7 EDL933 and the major non-O157 EHEC.** Biofilm formation on immobilized collagen I or a reconstituted ECM was performed in LB (A) and in DMEM (B) respectively. BSA was used a negative control for specific adhesion to ECM proteins. The development of the sessile biomass was assayed at 24h using the crystal violet staining method. Tested bacterial strains are listed in Table 1.

DISCUSSION

Worldwide, many foodborne outbreaks linked to the consumption of meat product contaminated by EHEC strains are regularly identified. EHEC infection principally affects young children where it generally leads to very serious complications, even death. Despite the existence of strict hygiene rules and efficient techniques for EHEC detection, contaminated food is regularly found. The serotype the most commonly isolated from human infection is O157, however, non-O157 EHEC strains represent also a major risk to human health and include O26, O45, O103, O111, O121 and O145 serotypes as well as O104, a serotype responsible for the main EHEC outbreak in 2011 (EFSA et al. 2013 USDA, 2012). Primary bacterial contamination occurred on slaughtering, especially when bacteria can be transferred from the hide to the carcass (Rhoades *et al.*, 2009). Due to the large number of human cases and the mortality rate in the consumption of meat contaminated by EHEC, understanding bacterial molecular attachment properties of the major serotypes to specific structures of meat is a prerequisite to improve safety.

As previously observed with a non-toxigenic isogenic mutant strain (Chagnot *et al.*, 2013a, Gobert *et al.*, 2007), *E. coli* O157:H7 EDL933 adhered and formed biofilm specifically to collagens I and III in LB but non-specifically to ECM proteins in DMEM. While validating the use of *E. coli* O157:H7 Δ*stx* for such investigation, it further evidenced the importance of handling the bacterial cells for bacterial adhesion assay. Shaking, centrifugation as well as washing procedures, involving repeated pipetting and vortexing to resuspend bacterial cells, were early suggested to damage cell-surface molecular determinants potentially involved in bacterial adhesion abilities (An *et al.*, 1997, Fletcher *et al.*, 1973). Although, it could hinder the true ability of the bacterial cells to adhere to ECM proteins, this crucial aspect has not been taken seriously into consideration and was actually completely overlooked until very recently (Dourou *et al.*, 2011, Zulfakar *et al.*, 2012). Here, the importance of applying the minimal mechanical treatment protocol was undoubtedly evidenced, indicating it must be carefully and thoughtfully considered by future investigations for rigorous assay of bacterial adhesion ability.

As such, we evidenced there are significant differences in bacterial adhesion abilities to the tested ECM proteins between O157 and non-O157 *E. coli* strains, which clearly contrast with the conclusions from a previous investigation (Zulfakar et al., 2012) but corroborate the findings that *E. coli* O157:H7 bacterial cells bind collagen (Medina, 2001). The present study focused on experimental conditions where *E. coli* O157:H7 showed specific adhesion and non-specific adhesion to ECM proteins, which were carefully chosen to correspond to the ECM components of

skeletal muscle tissue. However, it was shown that the type of ECM proteins, the growth medium, the temperature and the pH greatly influence the adhesion ability of *E. coli* O157:H7 (Chagnot *et al.*, 2013a, Chagnot *et al.*, 2012). Of course, further in-depth investigations would be required to characterize the influence of those key parameters on the adhesion and biofilm formation abilities of non-O157 EHEC to skeletic muscle ECM components.

The bacterial adhesion and biofilm formation results showed disparity between EHEC O157 and non-O157 serotypes. This implies that different surface determinants involved in adhesion are differentially expressed. As recently reviewed (Chagnot *et al.*, 2012, Chagnot *et al.*, 2013b), various cell-surface proteinaceous determinants could be responsible for bacterial adhesion to ECM proteins, MSCRAMM (Microbial Surface Components Recognizing Adhesive Matrix Molecules). In diderm-LPS bacteria like EHEC, it can be either single proteins or supramolecular protein structures, (i) anchored to the outer membrane by β-barrel mainly secreted by the Type V secretion system (T5SS) such as autotransporters (Ag43, EhaA…), or (ii) secreted and assembled by few secretion systems into supramolecular structures, such as flagella (T3bSS) and different types of fimbriae, *e.g.* Type 4 pili (T2cSS), pili T (T4aSS), pili F (T4bSS), injectisome (T3aSS), Type 1 pili (T7SS), curli (T8SS), *etc*..., (Chagnot *et al.*, 2013b). Regarding non-specific bacterial adhesion, it was shown to be associated with autoagreggation in *E. coli* O157:H7 EDL933 (Chagnot *et al.*, 2013a). The Ag43 is a surface adhesin expressed by many strains of *E. coli*, including pathogenic strains. Its involvement in the phenomenon of autoagreggation in *E. coli* has been demonstrated several times (Torres *et al.*, 2002). In *E. coli* K12, the expression of the Ag43 is phase variation dependent (Owen *et al.*, 1996), but its regulation is not fully understood as yet and has been poorly investigated in pathogenic *E. coli* as O157:H7 (Torres *et al.*, 2002). In addition to Ag43, other surpramolecular surface protein structures could play a role in the autoaggregation of *E. coli* strain such as Type 1 pili (King *et al.*, 2011, Ulett *et al.*, 2007, Schembri *et al.*, 2001), curli (Tree *et al.*, 2007) and type 4 HCP pili (Xicohtencatl-Cortes *et al.*, 2009). Further studies to investigate the protein determinants implicated in specific and non specific adhesion as well as biofilm formation on immobilized ECM proteins are required to better understand the behavior of O157 and non-O157 strains to contaminate meat but also other food matrices.

A better understanding of the molecular and cellular mechanisms involved in majors EHEC adhesion to meat is necessary to limit the risk of food outbreak, causing serious diseases (SHU and PTT) especially for young children (Hermos *et al.*, 2011). The discrepancies in the colonization abilities of EHEC to ECM proteins could ultimately be taken into consideration to evaluate the risk

of contamination of different types of food products and eventually lead to the development of more efficient preventing strategy of carcass contamination by bacterial pathogens in the meat industry.

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TABLE 1: List of O157 and non-O157 EHEC bacterial strains.

Strain	Somatic antigen	Flagellar antigen	Origin	Virulence profile	Reference[a]
EDL933	O157	H7	Clinical	*stx1, stx2, eae, hly*	(Riley et al., 1983)
ED180	O26	H11	Clinical	*stx2, eae*	Lothar Beutin
12047(Fred5)	O45	H2	Clinical	*stx1*	(Voitoux et al., 2002)
CH087	O103	H2	Clinical	*stx1, eae, hly*	(Pradel et al., 2008)
ED191	O111	H2	Clinical	*stx2, eae, hly*	Lothar Beutin
32316	O121	H19	Clinical	*stx2, eae*	This study
PH27	O145	H28	Clinical	*stx1, eae*	(Posse et al., 2008)
CB13348 (LB226692)	O104	H4	Clinical	*stx2*	Lothar Beutin

[a] Lothar Beutin collection (Nationales Referenzlabor für *Escherichia coli*, Berlin, Germany)

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ARTICLE n°3

Caroline CHAGNOT, Mohamed Amine ZORGANI, Nelly CACCIA, Thierry ASTRUC and Mickaël DESVAUX.

Differential involvement of Ag43 in bacterial adhesion and biofilm formation of *E. coli* O157:H7 to the extracellular matrix

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Differential involvement of Ag43 in bacterial adhesion and biofilm formation of *E. coli* O157:H7 to the extracellular matrix

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Keywords: Enterohemorrhagic *Escherichia coli*, Food contamination, Bacterial adhesion, Biofilm formation, MSCRAMM, Extracellular matrix, aggregation

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ABSTRACT

Enterohemorrhagic *Escherichia coli* (EHEC) O157:H7 is the most prevalent foodborne serotype associated with life-threatening hemorrhagic colitis and the hemolytic-uremic syndrome, which essentially affect young children. Major food vector of EHEC contamination is ground beef where primary bacterial contamination occurs during the slaughter. The extracellular matrix (ECM) is the most exposed part of the muscle and is an important support for bacterial adhesion. In EHEC, various surface-exposed protein determinants can be expressed and potentially involved in food contamination and further human infection. Antigen 43 (Ag43) is an autotransporter protein responsible for the autoaggregation and biofilm formation of many *E. coli* strains. However, the biological significance that Ag43 plays in EHEC adherence to meat extracellular matrix (ECM) proteins remain to be elucidated. Investigating the autoaggregation, bacterial adhesion and biofilm formation, the involvement of Ag43 was demonstrated in *E. coli* O157:H7 EDL933 but not the Sakai strain. The results suggest that Ag43 could play an important role in interaction between bacteria and meat and thus for food contamination by EHEC.

INTRODUCTION

Escherichia coli is a Gram negative bacterium, commonly found in the gut of humans and animals. Despite most strains of *E. coli* are harmless, some intestinal strains are diarrheagenic. The enterohemorrhagic *Escherichia coli* (EHEC) are one of the six *E. coli* enteropathotypes (Karmali *et al.*, 2010). The EHEC are anthropozoonotic pathogen responsible for foodborne infections. The main reservoir of EHEC is the digestive tract of ruminants. In most cases, the human infections result from the faecal contamination of food products. The food vector of contaminations is essentially beef meat, but sheep meat, raw milk or vegetables are also incriminated. These EHEC are responsible for bloody diarrhea and can induce serious human diseases. In children less than 15-years old, this bacterial infection can degenerate into hemolytic uremic syndrome (HUS) or thrombotic thrombocytopenic purpura (TTP), which quite often results in serious renal sequel and can sometimes be fatal. The main virulence factors of EHEC responsible for those symptoms are the Shiga-toxins. While there are over 300 different serotypes of Shiga-toxin producing *E. coli* (STEC), only a handful has been evidenced to be responsible for the major outbreaks or sporadic infections worldwide and can this truly be considered as pathogens, i.e. EHEC. During outbreaks involving consumption of contaminated minced meat, the *E. coli* O157:H7 serotype is the most frequently isolated. From the first major outbreak in the United States of America in 1982 involving contaminated burgers, the reference strain *E. coli* O157:H7 EDL933 was isolated and the significance of EHEC as a serious public health problem was recognized (Riley *et al.*, 1983). A second memorable outbreak occured in Japan in 1996 involving white radish sprouts and the isolated strain *E. coli* O157:H7 Sakai (Michino *et al.*, 1999)

In beef meat products, primary bacterial contamination involves the adhesion of bacteria to the surface of the meat usually at the stage of slaughtering, especially dehiding (Aslam *et al.*, 2003). The extracellular matrix (ECM) is the most exposed portion of the skeletal muscles in beef carcass. It is a complex protein matrix mainly composed of fibrillar proteins: collagen, elastin, laminin and fibronectin (Chagnot *et al.*, 2012, Purslow, 2005, Purslow, 2002, Thorsteinsdottir *et al.*, 2011). Previous studies have shown the ability of the *E. coli* O157:H7 to adhere and colonize the proteins of the muscle ECM but the bacterial proteins responsible of those interactions are not elucidated. The ability of bacteria to colonize biotic matrices, such as meat, and before being ingested and cause infection is linked to their ability to express surface adhesins able of interacting with ECM components. Those cell-surface proteinaceous determinants are called MSCRAMM (Microbial Surface Components Recognizing Adhesive Matrix Molecules) proteins. Their localization at the

surface of the bacterial cell necessarily involves the transport of those proteins *via* secretion systems, that is their translocations through two biological membranes in diderm-LPS bacteria (archetypal Gram-negative)(Desvaux *et al.*, 2006, Desvaux *et al.*, 2009). In diderm-LPS bacteria such as *E. coli*, MSCRAMM proteins are either (i) anchored to the outer membrane by β-barrel mainly secreted by the Type V secretion system (T5SS), or (ii) secreted and assembled by different secretion systems into supramolecular structures, such as flagella (T3bSS) and different types of pili, *e.g.* Type 4 pili (T2cSS), pili T (T4aSS), pili F (T4bSS), injectisome (T3aSS), Type 1 pili (T7SS), curli (T8SS)... (Chagnot *et al.*, 2013b). Of note, the expression of those proteins can be highly complex since regulated at very various levels (genetic, trancriptional, post-transcriptional, translational, post-translational translocational and/or post-translocational), which further depend on environmental conditions as observed for the adhesion abilities of *E. coli* 0157:H7 to ECM proteins respective to different culture conditions (Chagnot *et al.*, 2013a).

In *E. coli* O157:H7, several MSCRAMM proteins have been identified but their role in adhesion to muscle tissue of meat remains unclear. Among the 17 pili encoded by the genome of *E. coli* O157:H7, some are involved in adhesion, cell aggregation, biofilm formation and motility (Mandlik et al., 2008). Among the pili secreted and assembled through the chaperone-usher pathway (CUP) or T7SS (Desvaux *et a*l, 2009), F9 fimbriae have the ability to bind to elastin, fibronectin and collagen (Low *et al*., 2006). The extracellular-nucleation and precipitation pathway (ENP) or T8SS (Desvaux *et al.*, 2009) secrete and assemble pili called curli, which interact with laminin and fibronectin (Saldana *et al.*, 2009a). The HCP (haemorrhagic coli pili), *i.e.* a Type 4 pili assembled by the T2cSS, have the ability to bind to different ECM proteins such as fibronectin, laminin and collagens . The injectisome, *i.e.* T3aSS, may also be involved in the adhesion although its involvement in the recognition of ECM components has not been demonstrated as yet. The flagella, secreted and assembled by the T3bSS, is a supramolecular structure not only implicated in bacterial motility but also in adhesion as shown with H7 flagella, which allows specific adhesion to collagen, laminin and fibronectin (Erdem *et al.*, 2007). Besides those supramolecular protein structures, 9 autotransporters are encoded in the genome of *E. coli* O157:H7 and some have been characterized as involved in the specific interaction with ECM proteins such as collagen or laminin (Wells *et al.*, 2009, Easton *et al.*, 2011). The antigen 43 (Ag43) is involved in autoaggregation and biofilm formation (van der Woude *et al.*, 2008, Klemm *et al.*, 2006). In any case, the role of those cell-surface proteinaceous determinants and contribution in the colonization of muscle ECM has never been questioned in the literature. Besides, their characterization quite often results of heterologous protein expression and conditions of their expression in a *wild type* strain remain to be

demonstrated. Focusing on conditions where non-specific bacterial adhesion was reported to occur in *E. coli* O157:H7, this study then intended to identify their contribution to the adhesion and biofilm formation of skeletal muscle ECM.

.

MATERIAL AND METHODS

Bacterial strains and culture conditions

E. coli K12 MG1655 (Bachmann, 1996) and the non-toxigenic versions of *E. coli* O157:H7 EDL933 and Sakai strains were used in this study (Gobert *et al.*, 2007, Chaucheyras-Durand *et al.*, 2006), *i.e.* the strains *E. coli* CM-454 (for Christine Martin from INRA UR454) and *E. coli* CS (for Chihiro Sasakawa) (Table 1); those non-toxigenic strains result from gene knock-out on both the Shiga-toxin genes *stx1* and *stx2*. In addition, some isogenic mutants obtained from *E. coli* O157:H7 CM-454 were described below. All bacterial strains used in this study are listed in Table 1.

Bacteria were cultured in different relevant nutrient media as previously determined (Chagnot *et al.*, 2013a), that is either a chemically defined medium, *i.e.* DMEM (Dulbecco's modified eagle medium, Gibco) or a complex undefined medium, i.e. LB (lysogeny broth) (Bertani, 2004). From -80°C stock culture previously grown in the respective medium, strains were placed on the relevant agar medium and incubated overnight at 39°C (bovine temperature) (Bertin *et al.*, 2011). A preculture was set up from one bacterial colony grown in the respective nutrient broth medium at 39°C in an orbital shaker at low speed (70 rpm) till stationary phase. When necessary, antibiotics were added at the following concentrations: chloramphenicol (25 µg ml^{-1}), kanamycin (50 µg ml^{-1}).

Construction of in-frame deletion mutants

Isogenic mutant strains in genes encoding for MSCRAMM proteins were obtained from *E. coli* O157:H7 CM-454 using the one-step PCR-based method for inactivation of chromosomal genes (Datsenko *et al.*, 2000). Six genes were targeted, namely (i) *csgA* (*z1676*) encoding the main curline subunit of amyloid curli fibers, (ii) *ppdD* (*z0118*) encoding the major pilin subunit of Type 4 HCP, (iii) *escN* (*z5119*) encoding the T3SS ATPase necessary for secretion and assembly of the injectisome, (iv) *fliC* (*z3013*) encoding the flagellin of H7 flagellum, (v) *z2200* encoding the major pilin subunit of F9 fimbriae homologous to Type 1 pili, and (vi) *cah1* (*z1211*) and *cah2* (*z1651*), two genes 100% identical at the nucleotide sequence level both encoding an identical protein belonging to the Ag43 autotransporter family. Basically, an antibiotic resistance cassette encoding for a chloramphenicol acetyltransferase (Cm^R) was amplified by PCR from plasmidic DNA (pKD3) using primers, which have 50 bp at their extremity 3' corresponding to (i) the sequence just

upstream of the initiation codon of the CDS (coding DNA sequence) of the gene to delete for the forward primers (Fw), and (ii) the sequence just downstream of the stop codon for the reverse primer (Rv). Following high-fidelity PCR using TaKaRa LA Taq DNA polymerase, the amplicon was purified using a QIAquick PCR Purification Kit (Qiagen). The amplicon was electroporated into *E. coli* O157:H7 CM-454, which was first grown in the presence of 10 mM arabinose to induce the expression of the phage recombinase from pKD46. To select gene knockout events, the bacterial colonies were screened on LB plates containing chloramphenicol. The isogenic mutants were identified by colony PCR with outFw/outRv primers using GoTaq DNA polymerase (Promega) (Table S1) and were further confirmed by DNA sequencing (GATC-Biotech) on both strands using those primers. However, to discriminate the Δcah1 and Δcah2 mutants, two different outRv primers were designed. The two identical *Z1211* and *Z1655* genes are located at two region designated OI # 43 (o-iland) and OI # 48 with 97% identical on approximately 87kb. Reverse primers were chosen to 11kb downstream of the gene and the TaKaRa DNA polymerase was used to amplified the large size of the amplicon to indentified the mutants by colony PCR and sequencing.

Coating of microtiter plates with ECM proteins

Preparation of 96-wells polystyrene microtitre plates (Falcon) surface-coated with ECM proteins as previously described (Chagnot *et al.*, 2013a). Briefly, the skeletal muscle ECM proteins consisted of collagen I (Millipore, 08-115), III (Millipore, CC078) and IV (Sigma, C7521), laminin-α2 (Millipore, CC085), elastin (Sigma, E1625) and insoluble fibronectin (i-fibronectin, Sigma, F2518) and a reconstituted ECM including collagens, laminin, fibronectin, and elastin, i.e. MaxGel ECM (Sigma, E0282). BSA (bovine serum albumin (Sigma, A3803) was used as a control for specific adhesion to ECM proteins. Basically, ECM proteins were solubilised in 0.1 M carbonate coating buffer (pH 9.6), 250 µl were dispatched at a saturating concentration of well surface (50 µg ml^{-1}) and incubated overnight at 4°C. The wells were washed 3 times with TS (Tryptone Salt) at room temperature (rt) prior to be used for bacterial adhesion or biofilm formation assays.

Bacterial adhesion assay

Preculture was diluted 1:100 and grown as described above (39°C and low orbital shaking). The two media LB and DMEM were adjusted with NaOH (0.1 M) to reach a pH of 7 at the time of sampling, i.e. during the exponential growth phase at an OD_{600nm} of 0.5 (about 10^8 CFU ml^{-1}). Chloramphenicol was added and mixed gently at a final concentration of 90 µg ml^{-1} to prevent *de*

novo protein synthesis and stop further bacterial growth. As previously mentioned (Chagnot *et al.*, 2013a) and to preserve cell surface supramolecular structures, no vigorous shaking, vortexing or centrifugation was ever applied. Cutted tips were used to deposited cell suspension (200 µl) in relevant protein-coated wells of the microtitre plates. As a control, some wells were filled with sterile growth medium. Microtiter plates were incubated statically at 25°C temperature for 3 h. After incubation, bacterial suspension was removed by pipetting. To remove loosely attached cells, wells were first washed with TS (trypton salt). Adherent bacteria were fixed with 200 µl absolute ethanol during 20 min. Wells were then emptied by pipetting and dried for 30 min prior to staining with 200 µl of an aqueous-solution of crystal violet (0.1 % w/v) during 15 min. After to be emptied, wells were washed a second time with TS to remove the excess of unbound crystal violet dye, and dried for 30 min. For 1 min, under orbital shaking, the bound dye was solubilized from stained cells using 200 µl of an aqueous solution of acetic acid (33 % v/v). Finally, contents of each well (150 µl) were transferred to a clean microtiter plate and a microtiter plate reader was used to measure absorbance at 595 nm. The readings were corrected by substracting the average absorbance from control wells.

Autoaggregation assay

Preculture was diluted 1:100 and grown as described above. Based on a previously described assay (Hasman *et al.*, 1999), cell suspension was adjusted to approximately the same $OD_{600\,nm}$, chloramphenicol was added at a final concentration of 170 µg ml^{-1} and each culture was placed in conical tubes. No vigorous shaking, pipetting or vortexing was ever applied to preserve cell surface determinants potentially involved in autoaggregation. The tubes were incubated statically and vertically at 25°C. Samples of 100 µl were taken from the top of the tube at different time points to measure the $OD_{600\,nm}$. To confirm the autoaggregation, observations in phase-contrast microscopy were performed after 24 h of incubation.

Biofilm formation assay

This assay was based on a previously described protocol (Chagnot *et al.*, 2013a). Preculture was diluted 1:100, 200 µl of bacterial cell suspension were dispatched in protein-coated wells of the microtitre plate and further incubated at 25°C statically. Wells filled with sterile growth medium were used as control. To estimate the attached biomass after 24 h of sessile development, wells were subjected to the same sequential procedure as described for the bacterial adhesion assay,

namely, washing with TS, fixation with absolute ethanol, air drying, staining with an aqueous crystal violet solution, washing with TS, air drying, dye recovery with an aqueous acetic acid solution and reading of the absorbance at 595 nm

Image acquisition and analysis from microscopic observations

Sample preparation was inserted on the stage plate to take one image for each well. As follows, this operation was repeated 12 times in order to get satisfying statistical information. Field of view was chosen in the center of the well in order to avoid artifacts such as edge optical aberrations or biased bacteria spatial distribution. Fields of bacteria were imaged using a 60x magnification (40x objective with x1.5 intermediate magnification).

Observations were performed in phase contrast transmitted light. The corresponding images were acquired using an inverted phase-contrast microscope (Olympus IMT-2) coupled to a cooled CCD camera (Olympus DP30BW) and driven by the Cell^A software v3.2 (Olympus France SAS, Rungis, France). Images were acquired with a 40x objective allowing phase contrast microscopy (Olympus LWD-CD-PLAN40FPL, 0.55, 160/1). Images were processed with the public-domain image processing and analysis program ImageJ v1.43 (NIH-RSB, Bethesda, MD, USA) (http://rsb.info.nih.gov/nih-image/about.html).

Statistical analysis

Statistical analysis was performed from Excel using XLSTAT v2009.3.02. Data of assays result from at least five independent experiments, i.e. five biological replicates. For each experiment, a value was calculated from the average of repetitions performed in triplicate at fewest. The mean values from the biological replicates were compared to the mean values obtained with BSA used as a control. Data were statistically analyzed following Student's t-test with differences considered significant ($p<0.05$, *), very significant ($p<0.01$, **), highly significant ($p<0.001$, ***) or very highly significant ($p<0.0001$, ****).

RESULTS

Bacterial autoaggregation depends on the *E. coli* strains and is influenced by the growth media

From previous investigation, autoaggregation of the *E. coli* O157:H7 was for the first time reported when bacteria were grown in DMEM (Dulbecco's Modified Eagle Medium), which mimic the trend observed in bovine small instestine content, but not in LB. Such an occurrence was questioned for the reference *E. coli* K12 MG1655 and two well-known *E. coli* O157:H7 strains, i.e. EDl933 isolated from meat and Sakai isolated from vegetables. *E. coli* K12 MG1655 sedimented in both DMEM and LB, which resulted in the decrease of the OD_{600nm} overtime (Figure 1A). Microscopic observations revealed the bacterial sedimentation was associated with a phenomenon of cell autoaggregation (Figure 1B); where the sedimentation occured more rapidly was also associated with more cell aggregates at 24 h in DMEM compared to LB. As previously observed, the non-toxigenic *E. coli* O157:H7 EDL933 Δ*stx*, *i.e. E. coli* O157:H7 CM-454 strain (Table 1), sedimented in DMEM but not in LB over 48 h (Figure 1C). Microscopic examinations revealed it was associated with bacterial cell autoaggregation (Figue 1D); it appeared clearly that the bacterial cells aggregated and flocculated in DMEM and no cell aggregate could be observed in LB. Similarly, the non-toxigenic *E. coli* O157:H7 Sakai Δ*stx*, *i.e. E. coli* O157:H7 CS strain (Table 1), aggregated and flocculated leading to sedimentation in DMEM but not LB (Figure 1C and 1F). However, cell sedimentation at the bottom of the tube was not completed over 48 h and was not as quick as *E. coli* O157:H7 CM-454; actually, *E. coli* O157:H7 CS formed fewer aggregates in DMEM (Figure 1F).

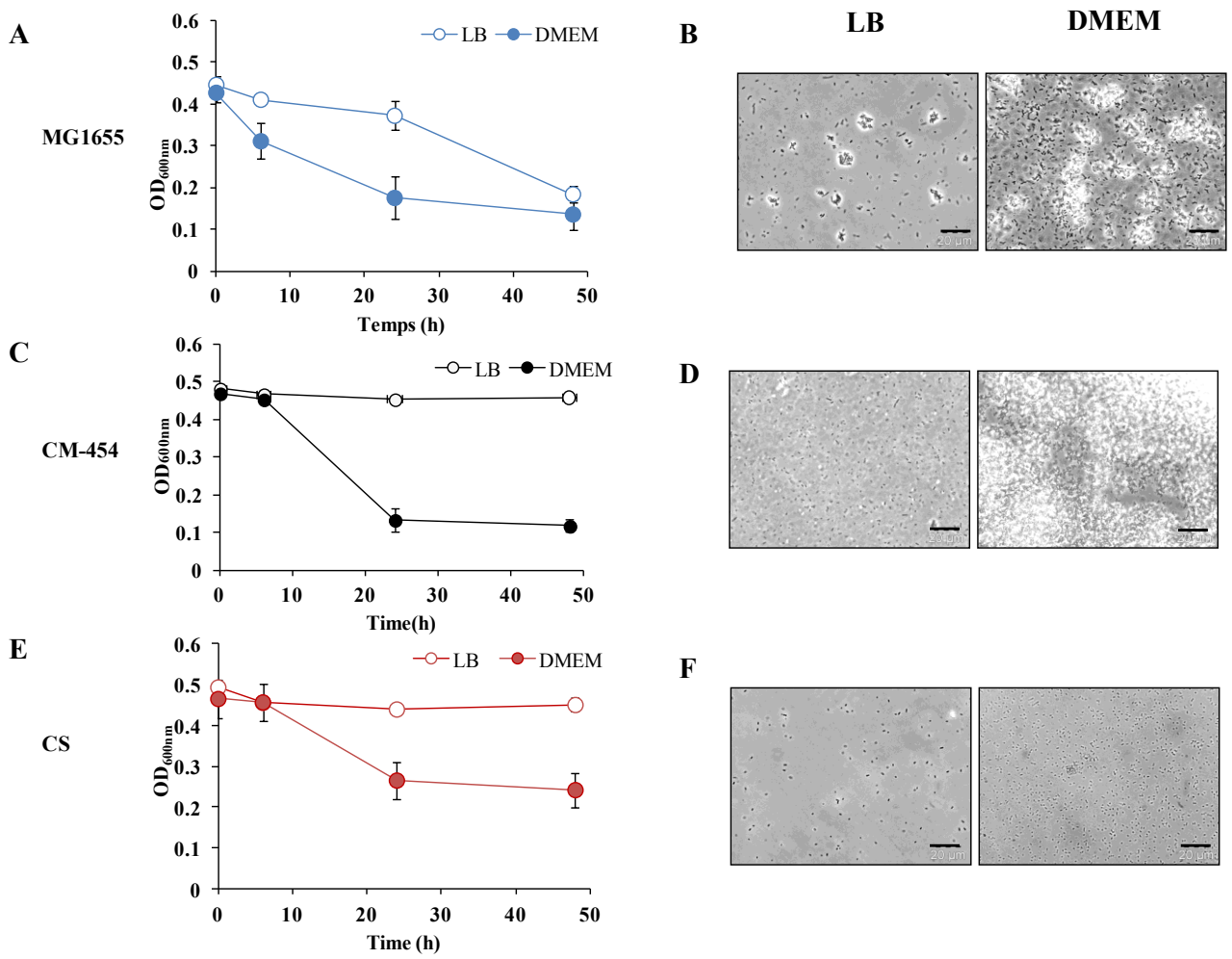

Figure 1:Autoaggregation of *E. coli* strains. Sedimentation assay was performed on *E.coli* strain (A) MG1655, (C) CM-454, and (E) CS, grown in DMEM and LB media at 25°C. Autoaggregation assay was visualized by phase contrast microscopy, after 24 hours of incubation at 25 °C. Bacterial cells for the assay were first grown at 39°C.

Autoaggregation in *E. coli* is associated with non-specific adhesion ability and biofilm fomation capacity to ECM

Since autoaggregation resulted in cell sedimentation, the adhesion ability of the three strains of *E. coli* was further investigated. In LB, *E. coli* K12 MG1655 adhered specifically to different ECM proteins and reconstituted ECM (Figure 2A) but not in DMEM, where bacterial adhesion was low and non-specific since it was similar on ECM proteins and BSA (Figure 2B). As previously observed (Chagnot *et al.*, 2013a), *E. coli* O157:H7 CM-454 adhered specifically to ECM proteins in LB (Figure 2A) and non-specifically to BSA and ECM proteins in DMEM (Figure 2B). *E. coli* O157:H7 CS, though, could not adhere to any of the ECM proteins tested in LB and DMEM either (Figure 2). It seems, therefore, the specific or non-specific adhesion ability is not strictly associated

with the autoaggregation, which varies as a function of the bacterial strain and growth medium. Remarkably, the high and non-specific adhesion of *E. coli* O157:H7 CM-454 to ECM in DMEM medium is associated with the autoaggregation. From there, biofilm formation ability was investigated (Figure 3). In LB, *E. coli* K12 MG1655 was the only one to be able to form biofilm with a maximum sessile biomass at 48 h of sessile growth. In DMEM, both *E. coli* K12 MG1655 and *E. coli* CM-454 *E. coli* could form biofilm with a maximum sessile biomass at 48 h of sessile development. *E. coli* O157:H7 CS, however, could neither form a biofilm in LB or DMEM. In any cases, it appeared that biofilm formation was associated with the autoaggregation.

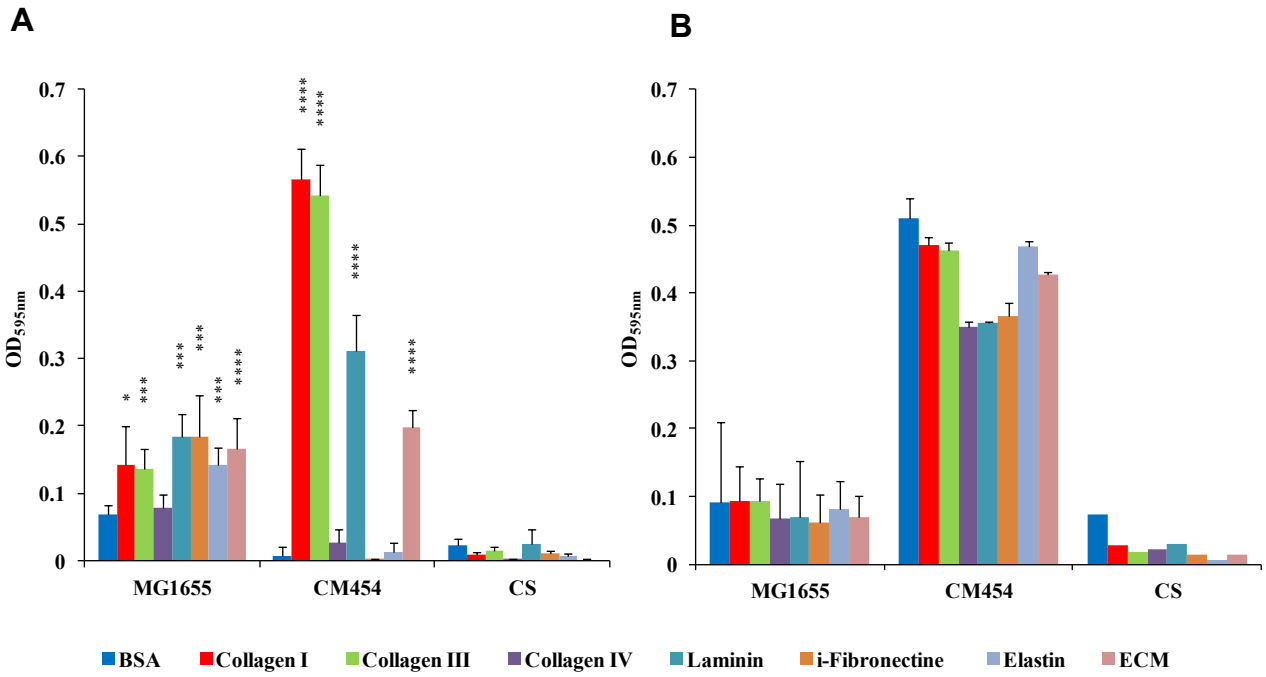

Figure 2: Adhesion to immobilized muscle ECM proteins of *E. coli strains* MG1655, *E. coli* O157:H7 CM-454 and CS grown in different media. Specific bacterial adhesion assay to the main ECM fibrous proteins present in meat was performed in (a) LB medium, and (b) DMEM at 25°C using BSA as a control and measured by the crystal violet staining method. Bacterial strains were first grown at 39°C.

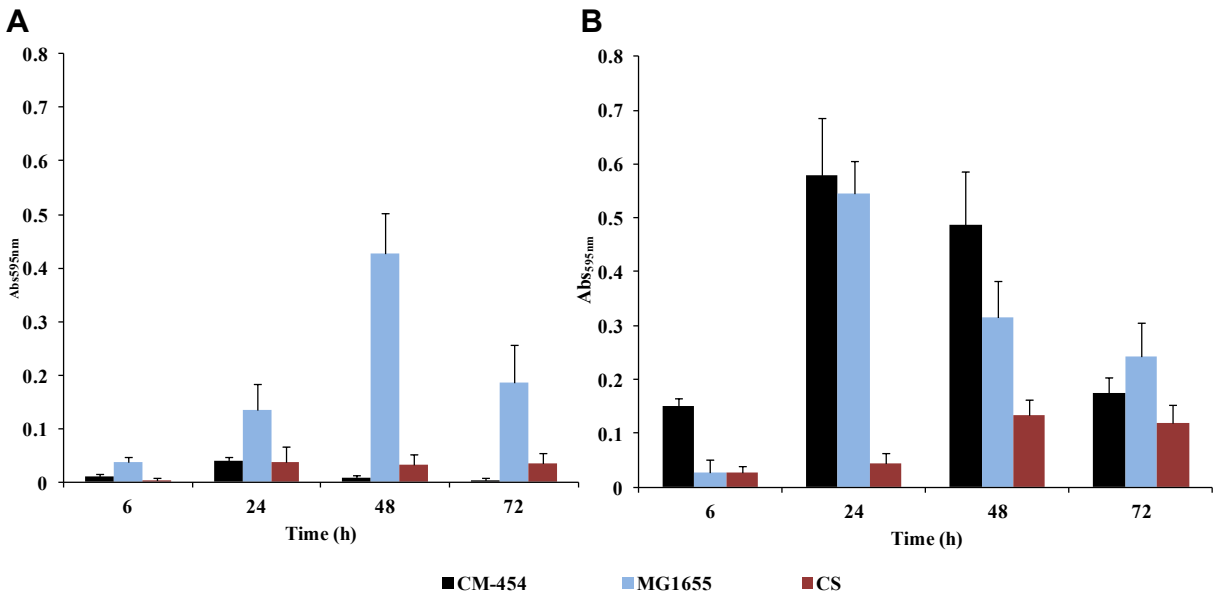

Figure 3: Colonization of BSA by *E. Coli* CM-454, MG1655 and CS strains. A: Kinetics of biofilm formation was performed in LB (A) and DMEM (B) medium at 25°C and measured by the crystal violet staining method. Bacterial strains were first grown at 39°C**.**

Ag43 is the major protein determinant implicated in bacterial adhesion of *E. coli* O157:H7 CM-454 but not of *E. coli* O157:H7 CS

To identify the molecular determinants involved in the surface colonization properties of *E. coli* O157:H7 CM-454, a functional genetic approach has been implemented. From literature survey, six cell surface proteins or supramolecular protein structures were targeted as potentially involved in autoaggregation and surface colonization to the ECM, namely the curli, the Type 4 HCP, the injectisome (T3aSS), the flagella (T3bSS), the F9 fimbriae, and the Ag43.

Once constructed, those isogenic mutants of *E. coli* O157:H7 CM-454 (Table 1) were phenotypically characterized for bacterial adhesion in DMEM. No difference could be observed for the bacterial adhesion ability to BSA or ECM of the *csgA*, *ppdD* and *EscN* mutants when compared to *E. coli* O157:H7 CM-454 (Figure 4). However, *E. coli* CM-454 Δ*z2200*, Δ*FliC* and Δ*cah1*Δ*cah2* showed a significant decrease of the amount of adhered biomass both on BSA and ECM. The most prominent reduction of the levels of adhered biomass was observed with *E. coli* CM-454 Δ*cah1*Δ*cah2*. Interestingly, single mutations in one of the two identical genes encoding the Ag43 did not show the same effect and the decrease in the adhesion ability was essentially attributed to the locus *cah1* (*z1211*). Despite the presence of the very same gene encoding Ag43 (but corresponding to the *cah2* locus), *E. coli* O157:H7 CS could not adhere in the tested conditions (Figure 2).

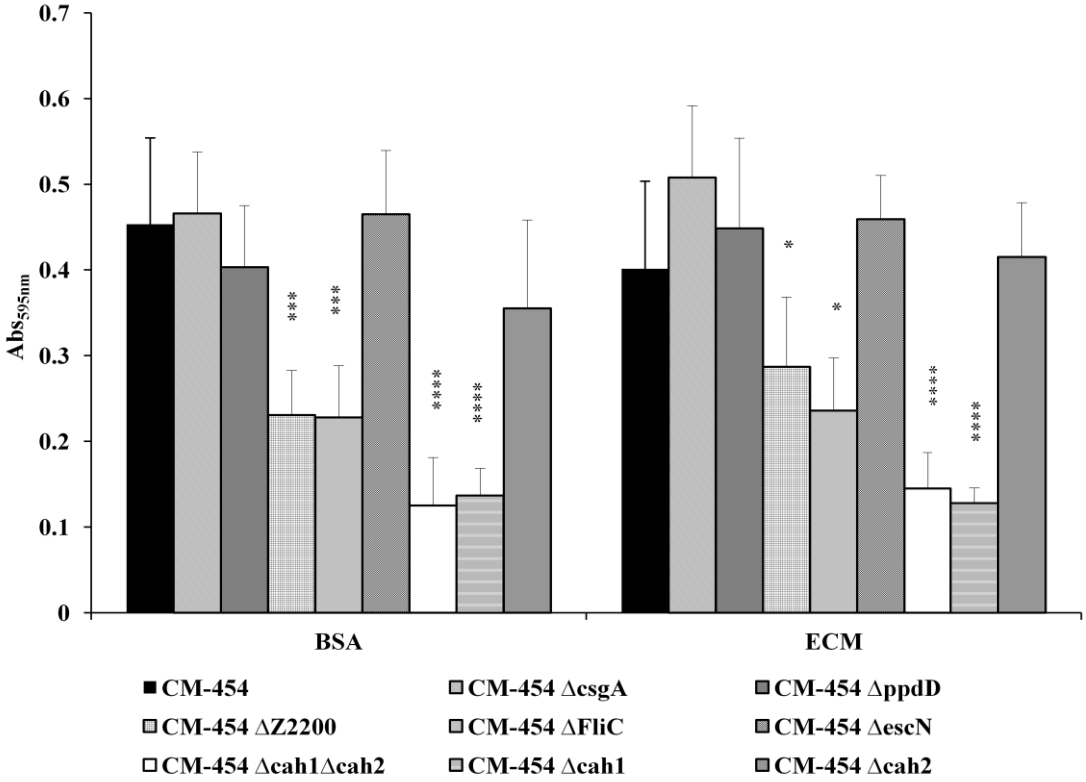

Figure 4: Role of the main MSCRAMM proteins mutant on adhesion in DMEM. The non-specific adhesion assay to BSA and ECM (MaxGel) were performed in DMEM at 25°C using the wild type *E. coli* O157:H7 CM-454 strains as a control and measured by the crystal violet staining method. Bacterial strains were first grown at 39°C**.**

Ag43 is the major determinant of the autoaggregation and biofilm formation in *E. coli* O157:H7 CM-454 but not *E. coli* O157:H7 CS or *E. coli* K12 MG1655

The involvement of Ag43 in autoaggregation phenomenom is very well-known but it has been essentially investigated in *E. coli* K12 MG1655. As previously reported, *E. coli* K12 MG1655 was impaired in its sedimentation and autoaggregation abilities upon deletion of *flu* (Figure 5). Similarly, the absence of Ag43 upon deletion of both *cah1* and *cah2* resulted in the loss the sedimentation and autoaggregation capacity of the strain when compared to *E. coli* O157:H7 CM-454 in DMEM. More surprisingly but in agreement with bacterial adhesion, this variation of the phenotype was essentially attributed to the locus *cah1* (*z1211*) since no difference could be observed with the isogenic *E. coli* O157:H7 CM-454 strain (Figure 5).

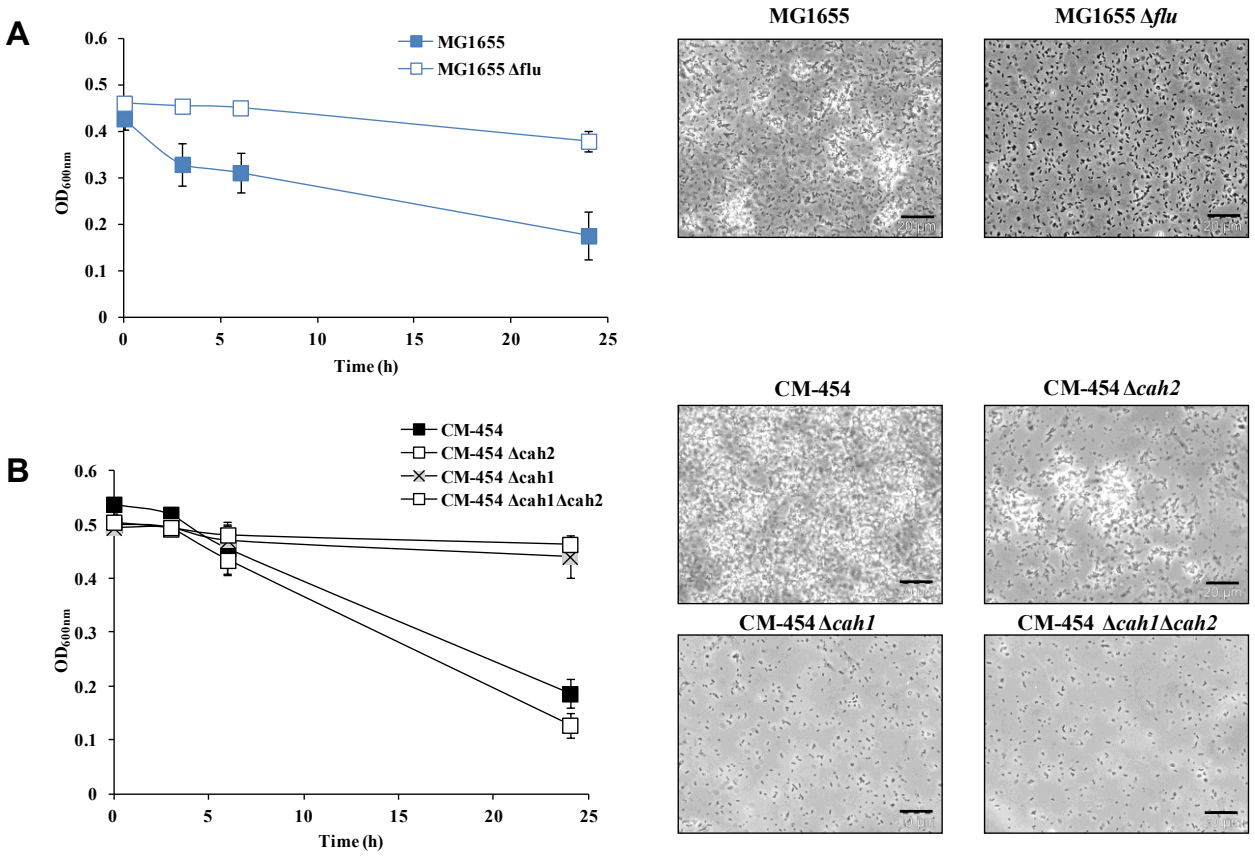

Figure 5: Autoaggregation comparison of mutants ang43 with wild strains. Sedimentation and autoaggregation were performed in DMEM at 25° C. (A) the mutant MG1655 Δflu was compared to the wild-type strain MG1655 and (B) the mutant CM-454 Δ*Z1651*, CM-454 Δ*Z1211* and CM-454 Δ*Z1211* Δ*Z1651* were compared to the wild type CM-454 strain. The observation of the bacteria autoaggregation (mutant / wild type) was visualized by phase contrast microscopy, after 24 hours incubation.

From there, the role of Ag43 in biofilm formation was further investigated. Despite the absence of Ag43, low difference in the amounts of adhered biomass could be observed in the course of sessile development for *E. coli* MG1655 Δ*flu* when compared to the *wt* (Figure 6A). From the start and on the contrary, the *cah1* and *cah2* double mutant exhibited much lower amount of adhered biomass in the course of sessile development, which difference will remain highly significant all along biofilm formation compared to *E. coli* O157:H7 CM-454. At a lesser extent, single mutants of *cah1* or *cah2* also showed lower ability to colonize surface especially at 24 and 48 h of sessile development (Figure 6B).

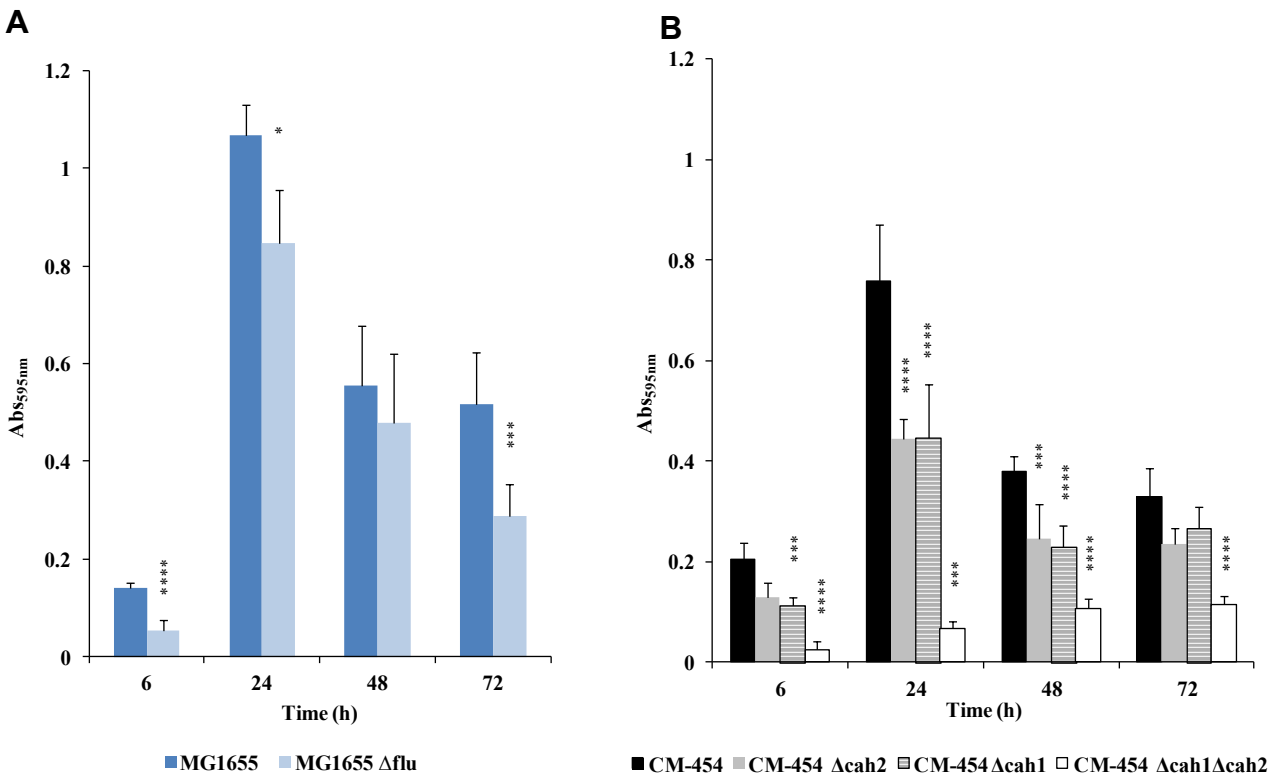

Figure 6: Role of Ag43 in biofilm formation in DMEM. Comparison of phenotype of mutants agn43 (A) *E. coli* O157:H7 CM-454 Δ*Z1651*, CM-454 Δ*Z1211* and CM-454 Δ*Z1211* Δ*Z1651* and in (B) MG1655 Δ*flu*. with the wild type strains respectively CM-454 and MG1655 in the formation of biofilm on BSA in DMEM at 25 °C. The biomass formed was estimated by Crystal violet staining. Bacterial strains were first grown at 39°C**.**

DISCUSSION

During the slaughter, bacteria can adhere to the meat surface and cause contamination. To identify some of the molecular determinants involved in this process, the adhesion of *E. coli* to ECM, the most exposed portion of the meat, was studied. As with bovine small instestine content, bacterial autoaggregation was demonstrated in DMEM (Chagnot *et al.*, 2013a). This previous study highlighted that the autoaggregation of *E. coli* O157:H7 EDL933 depended on the nature of the media, which induce differential expression either of the molecular determinant involved in aggregation or of determinants hindering cell aggregation, e.g. exopolysaccharides or pilus (Hasman *et al.*, 1999, Ulett *et al.*, 2007, Schembri *et al.*, 2001). As reported in *E. coli* K12 MG1655, the present investigation demonstrated the Ag43 was the main determinant responsible for the autoaggregation of the non-toxigenic *E. coli* O157:H7 EDL933 strain. In the same culture conditions, though, the non-toxigenic *E. coli* O157:H7 Sakai could neither autoaggregate nor adhere. Actually, *E. coli* K12 MG1655 and those *E. coli* O157:H7 encode homologs of Ag43 but belong to two distinct subfamilies (van der Woude *et al.*, 2008). While the expression of the Ag43 subjected to phase variation has been well investigated in *E. coli* K12 (Owen *et al.*, 1996), its regulation is not fully understood as yet and has been little investigated in pathogenic *E. coli* or O157:H7 (Torres *et al.*, 2002). It could be hypothesized that the regulation of their expression differ between different *E. coli* strains and/or autoaggregation could be hindered by other determinants differentially expressed in *E. coli* O157:H7 Sakai such as exopolysaccharides or supramolecular protein structures, e.g. some pili (Hasman *et al.*, 1999, Ulett *et al.*, 2007, Schembri *et al.*, 2001). In addition, *E. coli* O157:H7 EDL933 and *E. coli* O157:H7 Sakai encode the very same Ag43 protein but two identical copies present at two distinct loci are present in *E. coli* O157:H7 EDL933 against one in *E. coli* O157:H7 Sakai. Interestingly, single deletions in each of those two loci encoding the Ag43 in the non-toxigenic *E. coli* O157:H7 EDL933 resulted in different phenotypes with regards of bacterial adhesion. Genes *cah1* and *cah2* are located at two different chromosome regions designated OI # 43 (O- island) and OI # 48, respectively (Perna *et al.*, 2001). The mechanism of gene duplication in these O -islands is unknown but the event is quite recent considering the nucleotide sequence is nearly identical. Considering, gene duplication is assumed to play an important role in evolution, where differential gene regulation can occur and/or a copy of the gene retaining its original function while the other copy can diverge to acquire new functions (Torres *et al.*, 2002). Those different and complex aspects over the regulation of the expression of Ag43 in EHEC would undoubtedly required further investigations.

While Ag43 appeared to play a major role in the autoaggregation and bacterial adhesion of the non-toxigenic *E. coli* O157:H7 EDL933 strain, phenotypic characterization of the isogenic deletion mutants revealed the F9 pili and flagella H7 also played a role in bacterial adhesion. Actually, Type 1 pili homologous of the fimbrae F9 was reported to have a role in the autoaggregation and adhesion in *E. coli* (King *et al.*, 2011, Ulett *et al.*, 2007). Regarding the flagella, the absence of mobility can promote cell sedimentation and bacterial adhesion to surfaces. No effect on bacterial adhesion could be observed upon inactivation of assembly of the Type 4 HCP, curli or injectisome (Xicohtencatl-Cortes *et al.*, 2009, Vidal *et al.*, 1998). Though, their expression is highly dependent on environmental conditions including the growth medium.

Furthermore, this work revealed Ag43 was of great importance for biofilm formation by the non-toxigenic *E. coli* O157:H7 EDL933 strain. Of note, a low difference could be observed in the presence or the absence of *flu* in *E. coli* K12 MG1655. Actually, in the course of cell development other determinants can become more prevalently involved and hinder the role of Ag43 as it was shown for fimbrae for *E. coli* K12 MG1655(Reidl *et al.*, 2009). This can also explain the differences between about the respective involvement of *cah1* and *cah2* in bacterial adhesion compared to biofilm formation, which could more prominently other supramolecular strucutures such as F9 pili.

In the end, the Ag43 can play an important role in colonization of different biotic and abiotic surfaces since it induces autoaggregation, non-specific adhesion and promotes biofilm formation. Considering EHEC O157:H7 EDL933 and *E. coli* O157:H7 Sakai have been clinically isolated and human infection further associated with their presence in meat and vegetable products respectively, one might hypothesized their ability to colonize those food products is different and associated to different adhesion and biofilm formation determinants such as Ag43. Also, its involvement in the colonization of the intestinal gut of ruminants in relation to carriage in cattle or of the human intestinal tract of human during an infection would merit in-depth investigations. Some very promising approach lies in the development of vaccines where the passenger domain of Ag43 was shown to contribute to immune defense responses against ETEC (enterotoxigenic *E. coli*) infection (Harris *et al.*, 2011). Understanding the regulation of the expression of the Ag43 in *E. coli* O157:H7 could not only have repercussion on fundamental aspects but also in public health and food industry. Indeed, a better understanding of the molecular mechanisms involving the Ag43 in the adhesion of EHEC to the meat industry is necessary to limit the risk of food contamination via the development of new practices or innovative prevention methods especially at the stage of slaughter.

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Table 1: Plasmids and bacterial strains used in this study

Name	Revelant characteristics	References
Plasmid		
pKD46	Vector containing the Red recombinase of phage λ (Amp^R)	(Datsenko *et al.*, 2000)
pKD3	Vector containing the *cat* gene (Cm^R)	(Datsenko *et al.*, 2000)
E. coli strain		
CM-454	Non-toxigenic *E. coli* O157:H7 EDL933 (Δ*stx2AB, stx1AB::kan*)	(Gobert *et al.*, 2007)
CS	Non-toxigenic *E. coli* O157:H7 Sakai RIMD0509952 (*stx2A::kan* Δ*stx1A*)	(Chaucheyras-Durand *et al.*, 2006)
MG1655	Reference strain of *E. coli* K12 wild type (*wt*)	(Bachmann, 1996)
CM-454 pKD46	*E. coli* CM-454 with pKD46	This study
CM-454 Δ*csgA*	CM-454 deleted of *z1676* encoding the major subnit of curli	This study
CM-454 Δ*ppdD*	CM-454 deleted of *z0118* encoding the major subnit of pili HCP	This study
CM-454 Δe*scN*	CM-454 deleted of *z5119* encoding the major subnit of injectisome	This study
CM-454 Δ*z2200*	CM-454 deleted of *z2200* encoding the pili F9 (Δstx, Kan^R, Cm^R)	This study
CM-454 Δ*fliC*	CM-454 deleted of *z3013* encoding the major subnit of H7 flagella	This study
CM-454 Δ*cah1*Δ*cah2*	CM-454 deleted of both *z1211* and *z1651* encoding the Ag43	This study
CM-454 Δ*cah1*	CM-454 deleted of *z1211* encoding the Ag43	This study
CM-454 Δ*cah2*	CM-454 deleted of *z1651* encoding the Ag43	This study
MG1655 Δ*flu*	*E. coli* MG1655 deleted of *flu* encoding the Ag43	(Reisner *et al.*, 2003)

SUPPLEMENTARY MATERIAL

Table 1S. Oligonucleotide used in this study

Name	Oligonucleotide sequence (5'-3')
*csgA*Fw	TGCAGAACGGAATTGCACACAACAGACTGATACTTCTCTGTCTGATGAAGGTGTAGGCTGGAGCTGCTTC
*csgA*Rv	CAGAAAGTCATATTCAGCGTAGCCTGACCGTTATAGCCTTCAGCGCTGCTCATATGAATATCCTCCTTAGT
*ppdD*Fw	GCTGATGGCGTTTGCCAAAGTAGCACCAACCAAATCAAGGAGCGAAACAGGTGTAGGCTGGAGCTGCTTC
*ppdD*Rv	TGATAACGCAGGCACAGGGCCGTGAGCTGTGGAATATTCATTGCCGCTCCCATATGAATATCCTCCTTAGT
*escN*Fw	TTTTCAGGAGGTTGGGAATAATATCGAACTTAAAGTATTAGGAACGGTAAGTGTAGGCTGGAGCTGCTTC
*escN*Rv	CAATCGGTTCGCTCTGCTTTTACGAATAGATAAAATTCTGTCCAACATACCATATGAATATCCTCCTTAGT
*z2200*Fw	ATGAAACTCAAACATGTTGGTATGATTGTCGTTTCTGTGTTGGCGATGTCGTGTAGGCTGGAGCTGCTTC
*z2200*Rv	TTATAGATATTGAACTTTGAAGGTCGCATCTGCGTTTGCAGTACCAGCTACATATGAATATCCTCCTTAGT
*fliC*Fw	GGTGGAAACCCAATACGTAATCAACGACTTGCAATATAGGATAACGAATCGTGTAGGCTGGAGCTGCTTC
*fliC*Rv	ATCAGGCAATTTGGCGTTGCCGTCAGTCTCAGTTAATCAGGTTACAACGACATATGAATATCCTCCTTAGT
*cah*Fw	TGCAGAACGGAATTGCACACAACAGACTGATACTTCTCTGTCTGATGAAGGTGTAGGCTGGAGCTGCTTC
*cah*Rv	CAGAAAGTCATATTCAGCGTAGCCTGACCGTTATAGCCTTCAGCGCTGCTCATATGAATATCCTCCTTAGT
*csgA*outFw	GTCTGCCAGGGGAAATGTC
*csgA*outRv	GCCGGTCGGCTTCATCACA
*ppdD*outFw	CTACTGGAAGTGTCTGGC
*ppdD*outRv	GATGCGGTAGGCATTGTC
*escN*outFw	CATGAATCCAACGGGGAACG
*escN*outRv	CAGCGATGCACAATATGCCT
*z2200*outFw	CTGACACTTCCCTTGTTCG
*z2200*outRv	GCATCTGATGCATTCTCCATG
*fliC*outFw	GTTATCGGCCTGAATTGCG
*fliC*outRv	CCACAGCGAGTGTTTATCC
*cah1/2*outFw	GTCTGCCAGGGGAAATGTC
*cah1*outRv	TCTTCCGTAGTCGCTGATTG
*cah2*outRv	CTATCCACCATTGGGTATCG

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Résultats partie II

Tropisme bactérien en fonction des caractéristiques métaboliques et contractiles du muscle

La capacité d'adhésion des EHEC à la viande a majoritairement été étudiée sur des protéines purifiées de l'ECM, cependant très peu d'études ont été réalisées sur du muscle ou de la viande. Quelques travaux scientifiques ont ciblé cette adhésion (Li *et al.*, 1999, Benito *et al.*, 1996, Piette *et al.*, 1989) mais il n'existe aucune étude du tropisme bactérien pour un type de muscle ou un type de fibre musculaire. Or, des différences sensibles de composition, d'environnement physicochimique et de structure existent entre les différents types de muscle, elles pourraient influer sur l'adhésion des bactéries.

Le muscle squelettique est un assemblage de tissus musculaire, conjonctif, vasculaire et nerveux. Les fibres musculaires sont généralement divisées en quatre types, appelés type I, type IIA, type IIX et type IIB qui contiennent respectivement les isoformes de chaines lourdes de myosine I, IIa , IIx et IIb (Bar *et al.*, 1988, Schiaffino *et al.*, 1989). Elles se caractérisent également par leur métabolisme oxydatif (I, IIA) ou glycolytique (IIX, IIB) et par leurs vitesse de contraction lente (I) ou élevée (IIA, IIX et IIB). Grace à des anticorps monoclonaux dirigés contre les isoformes de chaines lourdes de myosine, il est possible de caractériser les quatre types purs de fibres musculaires I, IIA , IIX et IIB, mais aussi les types hybrides contenant plusieurs isoformes de la myosine , I- IIA , IIA - IIX ou IIX-IIB. Le type contractile influence sensiblement le contenu sarcoplasmique en enzyme du métabolisme. Les fibres de type I et IIA à métabolisme oxydatif présentent une teneur élevée en myoglobine (transporteur d'oxygene) et en mitochondries (respiration cellulaire). A l'inverse, les fibres IIX et IIB à métabolisme glycolytique ont un faible niveau de myoglobine et de mitochondries, mais un niveau élevé d'enzyme de la glycolyse dans le sarcoplasme (Lefaucheur, 2010, Chemello *et al.*, 2011). Les muscles squelettiques sont généralement composés de différentes proportions de chaque type de fibre directement lié à la nature du travail musculaire.

L'objectif de cette deuxième partie était de déterminer si les différences (métaboliques et contractiles) entre les fibres et par extension entre les muscles, peuvaient être à l'origine d'un tropisme bactérien favorisant la contamination d'un type de muscle particulier.

Dans un premier temps, mon objectif était de caractériser les types de fibres musculaires sur une coupe histologique, vierge de toute coloration ou additifs chimiques, basé sur les propriétés intrinsèques des tissus biologiques. La ligne DISCO du synchrotron SOLEIL a développé un microscope à fluorescence dans l'ultraviolet profond (DUV) couplé à une ligne de lumière synchrotron, fournissant un large spectre d'excitation (180 à 600 nm) et un spectre d'acquisition complet de chaque point scanné directement à l'intérieur des cellules vivantes avec une précision de l'ordre du micron (Jamme *et al.*, 2010). Cette technique de microspectroscopie à fluorescence DUV permet de caractériser finement les tissus biologiques (Jamme *et al.*, 2010) en utilisant la fluorescence naturelle dans l'UV de certaines biomolécules comme le tryptophane (Trp), la tyrosine (Tyr), le collagène ou des molécules telles que le NADH ou les vitamines. En explorant la réponse spectrale de fibres musculaires préalablement identifiées sur leur type, nous avons pu discriminer différents types de fibres musculaires et identifier les longueurs d'ondes d'émissions impliquées dans cette ségrégation. Cette approche à été appliquée à deux muscles modèles de rat, le soleus et l'EDL qui possèdent des types métaboliques et contractiles opposés. Elle a également été testée sur des muscles immédiatement prélevés après abattage (Article 4) et sur muscles prélevés à 24h *postmortem* (Article 5) afin d'évaluer l'effet des modifications structurales et physico-chimiques sur la réponse en spectroscopie UV.

Dans un second temps, l'étude de l'adhésion de la souche *E. coli* O157:H7 dans les conditions optimales d'adhésion définie dans l'article 1 a été étudié :

1) sur des coupes transversales sériées de muscles dont les cellules avaient préalablement été identifiées sur leur type par immunohistofluorescence,
2) sur des muscles entiers non lésés,

afin de mettre en évidence un tropisme éventuel de la bactérie pour un type de fibre ou un type de muscle particulier (Article 6).

ARTICLE n°4

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Discrimination of rat skeletal muscle fiber types by hyperspectral UV synchrotron microspectroscopy

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Discrimination of rat skeletal muscle fiber types by hyperspectral fluorescence UV synchrotron microspectroscopy

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Running title: Discrimination of rat skeletal muscle fiber types by hyperspectral UV synchrotron microspectroscopy

Key words: skeletal muscle, fiber type, myosin isoform**,** UV microspectroscopy, histology, synchrotron radiation

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ABSTRACT

The rat skeletal muscle consists of four pure types of muscle cells and their hybrids in different proportions, which differ in their contraction speed and their metabolic pathway. Intracellular composition is adapted to the fiber work and therefore to fiber types. These differences in fiber type's composition were exploited to discriminate them through their autofluorescence spectral features. Rat soleus and extensor digitorum longus (EDL) muscle fibers, previously identified on their cell types by immunohistofluorescence, were analyzed by synchrotron fluorescence microspectroscopy on serial muscle cross sections free of any coloration. Muscle fibers were excited at 275 nm showing differences in fluorescence emission intensity regarding the fiber types at 302, 325, 346 and 410 nm. On each muscle, pure fiber types were clearly discriminated by the ratio 410/325 for EDL ($p<0.01$) and 346/302 for soleus ($p<0.01$).

This study highlights the relevance of using autofluorescence spectral signal to identify the muscle fiber types without any staining or chemical product.

INTRODUCTION

Skeletal muscle contains about 75% water, 19% protein, 0.5–8% lipid and 1% glycogen, and is composed of several tissues such as myofibers, connective tissue, intramuscular adipocytes, vascular and nervous tissues. Myofibers which represent 75–90% of the muscle volume show heterogeneous population differing by structural, contractile, metabolic and physiological properties. Myofibers are generally divided into four types, called type I, type IIA, type IIX and type IIB (Pette *et al.*, 1990, Lefaucheur, 2010, Choi *et al.*, 2009). The type I and IIA fibers have an oxidative metabolism with H_2O and CO_2 as final products, while type IIX and IIB fibers use a glycolytic metabolism where pyruvate is anaerobically fermented into lactic acid. Myofibers are also characterized by their speed of contraction ranked as I < IIA < IIX < IIB (Schiaffino *et al.*, 1996). The underlying molecular basis of this typology resides in the polymorphism of myosin heavy chains (MyHC). Indeed, four adult MyHC isoforms have been identified in mouse, rat, guinea pig and rabbit skeletal muscles, i.e. types I, IIa, IIx and IIb isoforms (Bar *et al.*, 1988, Schiaffino *et al.*, 1989). These four isoforms allow to characterize the four main pure muscle fiber types I, IIA, IIX and IIB, but also hybrid fiber types containing several myosin isoforms, such as type I-IIA, type IIA-IIX or type IIX-IIB fibers. These hybrid fibers properties process the change from a pure type into another type, driven by a change in age, physical activity levels, or exposure to prolonged stress. The type modification always follows the order I → I-IIA → IIA → IIA-IIX → IIX → IIX-IIB → IIB and back in reverse (Pette *et al.*, 2000, Schiaffino *et al.*, 2011).

Composition in metabolic enzyme and structure proteins are adapted to the physiological function, and thus the type of fiber. Type I and IIA oxidative metabolism fibers have high content of myoglobin and mitochondria, and consequently higher content of oxidative phosphorylation enzymes (Lefaucheur, 2010, Chemello *et al.*, 2011) and cofactors of the respiratory chain such as NADH (Ren *et al.*, 1988, Mayevsky *et al.*, 2007). On the opposite, IIX and IIB fibers have low levels of myoglobin and mitochondria but higher levels of enzyme of the glycolysis chain (Lefaucheur, 2010, Chemello *et al.*, 2011). Overall a lot of differences in gene expression can be observed when comparing different fiber types (Chemello *et al.*, 2011, Choi *et al.*, 2009, Ogura *et al.*, 2008) suggesting numerous differences in protein composition depending on the fiber type. Muscles generally comprise various proportions of each muscle fiber type depending on the muscle function. The muscle fiber composition is of importance in a wide range of scientific fields, such as clinical research,

biomechanics studies or muscle food science. The proportions of the different myosin isoforms, and therefore the overall proportion of each type of fiber can be determined biochemically from muscle extract. However, this method cannot identify the distribution of each cell type and is unable to characterize hybrid fiber types.

Histological methods are most used to characterize the myofiber type on serial muscle cross sections. Initially, discrimination of different fiber types was based on differences in the sensitivity of the acto-myosin ATPase activity to pH preincubation. Three main fiber types have been conventionally determined by histoenzymology in adult skeletal muscle, i.e. types I, IIA and IIB (Brooke *et al.*, 1970b). By coupling the mATPase and succinate dehydrogenase activity myofiber could be classified according to contractile and metabolic properties (Ashmore *et al.*, 1971). Later, the use of monoclonal antibodies (mAbs) against myosin heavy chain (MyHC) isoforms enabled a more precise classification of fibers I, IIA, IIX and IIB (Picard *et al.*, 1998, Schiaffino *et al.*, 1989). One major interest in the use of mAbs is the delineation of hybrid fibers (I–IIA, IIA–IIX and IIX–IIB), which simultaneously express different isoforms of MyHC inside the same fiber (Pette *et al.*, 1990).

All these histological methods are cumbersome to implement, require serial sections with enzymology staining and/or immunolabelling colorations complementary to each other. All the data must then be taken into account to accurately identify the type of fiber. Some authors have developed methods of co-staining on the same tissue section to reduce the number of histological sections and therefore the duration of characterization (Raheem *et al.*, 2010, Gregorevic *et al.*, 2008). Others developed image analysis algorithms to automate the identification of cell types from digital images of muscle sections labeled for different myosin heavy chain isoforms (Meunier *et al.*, 2010). But despite these improvements, the techniques are time-consuming, expensive and require a specific qualification of laboratory personnel.

On the other hand, biological tissues can be explored thanks to their intrinsic properties. Skeletal muscle can indeed be characterized by exploiting its autofluorescence properties by front-face fluorescence spectroscopy (Dufour *et al.*, 2003, Sahar *et al.*, 2009). However, the low spatial resolution of this technique allows only an analysis of the whole tissue. Moreover, the sample excitation in the Deep Ultraviolet (200-350 nm) is arduous, making difficult the highlighting of some interesting fluorophores. The technique of deep ultraviolet fluorescence (DUV) microspectroscopy can overcome these constraints and is relevant to finely characterize biological tissues (Jamme *et al.*, 2010). As for front-face fluorescence, microspectroscopy does not need external specific probes or labeling but instead allows use of

the intrinsic fluorescence that exists for many biomolecules when excited below 350 nm wavelength range. In particular, changes in cellular metabolism can be assessed by observation of flavin adenine dinucleotide, nicotinamide adenine dinucleotide (NADH), tyrosine, tryptophan, or porphyrins (Jamme *et al.*, 2010). The coupling of a fluorescence spectrofluorimeter to a microscope allows a good spatial resolution. The DISCO beamline of the synchrotron SOLEIL developed a DUV fluorescence microscope coupled to a synchrotron beamline, providing fine tunable excitation from 180 to 600 nm and full spectrum acquisition from each point scanned, to study DUV excited fluorescence emitted from nanovolumes directly inside live cells or tissue biopsies (Jamme *et al.*, 2013)

Given the differences in composition of the different muscle fiber types, our goal was to explore the respective autofluorescence response of muscle fibers previously identified on their metabolic and contractile types.

MATERIALS AND METHODS

1. *Biological material*

Four 5 months old male Wistar rats (400-450g) were anesthetized by isoflurane gas before sacrifice by decapitation with a guillotine in accordance with institutional guidelines on animal experimentation. Immediately, the skin of the lower limbs has been removed and the hind limbs were dissected. Extensor Digitorum Longus (EDL) and Soleus muscles were taken from tendon to tendon, taking care not to cause any damage. Three of these rats were used for ultrastructural analyses and the last one was used for the DUV microspectroscopy measurements.

2. *Transmission Electron Microscopy (TEM)*

Sample preparation, observation and digital images acquisitions were done according to Astruc et al. (2010). Briefly, immediately after extraction from the rat leg, the muscles were cut transversely and a part of each muscle was immersed in 2.5 % glutaraldehyde in 0.1 M sodium cacodylate buffer, pH 7.2 during 24 hours, post-fixed in osmium tetroxide 1%, dehydrated through a graded series of ethanol and embedded in epoxy resin (TAAB, Eurobio France). Ultra-thin sections (90 nm) were stained with uranyl acetate and lead citrate. Observation and image acquisitions were done using a transmission electron microscope (Itachi HM 7650) equipped with a Hamamatsu AMT numeric camera system.

3. *Light microscopy (Histology and Microspectroscopy)*

3.1. Sample preparation

The muscle part remaining from TEM sampling was positioned on a cork plate with embedding medium (Tissue-Tek) and frozen in isopentane cooled to -160°C with liquid nitrogen (-196°C). Serial cross-sections (10 µm thick) performed using a cryostat (Microm, HM 560) were collected on glass slides for histological stains and on quartz coverslips (ref CNO.WIN-30-0.17-UV, Laser Components, 92190 Meudon, France) for DUV microspectroscopy fluorescence acquisitions. We voluntarily reduced the number of sections to limit erosion of the muscle block so as to identify the compounds and fibers from the first to the last studied section to enable reliable composition and morphology identifications. Sections intended for DUV microspectroscopy were not stained and stored at -20 °C under vacuum until analysis on the synchrotron beamline.

3.2 Muscle fibers and intramuscular connective tissue revelation

Sections were stained by Picrosirius red staining which reveal the intramuscular connective tissue in red and muscle fibers in yellow (Liu *et al.*, 1995).

3.3. Metabolic type of muscle fibers

The oxidative metabolism of individual muscle fibers was revealed by histoenzymology technique revealing the mitochondrial succinyl dehydrogenase activity (Nachlas *et al.*, 1957) which is an enzyme of the Krebs cycle and therefore characteristic of oxidative metabolism. The SDH catalyzes the conversion of succinic acid to fumaric acid and causes the reduction of the Nitroblue tetrazolium (NBT) which turns blue. Oxidative muscle fibers are stain dark blue while glycolytic one are pale blue. As SDH is located in mitochondria, it allows also to localize these organelles in the cells.

3.4. Fiber type determination

The identification of the different fiber types was performed by immunohistofluorescence using monoclonal antibodies specific of myosin heavy chains isoforms characteristic for the cell type. The fiber typing was made according to Shiaffino et al., 1989, with slight modifications. Briefly, slow and fast myosin heavy chains isoforms (MyHC) were identified using mouse monoclonal antibodies specific to MyHC isoforms BA-D5 (MyHC-I) (AGRO-BIO France), SC-71 (MyHC-IIA) (AGRO-BIO France), BFF3 (MyHC-IIB)(AGRO-BIO France). Myofiber subtypes I, IIA, IIB and hybrid IIX-IIB were deducted according to their response to the different antibodies and the unmarked cells corresponded to the types MyHC-IIX. The different primary MyHC antibodies were revelated by an Alexa Fluor 488 labelled goat anti-mouse IgG secondary antibody (A 11001, Invitrogen). The cell outlines were stained using a rabbit anti laminin primary polyclonal antibody (L9393 Sigma) and a goat anti-rabbit IgG Cyanine Cy3-labeled secondary antibody (111-165-008, Jackson).

To reveal the antigenic expression in muscle tissue, the cross-sections were incubated with both primary antibodies against MyHC and laminin in a humidified box overnight at

4°C. After washing, primary antibodies binding was revealed by incubating 1 hour in the dark at room temperature with both labelled secondary antibodies (Alexa 488 anti-mouse IgG and CY3 anti-rabbit IgG). Controls were performed without the first antibody to validate the results.

3.5. Observations and Images acquisition

Observations and images acquisitions were performed using a photonic microscope (Olympus BX 61) coupled to a high resolution digital camera (Olympus DP 71) and the Cell F software. Picrosirius red stained sections were observed and images were acquired in bright field mode while immunohistofluorescence images were acquired in fluorescence mode (Cyanine 3: 550/570 nm; Alexa Fluor 488: 495/519 nm).

3.6. Fiber type proportion

The proportion of each fiber type proportion was determined on the whole muscle cross section. The percentage of each muscle fiber type was calculated according to Meunier et al. (2010) using the image analysis Visilog 6.7 Professional Software (Noesis, Gif sur Yvette, France).

3.7. Spotting and identification of cell types

Myofibers types, identified by immunohistochemistry, were localised on the unstained serial sections intended for DUV microspectroscopy measurements. In this area, each myofiber characterised on its cell type was identified by a number. From 7 to 10 cells of each type were analysed in DUV microspectroscopy.

4. *DUV microspectroscopy analysis*

4.1. Spectra acquisitions

Synchrotron DUV microspectroscopy was performed on the DISCO beamline of the SOLEIL synchrotron radiation facility (Saint-Aubin, France) (Jamme *et al.*, 2010, Giuliani *et al.*, 2009). DUV monochromatized light (typically between 270 and 330 nm) was used to excite tissue sections through a 40x ultrafluar immersion objective (Zeiss, Germany). The

emission spectra were acquired from 290 to 540 nm and recorded with a 0.1 nm spectral resolution. The fluorescence spectrum arising from each excited pixel was recorded. On each selected cell, 20 acquisitions with 1 µm spatial resolution and 20s acquisition time per spectrum were made in the intracellular space. Among these 20 acquisitions, 10 were acquired in the central part of the cells and 10 were acquired on cell periphery to target mitochondria from the subsarcolemmal space and from superficial myofibrillar region located at the periphery of the intracellular space, near the sarcolemma (Nielsen et al. 2010). These last spectra acquisitions were performed several micrometers from the plasma membrane to avoid an overflow from the extracellular space. The 275 nm excitation wavelength was selected for a broad range excitation of intrinsic fluorochromes (Jamme et al, 2010).

4.2. Spectra treatments and statistical analysis

Autofluorescence spectra were spike and noise filtered using an in-house program written in MATLAB version 7.3 (The MathWorks, Natick, MA). The Unscrambler software (v9.8, Camo Software AS, Norway) was used to perform a baseline adjustment to zero and to apply unit vector normalization. Processed spectra were analyzed in principal component analysis (PCA). PCA was applied as an unsupervised approach, in order to handle this new set of data and reveal variances or combination of variables among this large multivariate data set. For this study, the fluorescence spectral domain was reduced to 290-540 nm. The number of possible components was always let sufficiently high (10). After analysis, the family label of each spectrum was revealed and the two first components were plotted. The mean characteristic spectrum of each group was also plotted in order to relate the separation to spectral feature. Score plots were used to show similarity maps allowing comparison of spectra regardless to sample categories. Loading plots derived from the first and second principal components X-loading plots were used to reveal and identify characteristics fluorescence peaks.

The fluorescence intensity for wavelength of interest identified on the PCA loadings were expressed as mean ± standard error of the mean (SEM). Variance analysis and mean comparisons were performed using one-way variance analysis and the Student-Newman-Keuls test under the XLSTATsoftware 2010 (Microsoft office, Redmond, USA).

RESULTS AND DISCUSSION

1. Muscle fiber characterization.

As expected, immunolabelling and enzymatic activity of SDH indicate that the soleus muscle has only oxidative metabolism fibers (66% type I and 34% type IIA) while the EDL muscle contains four types of pure fiber and hybrid IIX-IIB (4% of type I fibers, 18% fiber-type IIA, 24% type IIX fiber, 48% type IIB fiber and 6% type IIX-IIB) (Figure 1).

Whatever the muscle studied, the SDH activity is more pronounced in type IIA than in type I fibers (Figures 1 & 2), which is usually observed in rat muscles (Punkt *et al.*, 2004, Bloemberg *et al.*, 2012)

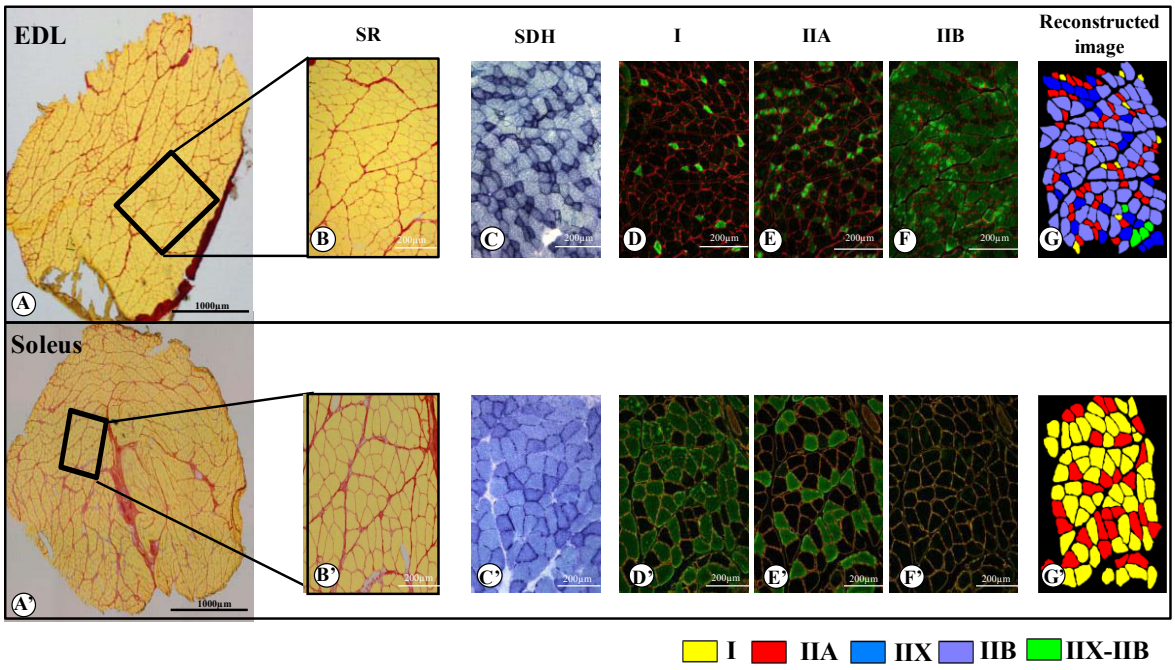

Figure 1: Histological muscles fiber type's characterization
Whole muscle cross sections of EDL (A) and soleus (A') muscles stained with Picrosirius red. Muscle fibers are stained in yellow and intramuscular connective tissue in red. For each muscle, all other sections are seriated, allowing multiple characterization of the same cells. (B & B'): higher magnification of an area of A & A'. (C & C'): SDH staining highlight the mitochondrial succinyl dehydrogenase ativity which is much more pronounced in oxidative fibers (I & IIA fiber types) than in glycolytic ones (IIB & IIX fiber types). (D-F & D'-F'): Each muscle fiber type was identified by immunohistofluorescence using monoclonal antibodies specific of MyHC isoforms (green fluorescence) and an anti-laminin antibody to highlight the cell periphery (orange fluorescence). (G & G'): From all immunohistofluorescence images, a virtual image is reconstructed showing the precise identification of each fiber type.

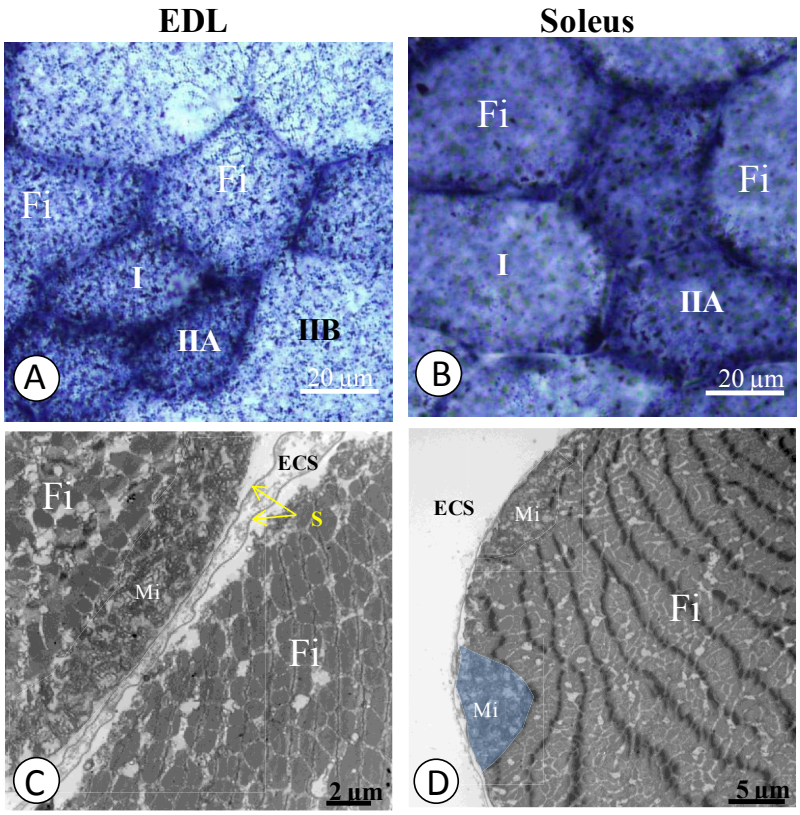

Figure 2: Mitochondria distribution in EDL and soleus muscles fibers
(A & B): Succinyl dehydrogenase (SDH) staining of transversal muscles sections of EDL and soleus respectively which fiber type was initially determined by immunohistofluorescence (light microscopy). Intensity of blue staining reflect the mitochondrial succinyl dehydrogenase activity and consequently the mitochondria location and density in the muscle cells. Glycolytic fibers are paler than oxydative one (A) that show dark blue coloration in the subsarcolemmal space which is discontinuous on soleus muscle fibers (B). Type IIA fibers express a higher SDH activity than type I fibers. (C & D): Transversal section of EDL and soleus muscle fibers (Transmission Electron Microscopy). Mitochondria packets are underlined in pale blue. Large amounts of mitochondria are seen on the subsarcolemmal space of the EDL oxydative fiber, but not in the glycolitic one (C). Mitochondria are distributed in discontinuous packets in the subsarcolemmal space of the soleus oxydative fibers (D), confirming the light microscopy results.
Fi: Fiber, ECS: extracellular space, Mi: mitochondria, I, IIA, IIB, IIX: type I, IIA, IIB, IIX fiber respectively.

Novak et al. (2010) made a survey from twenty articles with typing data on soleus and EDL muscles of male Wistar rats of 3 to 4 months old, close to the age of our own rats (5 months). From five different studies, Novak et al. calculated the fiber type's proportion of EDL muscle coming from Wistar adult male rats. They found in average 4% type I, 21% type IIA and 74% of fast glycolitic fibers including IIX, IIB and hybrid IIX-B fiber types which is very close to our results (4% type I, 18% type IIA and 78 % glycolytic fibers). Our results are also very close from the study of Bloemberg & Quadrilateral (2012) made on 5.5 month Sprague-Dawley rats (3.7 % type I, 20% type IIA fibers, 31% type IIX, 36% type IIB, and 9.3% of hybrid fibers).

Concerning the soleus muscle, the respective proportion of type I and type IIA fiber found in scientific literature are more heterogeneous with type I fibers ranging from 54 % to 90-97% (Cornachione *et al.*, 2011, Novak *et al.*, 2010, Punkt *et al.*, 1998)

Our results are closer to those of Cornachione et al. 2010, who observed on soleus muscle of adult female wistar rats about 54 % of type I fibers, 24 % of IIA fibers, the remaining fibers being considered as hybrid fibers.

The variation of percentage between studies, could be due to individual variability, the rearing method, the age of the animal (Pette *et al.*, 2000, Novak *et al.*, 2010) and the anatomical area of analysis (Punkt *et al.*, 1998). In our study, all rats were the same age, reared in the same conditions and sampling for analyses were always done on the middle part of the muscle.

2. DUV microspectrofluorescence

2.1. DUV Fluorescence spectra profile

An example of spectra acquisition area in the intracellular space and a typical spectrum is shown in Figure 3. The spectra analysis show two peaks at 332 and 408 nm with shoulders at 302 and 320 nm. The fluorescence emissionat 302 nm and 332 nm could be assigned to the tyrosine (tyr) (Pallu *et al.*, 2012, Jamme *et al.*, 2013), and tryptophan (trp) (Jamme *et al.*, 2013, Pallu *et al.*, 2012, Sahar *et al.*, 2009)respectively. The fluorophores emiting at 408 and 320 nm are not clearly identified. From the literature, fluorescence emission around 408 nm is usually assigned to collagen or elastin (Jamme *et al.*, 2013), but these proteins are specific of the extracellular space and are totally absent of the intracellular space where measurements were done. The excellent spatial resolution of the system (1 micron) excludes the possibility of recording such a strong signal from the extracellular space. This result suggests that an intracellular compound has an autofluorescence emission in the same wavelength range than collagen. It could be related to piridoxine, NADH, NADPH or some provitamines (Wagnieres *et al.*, 1998). The 320 nm emission fluorescence could correspond to shift of the tryptophan from 332 to 320 nm related to its insertion in a specific structural motif of proteins (Lakowitz, 2006)

2.2. Effect of spectra location on autofluorescence response

Whatever the considered muscle, no significant discrimination was observed between the spectra acquired in the periphery of the intracellular space (i.e. subsarcolemmal space) and those acquired in the central part of the cells. This result was initially surprising. Indeed, subsarcolemmal space and superficial myofibrillar region (closed to the subsacolemmal space) of oxidative metabolism type I and IIA fibers is known to contain much mitochondria (Filgueras *et al.*, 2012, Nielsen *et al.*, 2010, Yamada *et al.*, 2013). The mitochondrial inner membrane is rich in NADH responsible of transporting electrons. This NADH is autofluorescent when excitated at 260 nm (Jamme *et al.*, 2013) which is close to the excitation wavelength used in this experiment (275 nm) and we expected to highlight a peak located at about 460 nm corresponding to the maximal emission of NADH (Jamme *et al.*, 2013). The absence of peak at 460 could be due to the complete oxidation, during sample preparation, of this compound to NAD which is not fluorescent (Ren *et al.*, 1988, Mayevsky *et al.*, 2007). These authors show a state change of the compound from the reduced form (NADH) to the oxidized form (NAD) as a result of anoxia, occuring after animal sacrifice. However, NADH emission fluorescence could have shifted to about 408 nm because of the intracellular environment as suggested by Jamme et al. 2010. Indeed, SDH staining (Figures 1), which highlight mitochondria, confirmed a high density of these organelles at the edge of the oxidative muscle fibers but also demonstrated the high content of mitochondria in the whole sarcoplasm. It was confirmed by higher magnifications (Figure 2) showing that the subsarcolemmal mitochondria density was heterogeneous with packets of mitochondria spaced by subsarcolemmal areas containing low content in mitochondria. This mitochondria distribution decreases the probability to focus on high mitochondria density areas when targeting the subsarcolemmal space. This observation could explain the absence of discrimination between center and edge from the intracellular space.

2.3. Autofluorescence response regarding the fiber type

Our results show a clear discrimination of different cell types (Figures 4 & 5). Although the groups are well separated considering the whole data set (data not shown), the separation is even clearer by taking into account only the spectra acquired in the central part of cells. PCA score plots and loadings show a separation on PC2 of slow-twitch fibers (type I) from fast-twitch (type II) (Figure 4 A, B) on the 302 nm (Tyr) and 346 nm band and, in a lesser extent, a separation on PC1 of oxidative fibers (I, IIA) from glycolitic fibers (IIX, IIX

& IIB-IIB) (Figure 4 A) on 410 and 325 nm. Data on metabolic type are available only on EDL muscle since soleus muscle contains 100% of oxidative fibers.

Emission fluorescence at 302 nm is assigned to Tyrosine and suggest a large involvement of proteins in the response. Emission fluorescence at 325, 346 nm and 410 nm could originate from other intracellular compounds or could be due to shifts of tryptophan (from 332 to 325 and 346) and NADH (410) emission fluorescence.

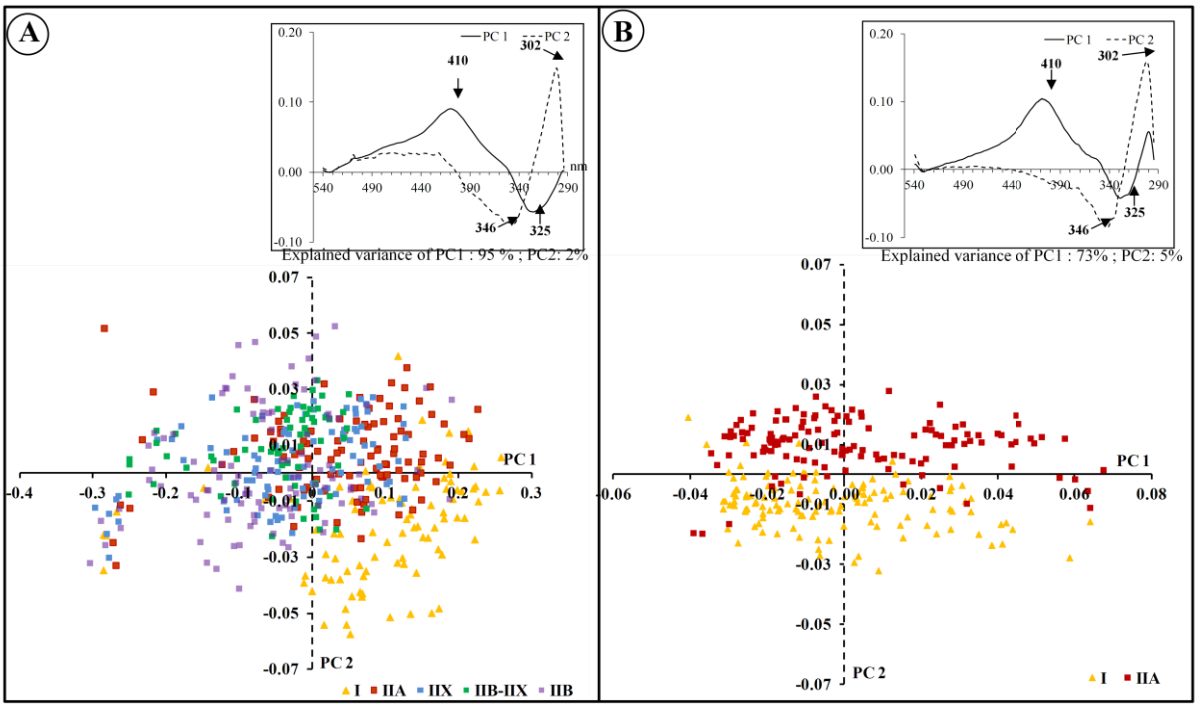

Figure 4: Autofluorescence response regarding the fiber type (spectra of fibers central part)
PCA score plots and loading of PC1 and PC2 from the four pure types of fiber (I, IIA, IIX, and IIB) and hybrids IIX-IIB in EDL (A) and from the two pure fiber types (I and IIA) in Soleus (B). On both muscles, the contractile type I (slow-twitch) and II (fast-twitch) are well separated along PC2. Compared to type I, type II shows higher fluorescence intensity at 410 nm and a lower one at 325 nm. In the score plot of the EDL (A), oxidative fibers (I and IIA) are separated from glycolytic fibers (IIX, IIB & IIX-IIB) along the PC1. Glycolytic fibers show higher fluorescence intensity at 302 nm and a lower one at 346 nm. The analysis does not discriminate glycolytic type fibers between them.

On EDL muscle, fluorescence intensity decreases with glycolitic metabolism and contraction speed at 410 nm while it increases at 325 nm (Figure 5). The differences between fibers types are amplified by the 410/325 ratio which decreases in the order I>IIA>IIX>IIX-IIB>IIB. However, the fluorescence intensity of hybrids IIX-IIB, although located between the IIX and IIB is not significantly different from pure types (IIX and IIB) that confine

themselves. In soleus muscle, compared to type IIA fibers, type I fibers show a higher fluorescence intensity of type at 346 nm and a lower one at 302 nm.

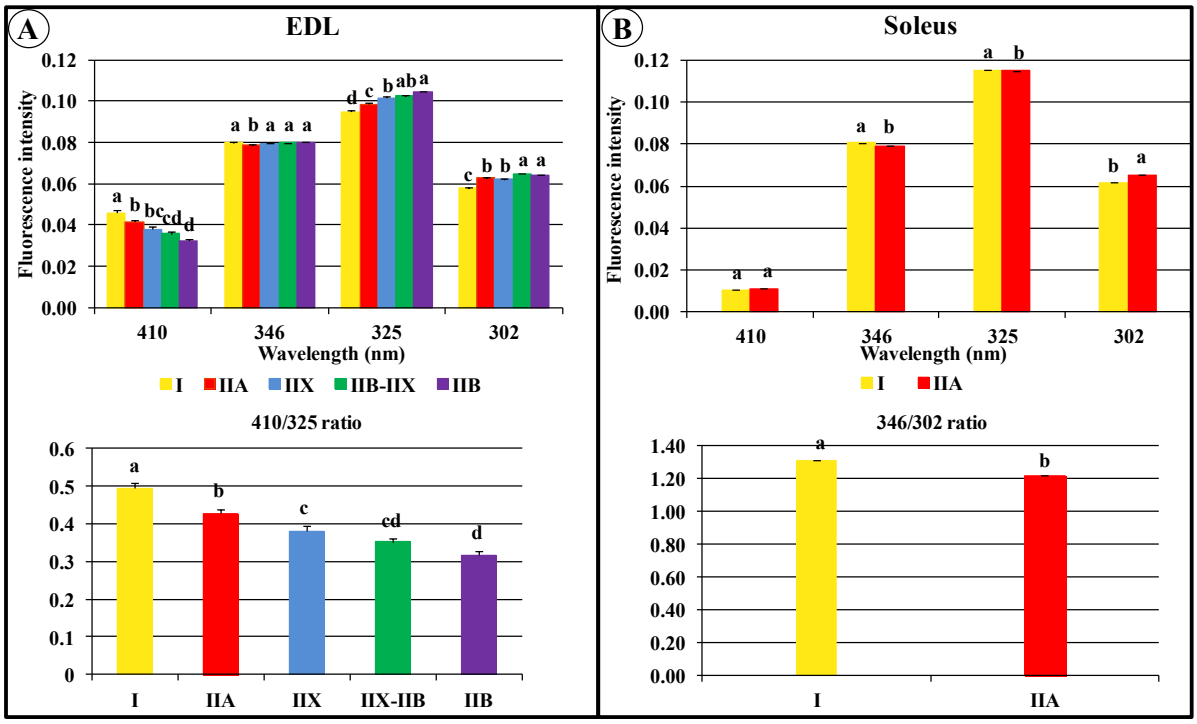

Figure 5: Fluorescence intensity of fiber types at wavelength evidenced by loadings of PCA
Fluorescence intensity corresponding to the descrimination peaks evidenced by PCA.
(A):On EDL muscle, fluorescence intensity decreases with glycolitic metabolism and contraction speed at 410 nm while it increases at 325 nm. The differences between fibers types are amplified by the 410/325 ratio. (B):Soleus muscle has only oxydative type fibers whith slow-twitch (Type I) and fast-twitch (Type IIA) fibers. Fluorescence intensity is lower at 346 nm and higher at 302 nm for type IIA fibers than for type I fibers. For a given wavelength (top histograms), different letters indicate a significant difference for $p<0.01$. For wavelength ratios comparisons (bottom histograms), different letters indicate a significant difference for $p<0.01$.

Whatever the considered muscle (soleus or EDL), compared to type IIA fibers, the fluorescence intensity of type I fibers is higher at 346 nm and lower at 302 nm.

Discrimination of contractile types (I versus II) on the center part of cells suggests differences in sarcoplasmic protein composition, including myofibrillar proteins. Some proteins varies on their isoform content depending on the contractile type, the best known being myosin. The comparison of the myosin isoforms aminoacid sequences of muscle fibers show more Tyr in type I than in type II fibers, which disagree with the higher autofluorescence emission at 302 nm observed in type II than in type I fibers. Although

myosin is the most abundant protein in myofibrillar material, other proteins have varied isoforms depending on the fiber type. Indeed, the myosin binding proteins or MBP such as C-protein, M-protein or titin (Gautel *et al.*, 1998, Wang *et al.*, 1991, Horowits, 1992, Obermann *et al.*, 1996) or the thin filament proteins such as tropomyosin, troponins (Bottinelli *et al.*, 1998, Danieli-Betto *et al.*, 1990) exist in different isoforms depending on the contractile type of the fiber. The differences in aminoacids composition, and especially in Tyr could explain a part of the autofluorescence variation between Type I and Type II fibers.

As the metabolism becomes more and more glycolytic, the fluorescence intensity at 410 nm decreases and increases at 325 nm (Figure 5). It is therefore likely that an intracellular compound discriminating metabolic type presents an autofluorescent response in the wavelength range of collagen and elastin (around 405 nm and 410 nm respectively) (Jamme *et al.*, 2013). The emission fluorescence at 325 nm and 346 nm are close to 333 nm which was assigned to Trp by various authors (Pallu *et al.*, 2012, Dufour *et al.*, 2003, Skjervold *et al.*, 2003b, Dufour *et al.*, 1998). The molecular environment of Trp is known to affect its fluorescence intensity and position (Lakowicz, 2006, Valeur *et al.*, 2001) explaining the slight shift observed in our case.

The preferential metabolic pathway, oxidative or glycolytic is associated to numerous enzymes and carriers of these respective pathways. The spectral differences observed could be due to the fact that compared to oxidative fibers, glycolytic fibers contain less of the fluorophore that autofluoresces at 410 nm and more that autofluoresce at 325 nm, both probably parts of unidentified macromolecules.

However, the autofluorescence of endogene fluorophores is much affected by their molecular and physicochemical environment than by their concentration. Indeed, the autofluorescence of a compound does not only depends on its concentration since the physicochemical environment has more influence on peaks positions and intensity (Lakowicz, 2006, Valeur *et al.*, 2001). Therefore, a large part of the differences observed in Tyr, the 325 nm and 346 nm (that could be assigned to different tryptophan insertion in proteins) and 410 nm autofluorescence wavelength emissions is probably a consequence of the differences in overall composition and molecular environment of different fiber types.

CONCLUSION

DUV fluorescence microspectroscopy coupled to synchrotron radiation has demonstrated its effectiveness for characterizing the muscle tissue. For the first time, muscle cells of different type have been discriminated directly on a tissue section exploiting the variation of their autofluorescent responses without adding exogenous markers.

Following an excitation at 275 nm, four main fluorescence emission bands were identified as possible discriminators of pure muscle fiber types: the 302 nm band assigned to Tyrosin and fluorescence peaks of unidentified fluorophore at respectively 325 and 346 nm that could correspond to tryptophan with different molecular environment, and 410 nm that could be a shift of NADH fluorescence emission. A muscle cell is adapted to suit different demands depending on whether it should resist fatigue, provide a violent effort or be most flexible. Its structure and composition are adapted to its function. Depending on the cell tasks, and therefore on the fiber type, the intracellular compartment presents differences in metabolic pathways, contractile proteins, and overall cytoskeleton appropriate to answer the cell needs. Therefore, the content of specific protein isoforms, soluble sarcoplasmic molecules, or even components of cellular organelles and sarcoplasmic reticulum could modulate the autofluorescence response of cells. Until now, no information enables us to say whether these changes in fluorescence intensity as a function of contractile and metabolic types are related to differences in concentration of fluorophores identified, including Trp and Tyr, or to their environment. However, identification of muscle fiber type is trivial in DUV fluorescence microspectroscopy choosing the appropriate excitation and emission wavelengths. Future developments using laser and laboratory microscopes (Zeskind *et al.*, 2007) could allow to characterize the fiber type quickly and at lower cost.

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ARTICLE n°5

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Postmortem changes of muscle fibers characterization by Deep UV Synchrotron microspectroscopy

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Postmortem changes of muscle fibres characterized by hyperspectral intracellular Deep UV autofluorescence

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Running title: *Postmortem* changes of muscle fibres characterized by hyperspectral intracellular deep UV autofluorescence

Key words: skeletal muscle, muscle fibre, *postmortem***,** meat, UV microspectroscopy, histology, synchrotron radiation

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ABSTRACT

After slaughter, the muscle cells undergo biochemical and physicochemical changes which may affect their autofluorescence characteristics. *Postmortem* glycogen metabolism was followed on rat Soleus and EDL muscles. The autofluorescent response of different EDL and Soleus muscle fiber types was investigated by Deep UV synchrotron microspectroscopy immediately after animal sacrifice and after 24 hours storage in a humid chamber. The content of glycogen initially low (23 micromoles glycosyl / g fresh muscle at 10 min *postmortem*) decreased until 18 micromoles /g fresh muscle at 24h *postmortem*. The pH of lower limb muscles reaches its minimum of 6.1 after 2h anoxia then gradually rose.

The spectral muscle fiber autofluorescence response showed discrimination depending on *postmortem* time (T0 versus T24h) on both muscles at 346 and 302 nm and to a lesser extent at 408 and 325 nm. Taken individually, all fiber types are discriminated but with variable accuracy, the type IIA showing better separation of T0/T24h than other fiber types.

These results highlight the relevance of using the autofluorescent response of muscle cells to rapidly assess their state of *postmortem* changes.

INTRODUCTION

Meat come from the skeletal muscle that generally containing different proportion of the four pure myofiber types : type I, type IIA, type IIX and type IIB composed of the respective myosin heavy chain isoforms (MyHC) I, IIa, IIx and IIb (Pette *et al.*, 1990)(Chagnot et al Article 4) . Hybrid fibers which are intermediate between two pure types contain two different myosin isoforms (I-IIA, IIA-IIX, IIX-IIB) are also found. The muscle fibers are characterized by their metabolism (oxidative or glycolytic) and their contraction speed (slow or fast). The type I are slow-twitch fibers while types II are all fast-twitch fibers. Type I and IIA fibers have a oxidative metabolism, IIX and IIB fibers have a glycolytic one.

After slaughter of farm animals, muscle undergoes significant metabolic, physical, structural and biochemical changes that determine the qualities of meat and meat products (Bendall, 1973, Greaser, 1986). After bleeding, the muscle is deprived of oxygen and nutrients. Muscle cells will try to survive by degrading their glycogen stock through anaerobic glycolysis. The lactate, which accumulates in cells, is accompanied by a drop in pH. When the energy reserves are depleted, muscle pH stabilizes at values generally comprised between 5.7 and 6.2 called ultimate pH. The kinetics of change in pH is species dependent and can range from 2 hours to 24 hours to poultry in cattle to rich the ultimate pH. After this first phase of *postmortem* changes, additional biochemical changes and significant ultrastructural alterations are observed and are related to the improvement of meat quality, especially meat tenderness (Ouali, 1990, Koohmaraie, 1994). The speed of these *postmortem* changes (also called meat maturation) is dependent on the species, and mostly on the metabolic and contractile type of considered muscles (Ouali, 1990). Degradation of myofibrillar structure is faster in the white fibers than in red ones.

Characterization of *postmortem* changes, related to meat quality, can be assessed by mechanical measurements, biochemical and / or ultrastructural analysis, but these methods are usually destructive and time consuming. Some authors have exploited the variation of physical properties of muscles to characterize differences in composition by fluorescence spectroscopy methods (Dufour *et al.*, 2003, Swatland, 1987, Skjervold *et al.*, 2003a)(Chagnot et al. Article 4). This highly sensitive method allows detecting fluorophores naturally present in muscle and whose properties are very sensitive to changes in their environment. Collagen, tryptophan, tyrosine or NADH are among the most abundant autofluorescent molecules in

muscle (Chagnot et al. Article 4) (Dufour *et al.*, 2003). The autofluorescence of collagen and tryptophan signal allows the discrimination of different muscles and the duration of *postmortem* meat storage (Dufour *et al.*, 2001, Frencia *et al.*, 2003).

More recently, the combination of a microscope to a UV fluorescence spectrometer coupled to synchrotron radiation has been used to characterize animal tissues at ultrastructural level (Jamme *et al.*, 2013, Jamme *et al.*, 2010, Pallu *et al.*, 2012, Petit *et al.*, 2010) Chagnot et al. Article 4). Chagnot and collaborator (Article 4) have discriminated the different types of muscle fibers in the rat muscle through their differences in intracellular compartment autofluorescence signal.

Our objective was to characterize at the cellular level the *postmortem* metabolism and structural changes of two rat muscles very different on their contractile and metabolic types and to explore the effect of a 24h maturation time on the muscle fibers autofluorescence response, taking into account their metabolic and contractile types.

MATERIALS AND METHODS

1. Animals and samples

The rat model was chosen because it allows a better control of rearing and slaughter conditions, it's easy to extract entire muscles,can be extracted entirely without lesion, achieve early samples in the laboratory by controlling the best kinetic sampling and temperature conditions.

Twenty four male Wistar rats, aged 5 months and weighting about 500 g, housed under controlled environmental conditions (room temperature 22°C; 12 h light-dark cycle, light period starting at 08:00 AM), fed *ad libitum* and given free access to water were sacrificed by decapitation following an isoflurane gas anesthesia in accordance with institutional guidelines on animal experimentation. Immediately after the slaughter, the lower limbs were carving and dissected under sterile conditions. Extensor Digitorum Longus (EDL), Tibialis Anterior, Gastrocnemus and Soleus muscles from right and left back legs were extracted from tendon to tendon avoiding any lesion.

2. Muscle postmortem kinetic

Muscles of three rats were immediately cryofixed at the end of dissection (10 minutes after decapitation). For the 21 other rats, muscles were suspended at 20 °C in a sterile moist chamber to prevent drying of the muscle and contamination from microorganisms. Lead ballast were attached at the caudal tendon of each muscle (1.5g for Soleus and EDL, 4.5g for Tibialis and Gastrocnemus) to preserve the anatomical muscle length (Figure 1S).

After respectively 30min, 1h, 2h, 4h, 8h, 12h and 24h *postmortem*, muscles of three rats were removed from their moist chambers and prepared for biochemical and imaging analysis.

3. pH measurements

As EDL and Soleus muscles are too small, pH measurements were done on Tibialis Anterior and Gastrocnemius muscles which metabolic and contractile types are closed to whose of EDL and Soleus muscle. pH measurements were done according to Fernandez et al., 2002, with adaptation to the small quantity of muscle available. Briefly, muscles were

weighed and ground in idoacetate buffer 5 mM (2.5ml / g of muscle) and pH was measured in the muscle suspension at room temperature.

4. Glycogen determination

Glycogen content was determined on each right EDL and Soleus rat muscles at each *postmortem* time by enzymatic procedures according to Bergmeyer, 1974, with slight modifications. About 0.15g of muscle tissue was homogenized with 1.8 mL of 0.55 M percloric acid. On two 0.5 ml aliquots, glycogen was hydrolysed in glucose with amyloglycosidase. Glucose was transformed in 6-phosphogluconolactone after hexokinase and G-6-P déshydrogenase action accompanied by NADH H+ production. NADH, proportional to glucose content, was determined by spectrophotometry at 340 nm and compared to standards to calculate the glucose concentration. Results were expressed in micromoles per gram of fresh tissue.

5. Histology

5.1.*cryofixation and histological sections*

Parts of left EDL and Soleus muscles from 0 (T0) and 24h (T24) *postmortem* time, were positioned on a cork plate with embedding medium (Tissue-Tek) and cryofixed by immersion in -160°C isopentane cooled with liquid nitrogen (-196°C). Based on a previously described protocol Chagnot et al (Article 4), serial cross-sections of entire muscles (10 µm thick) were realized using a cryostat (Microm, HM 560) and collected on quartz coverslips slide for DUV microspectroscopy fluorescence analyses. Sections were stored at -20 °C under vacuum until observation.

5.2. *Muscle fiber type determination*

The fiber typing was made according to Chagnot et al (Article 4). Fiber types were identified by highlighting the different myosin heavy chains isoforms (MyHC) thanks to specific mouse monoclonal antibodies BA-D5, SC-71, BFF3 (AGRO-BIO France). The different primary MyHC antibodies were revelated by an Alexa Fluor 488 labelled goat anti-mouse IgG secondary antibody (A 11001, Invitrogen). Moreover, the ECM protein, laminine, surrounding the muscle fiber, were stained using an anti laminin primary polyclonal antibody (L9393 Sigma) and a Cyanine Cy3-labeled secondary antibody (111-165-008, Jackson).

The myofiber response to the different MyHC antibodies allowed to identify fiber types I, IIA, IIB and hybrid IIX-IIB. IIX fiber types corresponded to the remaining unmarked cells.

Controls were performed without primary antibody to validate the results.

5.3. *Observations and Images acquisition*

Observations and images acquisitions were performed using a light microscope (Olympus BX 61) coupled to a high resolution digital camera (Olympus DP 71) and the Cell F software. Images were acquired in fluorescence mode (Alexa Fluor 488: 495/519 nm, Cyanine 3: 550/570 nm) according to Chagnot et al. (Article 4).

6. Deep UV fluorescence microspectrocopy

6.1. Spotting of cell types on unstained muscle serial cross-section

From 2 rat Soleus and EDL muscles (0 and 24h post-mortem), a unstained serial section was deposited on quartz coverslips, and 10 cells of each type (I, IIA, IIX, IIX-IIB, IIB) were spotted for fluorescence spectra acquisition by Deep UV microspectroscopy because fiber types identification by immunohistofluorescence was performed on other serial sections.

6.2. Spectra acquisitions

Synchrotron DUV microspectroscopy was performed on the DISCO beamline of the synchrotron SOLEIL radiation facility (Saint-Aubin, France) (Jamme *et al.*, 2010, Giuliani *et al.*, 2009). The spectra were recorded as previously described in Chagnot *et al*. (Article 4). Briefly tissue sections were excited at 275 nm under the microscope objective (Zeiss Ultrafluar 40x), the emission spectra were acquired from 290 to 540 nm. Full fluorescence spectra were recorded from each excited pixel. On each spotted cell, 20 acquisitions were made in the intracellular space (10 in the central region and 10 on periphery of the cell).

6.3. Spectra treatments and statistical analysis

The spectra treatments and statistical analysis were performed according to Chagnot *et al*. Article 4. Several EDL cells had 2 successive UV flash and we detected an effect of this

double flash on the fluorescence response during data analysis. Therefore, we decided to eliminate the double flash cells from the analysis. Then statistical analyses were done on 10 type I Soleus fibers, 10 types IIA Soleus fibers, 3 type I EDL fibers, 2 type IIA EDL fibers, 5 type IIX EDL fibers, 8 type IIX-IIB EDL fibers and 3 type IIB EDL fibers.

Autofluorescence spectra were spike and noise filtered using an in-house program written in MATLAB version 7.3 (The MathWorks, Natick, MA) and images from individual fluorescent component were produced. The Unscrambler software (v9.8, Camo Software AS, Norway) was used to perform a baseline adjustment to zero, to apply unit vector normalization and to analyze the processed spectra in principal component analysis (PCA). After analysis, the family label of each spectrum was revealed and the two first of the 10 components tested were plotted. The mean characteristic spectrum of each group was also plotted in order to relate the separation to spectral feature. Score plots were used to show similarity maps allowing comparison of spectra regardless to sample categories. Loading plots derived from the first (PC1) and second principal components (PC2) were used to reveal and identify characteristics fluorescence peaks.

Numerical variables of fluorescence intensity for wavelength of interest identified on the PCA loadings were expressed as mean ± standard error of the mean (SEM). Variance analysis and mean comparisons were performed using one-way variance analysis and the Student-Newman-Keuls test under the XLSTATsoftware 2010 (Microsoft office, Redmond, USA).

RESULTS AND DISCUSSION

1. *Postmortem* metabolism evolution.

The level of EDL and Soleus muscles glycogen is about 23 µmol / g fresh muscle at 10 minutes *postmortem* and decreased slightly with time for both muscles (Figure 1).

The initial rate of glycogen (around 23µmoles / g fresh muscle) is a little lower than the data from Calder & Geddes (1990) (Calder *et al.*, 1990) and Manabe et al. (2013) (Manabe *et al.*, 2013) who observed 30-40 µmol/ g of fresh muscle in the rat muscle. This difference is probably due to the delay of 10 minutes necessary for muscles dissection where a part of glycogen was depleted. Indeed 60% of muscle glycogen is degraded within 5 minutes after the death of the animal (Calder *et al.*, 1990). This hypothesis is validated by the initial pH (6.9), slightly lower than usual *in vivo* pH (7.2), as a consequence of glycogen degradation associated with a release of protons in the tissue.

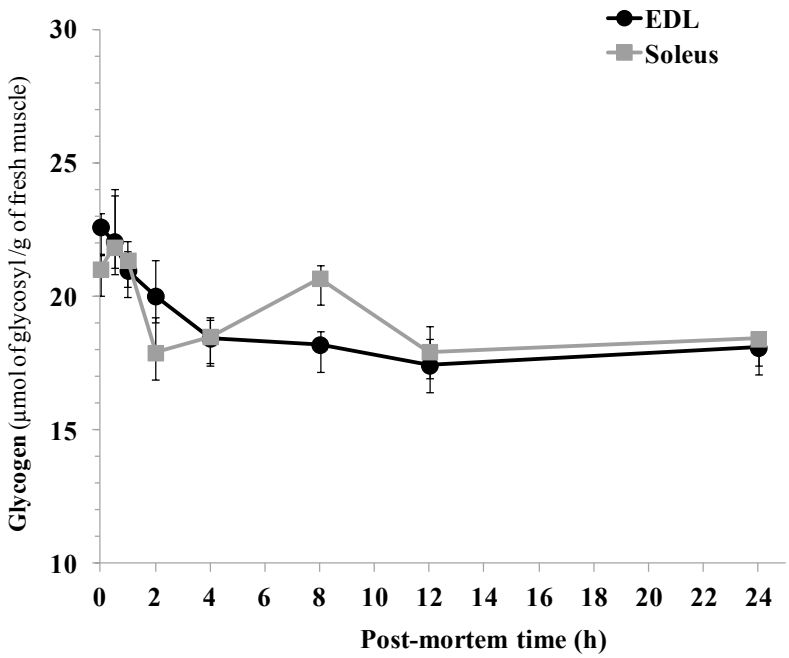


Figure 1: *Postmortem* evolution of glycogen content in EDL and Soleus muscles

The rat muscle glycogen content appears to be considerably inferior to what occurs in farm animal species with values of glycogen are about 60-70 umol / g in rabbits (Astruc *et al.*, 2002), 85-100 micromol / g in pigs (Fernandez *et al.*, 2002, Kocwin-Podsiadla *et al.*, 1995), 45-50 umol / g in turkey (Fernandez *et al.*, 2001), 80 to 100 umol / g in cattle (McVeigh *et al.*, 1982, Immonen *et al.*, 2000) and about 70 umol / g in sheep (Diaz *et al.*, 2014). Glycogen reserves remained relatively high in rat muscle (Figure 1), unlike in farm animals whose meat glycogen reserves fall to less than 10 micromoles / g after rigor mortis with a pH stabilization around 5.6-6.0 depending on the type of muscle (Bendall, 1973, Greaser, 1986).

From the sacrifice of animals, the pH decreases until 2 hours and re-increases faster for the Tibialis Anterior than for the Gastrocnemius muscle, but with a similar profile curve (Figure 2). The slight pH drop implies a small decrease in their glycogen content. The rise in pH after 2h *postmortem* is unexpected and suggests neutralization with a basic compound. Adenosine monophosphate degradation (AMP) to Inosine monophosphate (IMP), and protein degradation release NH3 which could induce this increase in pH. Muscle isolation and conditioning in closed moist chamber could promote the release and fixation of basic compounds newly formed with resulting a pH increase.

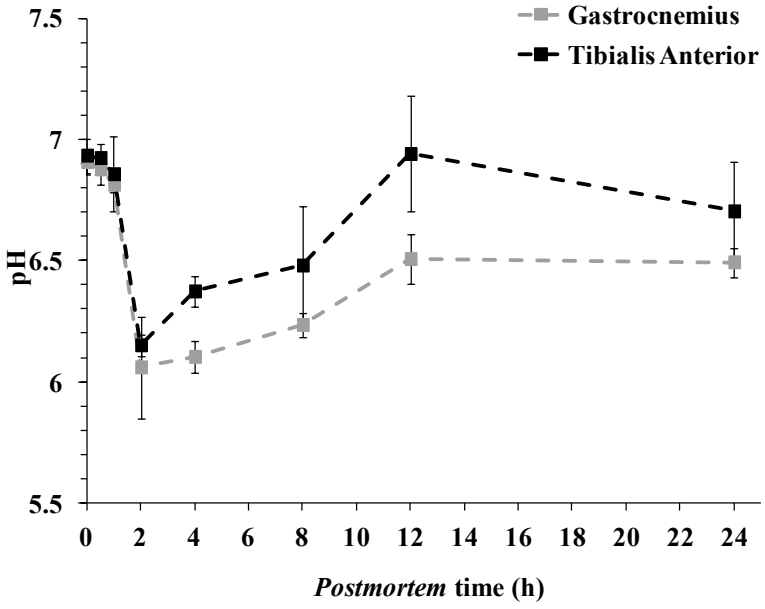


Figure 2: *Postmortem* pH evolution of Gastrocnemius and Tibialis anterior muscles

2. Fluorescence response of muscle fibers according to *postmortem* time

The results of PCA and variance analysis are presented in Figures 3-5. PCA and loadings indicate a separation of the two *postmortem* times (T0 and T24) essentially in the PC2 on the fluorescence intensities at 302 and 346 nm, and in a lesser extent, on the PC1 at 325 and 408 nm, whatever the considered muscle (Figures 3 and 4). In general, 302 nm and 408 nm fluorescence intensities were higher and 346 nm and 325 nm fluorescence intensities lower for T0 than T24 samples. Separation on the EDL PCA is less clear than in the Soleus, mainly because the EDL is composed of four types of fiber plus IIX-IIB hybrid fibers while the Soleus is composed of only I and IIA fibers.

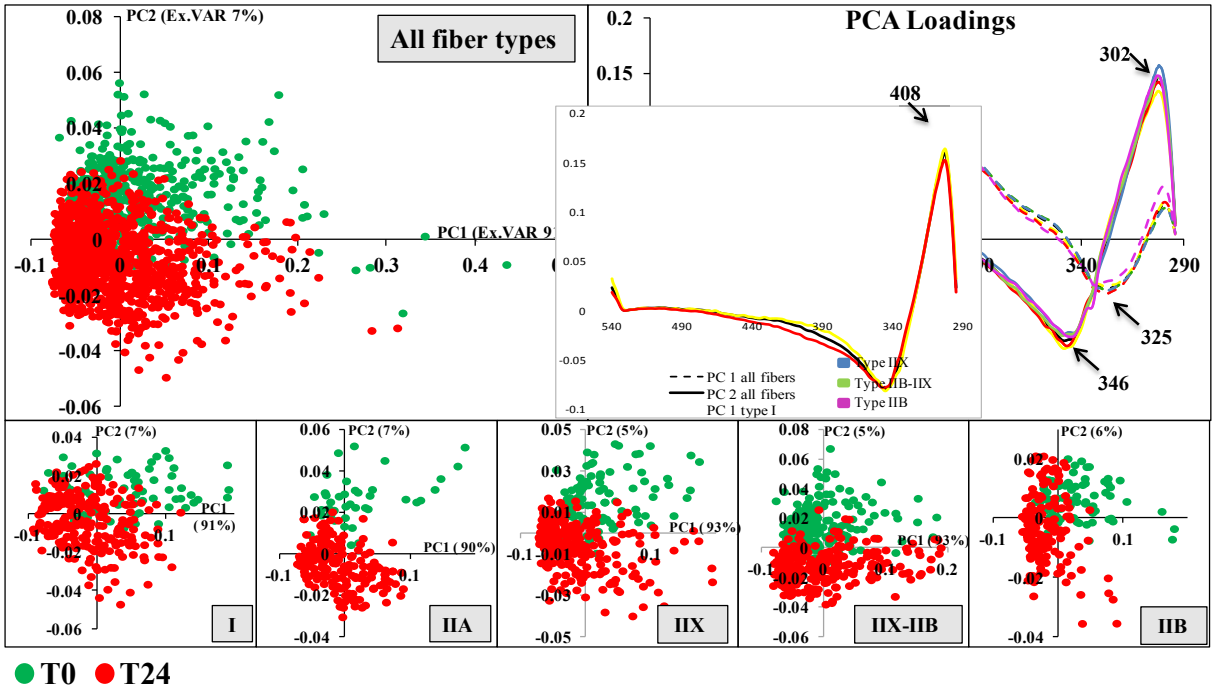


Figure 3: PCA score plots and loadings of fluorescence response of fresch (T0) and 24 hour *postmortem* (T24) EDL muscle
Postmortem time was most discriminated on the PC2 (346 and 302 nm bands) for IIA, IIX and IIX-IIB fibers and on the PC1 (408 and 346 nm bands) for I and IIB fiber types.
Ex.VAR: explained variance

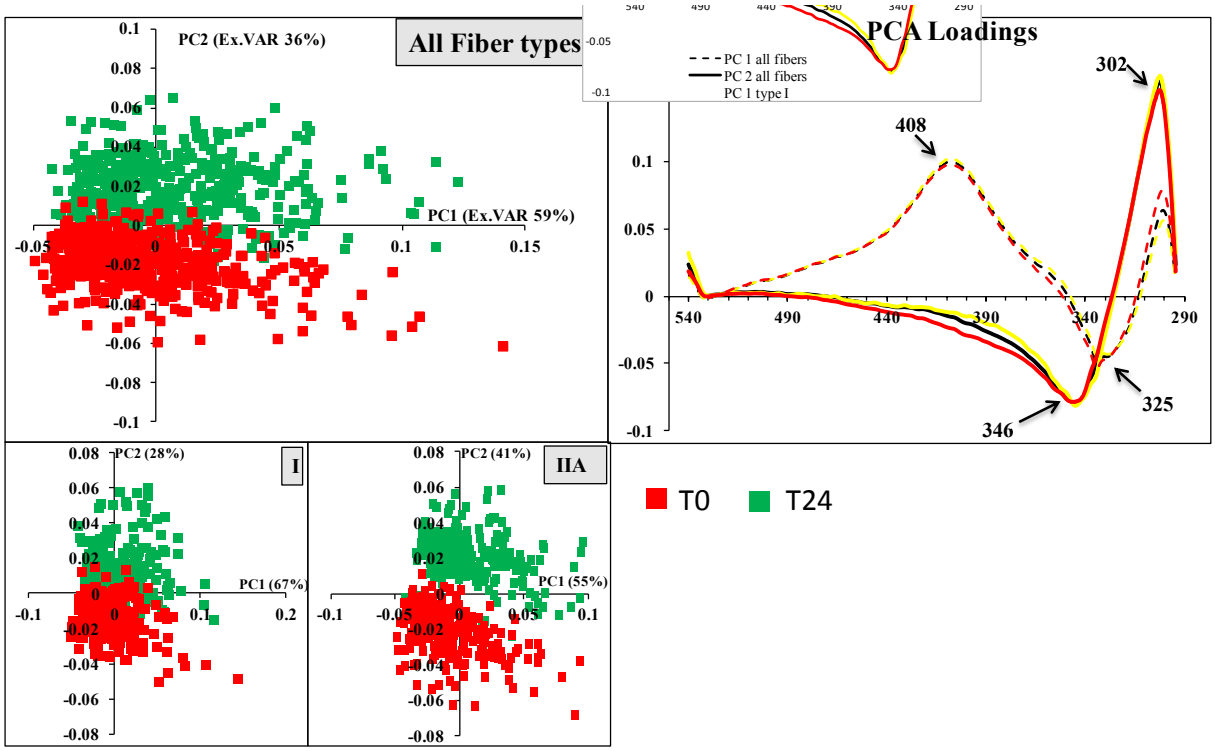


Figure 4: PCA score plots and loadings of fluorescence response of fresch (T0) and 24 hour *postmortem* soleus muscle
Ex.VAR: explained variance. *Postmortem* time was discriminated on the PC2 on the 346 and 302 nm bands.

Some cell types present a better separation of *postmortem* times like type IIA fibers which show excellent discrimination of postmortem times on both the EDL and Soleus muscles. IIB-IIX hybrid fibers and IIX fibers (absent in the Soleus muscle) also show a good separation regarding the *postmortem* time. *Postmortem* time of soleus muscle type I fibers discrimination is a slightly bit less marked than for type IIA but still very sharp. In contrast, *postmortem* time discrimination of EDL type I and type IIB fibers is not as sharp on PC2. For these fibers types, the *postmortem* time is discriminated on both PC2 at 302 and 346 nm and PC1 at 408 and 326 nm.

Discriminatory wavelengths of PC1 and PC2 are exactly the same for every 2 muscles (EDL and Soleus) and all types of fibers: 325 nm and 408 nm for PC1, 302 and 346 for PC2. So it seems that only few fluorophores are concerned in *postmortem* time discrimination.

Significant differences of emission fluorescence intensities regarding the *postmortem* time are amplified by the 346/302 and 408/325 ratios which are systematically lower and higher respectively for T0 compared to T24 muscle fibers. (Figure 5).

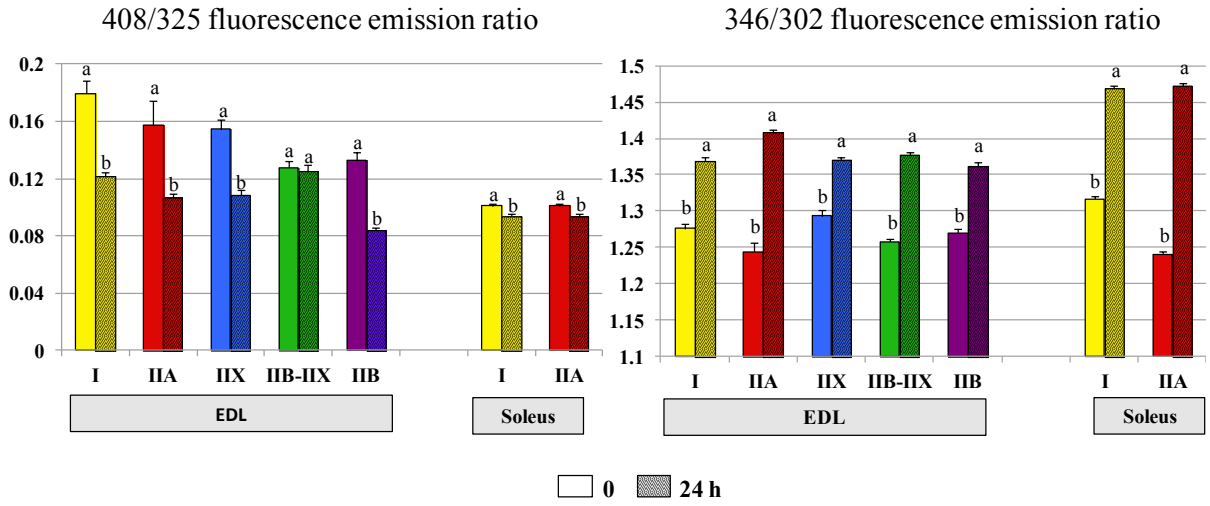


Figure 5: Ratios of fluorescence intensity wavelengths evidenced by loadings of PCA regarding the *postmortem* time
Fluorescence intensity ratio of wavelengths corresponding to discrimination peaks evidenced by PCA. 24hours *postmortem* time muscle fibers (T24) show higher 346/302 and lower 408/325 values than fresh muscles fibers (T0). Different letters indicate a significant difference for $p<0.01$ of the same fiber type from 0 and 24 hour *postmortem* time

The fluorescence emission at 302 nm is assigned to tyrosine (Jamme *et al.*, 2013, Pallu *et al.*, 2012). The assignment of fluorescence emissions at 326, 346 and 408 nm to specific compounds is less obvious. The tryptophan fluoresces between 325 and 360 nm depending on the cell type and the molecular environment (Jamme *et al.*, 2013, Pallu *et al.*, 2012)(Chagnot et al. Article 4). In living cells, it is assumed that it fluoresces at 335 nm (Jamme *et al.*, 2013). This wavelength (335 nm) is located between the two peaks of fluorescence at 326 and 346 nm and it is difficult to know which one corresponds to tryptophan. Finally, the compound which emitting fluorescence at 408 nm can not be identified accurately. Collagen and elastin fluoresce in this wavelengths range (Jamme *et al.*, 2013), but these proteins are located in the extracellular matrix, which excludes the involvement of these compounds in observed fluorescence as our spectral acquisitions are strictly intracellular.

The fluorescence peak at 408 nm could be attributed to NADH and / or pyridoxine since on living cells these compounds are suspected to fluoresce in this wavelength range (Jamme *et al.*, 2010, Jamme *et al.*, 2013). We do not work on living cells, but on histological sections of muscle cells that have been dried in air. Then, physicochemical environment is substantially different than in living cells, inducing shifts of the fluorescence maxima of NADH and tryptophan. In our experiment, all samples were prepared in exactly the same conditions and data are therefore comparable.

From animal slaughtering, the muscle undergoes structural, biochemical and physicochemical changes(Greaser, 1986, Bendall, 1973). The pH drop is accompanied by a glycogen decrease, a lactate accumulation in cells and muscle contraction. Quickly, the plasma membranes are perforated (Astruc, 2014) and gradually ionic gradients plasma membrane), nucleus and organelles (mitochondria, lysosome sarcoplasmic reticulum …) collapse leading to the ion concentration equilibrium in the cells. Muscle proteins are degraded into peptides under the action of endogenous proteolytic enzymes (Ouali, 1990, Koohmaraie, 1994). These phenomena substantially modify the intracellular composition with consequent changes on its pH, ionic strength and osmotic pressure. It is unlikely that the amino acid components of the peptides derived from protein hydrolysis are degraded.

Therefore, T0 and T24 differences in fluorescence intensity of amino acids are probably related to changes in their environment (Valeur *et al.*, 2001, Lakowicz, 2006). The differences are mainly seen on the PC2 loadings at 302 nm corresponding to tyrosine, and 346 nm. The latter band could match the trp whose molecular environment shift the peak of 335 nm (Jamme *et al.*, 2013) at 346 nm.

The level of discrimination seems dependent on both muscle (EDL or Soleus) and fiber type. Compared to type I fibers, the better *postmortem* time separation on IIA fibers suggested more marked environmental changes. Compared to type I fibers, fast-twitch type IIA fibers are associated with higher level of glycogen and therefore a higher lactate production and a lower ultimate pH. These differences may partly explain the better *postmortem* times separation on the PCA of type IIA fibers.

Postmortem time discrimination, less clear on the EDL than on the Soleus muscle is probably related to the fact that the EDL contains 4 types of fibers more IIX-IIB hybrids. In the EDL, the *postmortem* time of types I, IIB and IIX fibers is discriminated on both PC1 and PC2, the T24 showing a lower fluorescence intensity at 408 nm and a higher one at 325 nm than the T0.

Compared to T0, the decrease in fluorescence intensity at 408 nm for T24 reinforces our hypothesis that this band corresponds to NADH. Indeed, NADH (fluorescent) is completely oxidized to NAD (non-fluorescent) after a short period of anoxia, and all the more after 24 hours *postmortem* (Ren *et al.*, 1988, Mayevsky *et al.*, 2007), which would explain the difference in 408 nm intensity as demonstrated on the PC1 loading. The fact that the

discrimination at 408 nm is not as clear as some types of fiber may be due to the rate of oxidation of NADH depending on the fiber type.

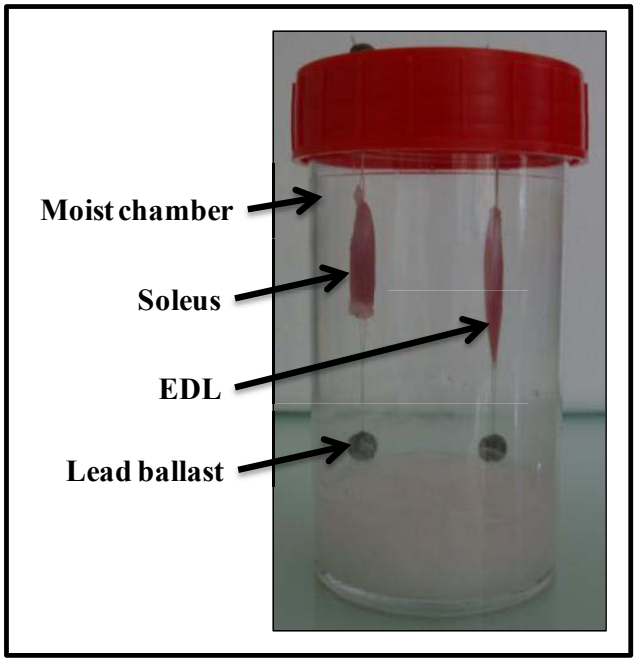
CONCLUSION

The muscle physicochemical and biochemical changes subsequent to its extended anoxia lead to a variation of the fluorescence characteristics of endogenous molecules emitting at 302 and 346 nm and to a lesser extent at 408 and 325 nm. The fluorescence at 302 nm is assigned to tyrosine. Compounds emitting fluorescence at 408, 346 and 325 nm cannot be identified with certainty, but are suspected to be tryptophan (346 nm), NADH (408 nm) and the 325 nm peak could be assigned to an enlargement of the tryptophan band (Lakowicz, 2006), reflecting a shift relative to tryptophan environment changes. The analysis of the fluorescence spectra allows the discrimination of 24 hours *postmortem* muscles from 10 minutes *postmortem* ones. The quality of discrimination depends on the fiber type; the most discriminated being the IIA fiber type suggesting a more efficient discrimination of *postmortem* time on muscle with high type IIA fiber proportion.

Our results demonstrate the benefit of exploiting the variation of autofluorescence muscle cells to rapidly characterize the effect of prolonged anoxia. These observations bring into perspective the possibility of using the DUV spectroscopy in fields as far apart as medicine or muscle food to diagnose the effect of muscle ischemia after a stroke or trauma or characterize the degree of maturation of meat.

SUPPLEMENTARY MATERIALS

Figure 1S: Positioning of the muscles in moist chamber



ACKNOWLEDGEMENTS

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ARTICLE n°6

Caroline Chagnot, Annie Vénien, Mickaël Desvaux and Thierry Astruc

Colonization of meat by *Escherichia coli* O157:H7: Investigating the bacterial tropism at *postmortem* for different skeletal muscle types and fibers

Pour publication dans Molecular Nutrition & Food Research

Colonization of meat by *Escherichia coli* O157:H7: Investigating the bacterial tropism at *postmortem* for different skeletal muscle types and fibers

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Running Title: Colonization of muscle by *Escherichia coli* O157:H7

Keywords: Enterohemorrhagic *Escherichia coli*, Food contamination, Bacterial adhesion, Surface colonization

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ABSTRACT

Enterohemorrhagic *Escherichia coli* (EHEC) are responsible for serious pediatric diseases and of great concern for the meat industry. Ruminants are the main reservoir of EHEC, which fecal contamination of foodstuff can be direct or indirect. Contamination of meat by EHEC occurs at slaughtering, essentially at dehiding stage where bacteria can be transfer from hides to carcasses. The skeletal muscles are constituted of two major biological tissues, i.e. the muscular and connective tissues. Four major pure types of muscle fibers (I, IIA, IIX, IIB), which differ in their metabolism and contraction speed, can be discriminated, whereas the endomysium, perimysium and epimysium are the different connective tissues forming the extracellular matrix (ECM) and found at different organizational levels of the muscle. Investigating the attachment of *E. coli* O157:H7 to meat, it clearly appeared that bacteria displayed differential tropism as a function of the muscle types. The *postmortem* evolution of muscle was further considered in relation to the different types of skeletal muscles and constitutive fibers. While *E. coli* O157:H7 adhered similarly to the different types of muscle fibers, bacterial adherence essentially occured at the ECM. By bringing further insight on the ability of *E.coli* O157:H7 to adhere to skeletal muscle, this investigation paves the way to develop innovative practices and strategies to reduce the contamination of meat by EHEC.

INTRODUCTION

In meat industry, the significance of enterohemorrhagic *Escherichia coli* (EHEC) as a serious public health problem was undoubtedly recognized. EHEC are foodborne pathogens, producing Shiga toxin responsible for serious pediatric diseases such as HUS (Hemolytic Uremic Syndrome) or TTP (Thrombotic Thrombocytopenic Purpura) (Karch *et al.*, 2005, Tarr, 2009, Gould *et al.*, 2009). Worlwide, many food poisoning associated with the consumption of contaminated meat products by EHEC have been reported. EHEC O157:H7 are the most frequently incriminated strains. Ruminants (mainly cattle) are the natural reservoir of these bacteria and the origin of contamination is essentially fecal (Chase-Topping *et al.*, 2008a). It is generally accepted that the muscle masses of healthy animals are sterile (Gil, l979) and that bacterial contamination of muscles occurs at slaughtering, mainly when the hide is removed or when good practices are not respected, *e.g.* evisceration accident (Chase-Topping *et al.*, 2008a). In the beef industry, the respect of good hygienic practices during slaughtering reduces contamination of carcasses but cannot guarantee the absence of *E.coli* O157:H7 from meat (Rhoades *et al.*, 2009). Bacterial survival and growth in meat tissues is dependent upon their ability to attach on meat surfaces.

Meat results from *postmortem* maturation of skeletal muscle that contains different proportion of the four types of myofiber, *i.e.* types I, IIA, IIX and IIB (Pette *et al.*, 1990), which differ by the type of myosin heavy chain isoforms (MyHC) (Chagnot *et al* article 4). Hybrid fibers contain two different myosin isoforms (I-IIA, IIA-IIX, IIX-IIB) but are found in lower proportion. The different muscle fibers are discriminated by their metabolism, *i.e.* oxidative (types I and IIA) or glycolytic (types IIX and IIB) and their contraction speed, *i.e.* slow twitch (types I) or fast twitch (types II). Structural difference had been also observed such as variation of fiber cross sectional area depending on the fibers specialization and/or muscle types (Realini *et al.*, 2013). The proportion of fiber types composing each muscle depends on its function. Muscle fibers are surrounded by connective tissue forming the extracellular matrix (ECM); it is divided into three types: (i) endomysium, the deep layer in contact with muscle fiber, (ii) the perimysium surrounding the fiber fascicle, and (iii) epimysium the most exposed part of muscle. Despite few difference of protein proportion observed, the three types of connective tissue are similar and are mainly composed of fibrillar collagens of type I and III (Gillies *et al.*, 2011).

E. coli O157:H7 has the ability to attach the beef meat (Chen *et al.*, 2007, Cabedo *et al.*, 1997, Rivas *et al.*, 2006a, Li *et al.*, 1999) and colonize muscle extracellular matrix (ECM) proteins such

as collagen (Auty *et al.*, 2005, Medina, 2001, Chagnot *et al.*, 2013a). Depending on growth conditions, specific and non-specific bacterial adhesion could occur on some ECM fibrillar proteins (Chagnot *et al.*, 2013a). Moreover, temperature and pH had a strong influence on initial bacterial adhesion (Chagnot *et al.*, 2013a). After slaughter, the muscle is converted to meat following of a succession of significant metabolic, physical, structural and biochemical changes (Bendall, 1973, Greaser, 1986), which could influence bacterial adhesion to carcasses.

Following the blooding and therefore oxygen and nutrient deprivation, muscle pH decrease from 7.2 to about 5.0-6.0 depending on initial content in muscle glycogen and lactate accumulates in the cells (Bendall, 1973, Monin *et al.*, 1985) leading to an increase in osmotic pressure in the muscle cells. Muscle temperature also decreases more or less quikly, depending on the cooling speed of the carcasses and on the muscle depth (Follett *et al.*, 1974). Intracellular water diffuses into the extracellular space, which leads to a decrease in cell size and an increase in the relative space of extracellular spaces (Offer *et al.*, 1988, Guignot *et al.*, 1993, Huff Lonergan *et al.*, 2010). White glycolytic muscles are generally more susceptible to exudation than red muscles (Lefaucheur, 2010).

After slaughter, the meat is deliberately stored for several days in a cold room to improve its texture and flavor. During this stage, the muscle undergoes news structural and biochemical transformations. The action of endogenous proteolytic enzymes leads, among other things, to the fragmentation of the muscle fibers. Maturation is characterized by an important series of ultrastructural changes. Mitochondria are deformed and their membranes are altered (Abbott *et al.*, 1977, Ouali *et al.*, 2013).Degradation of costumers, which are junctions of cytoskeletal proteins to the sarcolemma, lead to the detachment of the sarcolemma from the peripheral myofibrils (Taylor *et al.*, 1995, Taylor *et al.*, 1998, Waardenberg *et al.*, 2008). The sarcolemma itself perforates (Currie *et al.*, 1983, Heffron *et al.*, 1974, Rowe, 1989, Astruc, 2014). Myofibrils break at the N2 line, adjacent to the Z line (Davey *et al.*, 1970, Dutson *et al.*, 1974, Abbott *et al.*, 1977, Koohmaraie, 1994, Taylor *et al.*, 1995, Taylor *et al.*, 1998, Raynaud *et al.*, 2005). The hydrolyzed products are released into the sarcoplasm and substantially change its biochemical composition. The speed of these *postmortem* changes depends on the species, and mostly on the metabolic and contractile type of considered muscles, white glycolytic muscles being more sensitive to proteolysis than red ones (Ouali, 1990). The intramuscular connective tissue also undergoes some changes during the meat maturation (Bailey *et al.*, 1989, Nishimura, 2010). Recently, Nishimura (2010) suggested that *postmortem* degradation of proteoglycans destabilize the ECM and lead to a disintegration of the collagen fibers. All the modifications generated by the slaughter and *postmortem* changes in

muscles may modulate the adhesion of bacteria to their support, especially after water transfers that may convey soluble potential substrates in tissue areas more easily accessible to bacteria. The kinetics of *postmortem* proteolysis suspected of being different depending on the muscle fiber type could also be a factor of variation of bacterial adhesion.

This study focuses on the interactions between bacteria and muscle and particularly on the influence of metabolic and contractile fiber type as well as the *postmortem* structural modification on the adhesion of *E. coli* O157:H7.

MATERIAL AND METHODS

1. Biological material

1.1. Muscle Sampling and maturation processing

Two muscle models were chosen based on their very different metabolic and contraction speed features *e.i.*: (i) Soleus containing only oxidative fibers (types I and IIA), and (ii) Extensor Digitorum Longus (EDL) containing essentially the glycolytic fibers, IIX (24%) , IIB (46%) but also, in lower proportion, types I (4%) and IIA (18%) (Chagnot *et al.* Article 4). The rat model was used because it allows a better control of slaughter conditions and *postmortem* kinetics as well as the possibility of extracting the entire muscles without any lesion.

Five Wistar male rats were used (5 months old, about 500 g weights). After an anesthesia by isoflurane gas, they were sacrificed by decapitation in accordance with institutional guidelines on animal experimentation. Immediately after the slaughter, the lower limbs were carved and dissected under sterile conditions. From tendon to tendon, EDL and Soleus muscles were sampled without lesion. Muscles were suspended in a sterile moist chamber maintained at 20 °C to prevent contamination from microorganisms and prevent muscle alteration from drying. The artificial tension of muscle imposed by the animal carcass was mimicked by using lead ballast (1.5g) at the bottom muscle tendon. Based on a previous study Chagnot *et al* (Article 2), two different muscle *postmortem* times were considered, *e.i.* T0 (10min after slaughter) and T24 (24h after slaughter).

1.2. Bacterial strain

A detoxified strain of *E. coli* O157: H7 EDL933 was used in this study, the *E. coli* O157:H7 CM-454 (Chagnot *et al* Article 2)(Gobert *et al.*, 2007) transformed with the vector pSARE-Red1 expressing the fluorescent protein mRuby (Chagnot *et al.*, 2013a, Perna *et al.*, 2001). Strain was cultured in two different nutrient media: (i) DMEM (Dulbecco's modified eagle medium, Gibco) and (ii) LB (lysogeny broth) adjusted with NaOH (0.1 M) to reach pH 7 at the time of sampling corresponding to the pH of rat muscle at T0 and T24 (Chagnot *et al* Article 5). A preculture was set up from one bacterial colony grown in the respective nutrient broth medium added by 300 µg ml^{-1} of erythromycine at 39°C in an orbital shaker at low speed (70 rpm) till stationary phase.

1.3. Bacteria growth for the adhesion tests

Preculture was diluted 1:100 and grown as described above. Sampling was performed during the exponential growth phase at an OD_{600nm} of 0.5, *i.e.* about 10^8 CFU ml^{-1}. Chloramphenicol was added and mixed gently at a final concentration of 170 µg ml^{-1} to prevent *de novo* protein synthesis and growth during the time of contact of bacterial cells with muscle in the adhesion assay. No mechanical treatement was used to preserve cell surface supramolecular structures potentially involved in adhesion (Chagnot *et al* Article 2).

2. Bacteria adhesion to cutting muscles

2.1. Sample processing

Both right and left EDL and soleus muscles of two rats were used for bacterial adhesion assay on muscle sections and histology. At T0 and T24, parts of EDL and Soleus muscles were positioned on a cork plate with embedding medium (Tissue-Tek) and frozen to -160°C in isopentane with liquid nitrogen (-196°C). Based on a previously described protocol (Article 4 and 5), serial cross-sections (10 µm thick) were obtained using a cryostat (Microm, HM 560) and collected on glass slides.The sections were stored at -20°C under vacuum until use.

2.2. Histochemistry and image analysis

Sections were stained by Picrosirius red revealing the intramuscular connective tissue (epimysium, perimysium and endomysium) in red and muscle fibers in yellow (Liu *et al.*, 1995).

The fiber typing was made according to Chagnot *et al* Article 4. Briefly, to the identification of slow and fast myosin heavy chains isoforms (MyHC), mouse monoclonal antibodies specific to MyHC isoforms BA-D5, SC-71, BFF3 (AGRO-BIO France) were used in three differentes serial muscle cross sections. The myofiber response to the different antibodies permitted to deduct the subtypes I, IIA, IIB and hybrid IIX-IIB, the types MyHC-IIX corresponded to the remaining unmarked cells. The different primary MyHC antibodies were revelated by an Alexa Fluor 488 labelled goat anti-mouse IgG secondary antibody (A 11001, Invitrogen). Moreover, the ECM protein, laminine, surrounding the muscle fiber, were stained using an anti laminin primary polyclonal antibody (L9393 Sigma) and a Cyanine Cy3-labeled secondary antibody (111-165-008, Jackson).

The cross-sections were incubated with both primary antibodies against MyHC and laminin. After washing, both labelled secondary antibodies were incubated revealing the primary antibodies binding. Controls were performed without primary antibody to validate the results.

Observations and images acquisitions were performed using a photonic microscope (Olympus BX 61) coupled to a high resolution digital camera (Olympus DP 71) and the Cell F software. For histology assay, picrosirius red stained sections were observed and images were acquired in bright field mode while immunohistofluorescence images were acquired in fluorescence mode (Cyanine 3: 550/570 nm; Alexa Fluor 488: 495/519 nm. Images of immunohistochemistry processing was realised with the program FibTypFluo in visilog 5.4 (Noesis, France) software (Meunier *et al.*, 2010) creating a virtual image of the different fiber types composing the muscle section.

On a histological section without any staining, the perimeter of 1.5cm^2 square was traced with hydrophobic gel (PAP pen for immunostaining, Sigma), to maintain the bacterial solution on the slide. Then, 500 µl of bacterial suspension was deposited with cutted tips. Muscle sections were incubated statically in a humid chamber at 25°C for 30min. After incubation, bacterial suspension was removed by pipetting and washed by dipping two times, in milliQ water, to remove non-attached cells. The experiment was repeated twice on serial sections of both muscles in each growth condition (DMEM or LB) and *postmortem* time (T0 and T24).

At least 6 fields of view (magnification X2000) were analysed on each muscle cross-section. On each field of view, two images were systematically recorded in fluorescent and in bright field mode respectively, the first one revealing bacteria adhered (Cyanine 3: 550/570 nm) and thesecond one showing the muscle fibers previously identified on their fiber types by immunohistofluorescence. Then, images were processed with visilog 5.4 (Noesis, France) software. The pixels corresponding to bacteria were extracted by thresholding segmentation of the light gray levels and the muscle fibers image (bright field mode) and bacteria (artificial gray scale images) were superimposed. The relative area of bacteria was assessed by quantifying the number of these pixels in regard to the total number of pixels corresponding to the cell. Ten cells of the same fiber type were compared for different conditions of post-mortem time and medium of bacterial growth.

3. Bacteria adhesion to entire muscles

3.1. Sample processing

EDL and soleus muscles of 3 rats were incubated 30 minutes in 15 ml bacterial solution statically at 25°C. Muscles were washed by dipping three times, in milliQ water, to remove no-attached cells. Then muscles were deposit in steril Petri dish adapted to inverted fluorescence microscopy (ibidi). Left muscles were incubated in LB bacteria growth condition and right ones in DMEM bacteria growth condition

3.2. Observations of muscle surface

Surface of muscle were observed in fluorescence mode using an inverted phase-contrast microscope (Olympus IMT-2) coupled to a cooled CCD camera (Olympus DP30BW) optimized for high sensitivity fluorescence work and driven by the Cell A software v3.2 (Olympus France SAS, Rungis, France). Fluorescence light source was a mercury short arc lamp (HBO103W/2, OSRAM, Augsburg, Germany). Fluorescence acquisition was fitted with a "Cyanine 3" cube. Then, images were processed with the public-domain image processing and analysis program ImageJ v1.43 (NIH-RSB, Bethesda, MD, USA) (http://rsb.info.nih.gov/nih-image/about.html). The pixels corresponding to bacteria were extracted by thresholding segmentation of the light gray levels and proportion of bacterial covered surface was calculated.

4. Statistical analysis

Results are expressed as mean ± standard error of the mean (SEM). Data were analysed using using one-way variance analysis and the Student-Newman-Keuls test under the XLSTATsoftware 2010 (Microsoft office, Redmond, USA).

For the comparison of only two different populationsdata were analyzed following Student's t-test. Differences were considered as significant (p<0.05, *), very significant (p<0.01, **), highly significant (p<0.001, ***) or very highly significant (p<0.0001, ****).

RESULTS AND DISCUSSION

Bacterial tropism occurred for adhesion at the surface of different muscle types

Questionning the adhesion ability of *E. coli* O157:H7 to skeletal muscle, two model muscles with very distinct metabolic and contractile features were considered, *i.e.* the EDL (glycolitic muscle) and Soleus (oxidative muscle), which were extracted enterily and without any lesion. Respective to the bacterial cells, experimental conditions reported to induce specific and non-specific adhesion to ECM proteins were applied, *i.e.* adhesion at 25°C pH 7 using LB or DMEM as growth medium respectively (Chagnot *et al* Article 2).

Using LB, *E. coli* O157:H7 CM-454 was not covering evenly the surface of the muscles but adhered to specific structures of the epimysium by following the outline of the muscle fibers (Figure 1). Regarding the bacterial surface coverage, it was significantly higher for the Soleus than for the EDL. Besides the fact that bacterial growth in LB results in specific adhesion especially to collagens I and III for *E. coli* O157:H7 CM-454 (Chagnot *et al.*, 2013a), this localization trend at the surface of entire skeletal muscles could be explained by variation of the organisation or thickness of the epimysium. Indeed, it is well known that the epimysium is a highly nonlinear connective tissue, which exhibits longitudinal periodicities with approximately 1 µm spacing (Gao *et al.*, 2008, Gillies *et al.*, 2011). In DMEM and whatever the EDL or Soleus, the muscles were covered all over by *E. coli* O157:H7 CM-454, forming packed of bacterial cells in some area (Figure 1). Actually, DMEM was clearly shown to induce autoaggregation of *E. coli* O157:H7 EDL933 and *E. coli* O157:H7 CM-454 (Chagnot *et al.*, 2013a)(Chagnot et al Article n°2), resulting in non-specific bacterial adhesion to ECM proteins. Still, bacterial surface coverage was very significantly higher at the surface of Soleus (1 %) than EDL (0.6 %) (Figure 1). Thus, it clearly appeared that some kind of bacterial tropism occurred for adhesion to the surface of different types of muscles. This difference in surface coverage to EDL compared to Soleus both with bacterial cells grown in LB or DMEM could be explained by variation in the composition and proportion of different components of the muscle types, such as the muscle fibers.

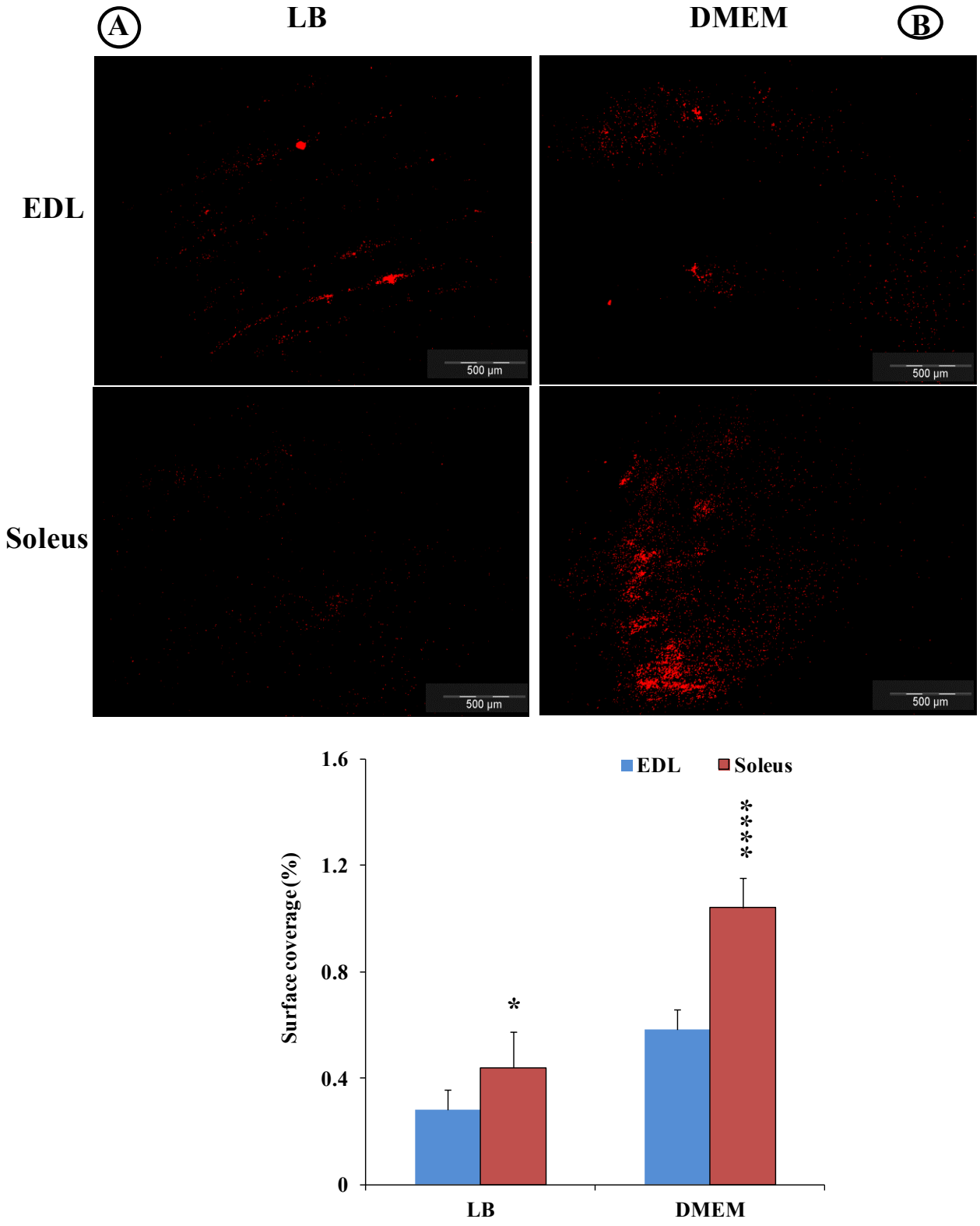

Figure 1: Adhesion of *E. coli* O157:H7 CM-454 to the surface of muscles as observed by epifluorescence microscopy.
Bacterial cells for the adhesion assay were first grown at 39°C in DMEM or LB media. Bacterial adhesion assay to the surface of EDL and Soleus muscle was performed at pH7 and 25°C. Epifluorescence microscopy data were expressed as the percentage of area covered by fluorescent *E. coli* O157:H7 CM-454 harbouring pSARE-Red1.

Postmortem evolution of muscle influenced _E. coli_ O157:H7 adhesion to muscle section

To further investigate the adhesion of *E. coli* O157:H7 CM-454 to cut skeletal muscles, serial cross-sections of EDL and Soleus were performed at two *postmortem* times.

At T0 in LB condition, for both EDL and Soleus muscles, surface coverage was quite low and did not exceed 0.5 % and 0.3% respectively (Figures 2A and 2C). However, 99.6 % and 98.5 % of adhered bacteria respectively were localized at the outer edge of the muscle cells (Figures 3, and 2C). In contrast and at T0 in DMEM, the surface coverages were much higher since it reached 2.8 % and 4.3 % for *E. coli* O157:H7 CM-454 adhering to Soleus and EDL muscles respectively (Figures 2C). In those conditions, about half of the bacterial cells were found at the periphery, whereas the other half were found on the muscle cells (Figure 3). Interestingly, the total amount of adhered biomass to Soleus or EDL muscles was higher with *E. coli* O157:H7 CM-454 previously grown in DMEM than with bacteria grown in LB (Figure 2C). Actually, the periphery of the muscle fibers corresponds to the endomysium (and/or perimysium), which constitute the ECM. From *in vitro* colonization investigations, it was previously shown that *E. coli* O157:H7 CM-454 adhere specifically to some ECM proteins in LB, especially collagen I and III, whereas non-specific bacterial adhesion to ECM proteins occured in DMEM (Chagnot *et al.* 2013). Actually, autoaggregation resulting in non-specific bacterial adhesion was previously observed in DMEM medium (Chagnot *et al.*, 2013a, Torres *et al.*, 2002). In those two conditions, it was here confirmed that ECM was an important support for adhesion of *E. coli* O157:H7 in the course of meat contamination.

At T24 in LB medium, the surface coverage of EDL (0.05%) and Soleus (0.1 %) by *E. coli* O157:H7 CM-454 was significatively lower than at T0 (Figure 2A). In DMEM, however, the surface coverage was similar at T0 and T24 for EDL and Soleus muscles respectively but the surface coverage was again very significantly higher than in LB (Figure 2B). Again, the ability of *E. coli* O157:H7 CM-454 to adhere to ECM was shown to be clearly distinct in LB and DMEM (Chagnot *et al.*, 2013a). Interestingly, both the EDL and Soleus muscles showed some structural changes at 24 h *postmortem*, namely the size of extracellular spaces increased and the meshing of the ECM became loosen (Figure 2B). Recently, *postmortem* degradation of proteoglycans was suggested to destabilize the ECM and lead to a dessolidarisation of collagen fibers (Nishimura, 2010). This *postmortem* structural modification could affect the specific adhesion of bacteria to collagen as observed in LB.

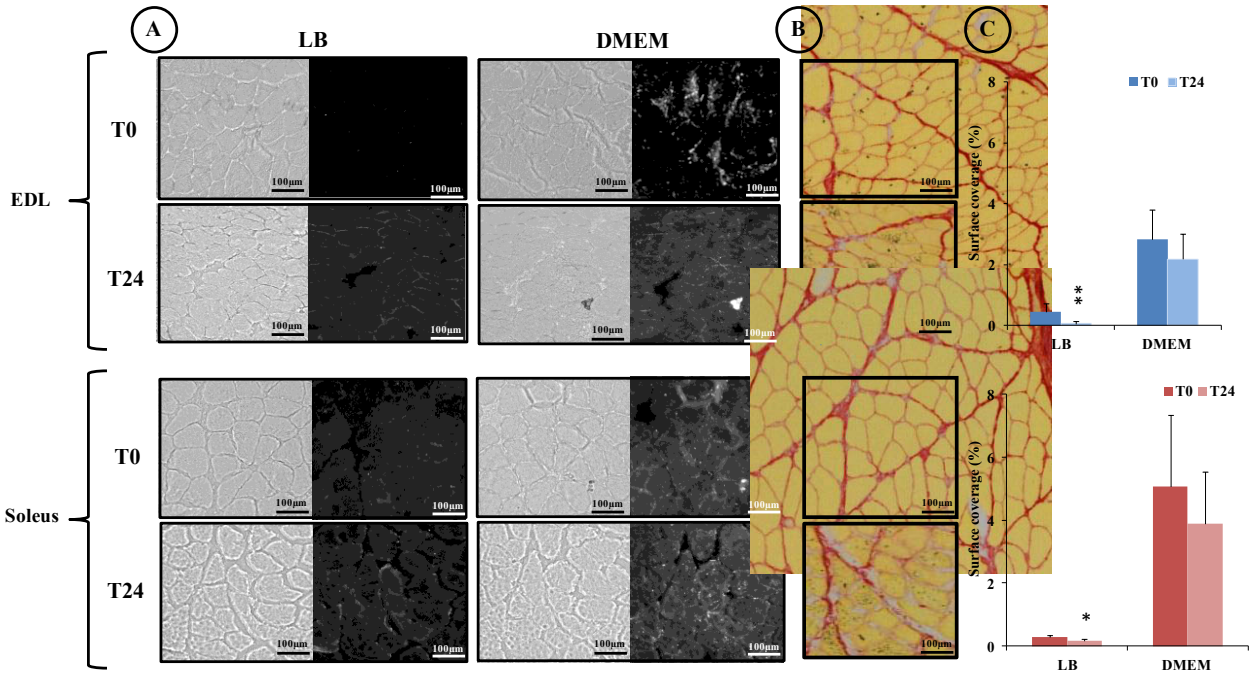

Figure 2: Bacterial adhesion to muscle cross sections as a function of the *postmortem* time as observed by epifluorescence microscopy.
(A) Bacterial adhesion to EDL and Soleus cross-section was assessed at two *postmortem* times: T0 (10 min after slaugther) and T24 (24 h after slaughter). On each muscle section, two images were recorded: (i) one in bright field mode one showing the muscle fibers and (ii) one in fluorescent revealing bacteria adhered (Cyanine 3: 550/570 nm). (B)Observation of structural evolution of connective tissue at T0 and T24 *postmortem* on serial cross sections of EDL and soleus muscles stained with Picrosirius red. (C)Epifluorescence microscopy data expressed as percent of area covered by fluorescent *E. coli* O157:H7 CM-454 strain. Bacterial adhesion assays were performed at 25°C using bacterial cells previously grown in DMEM or LB.

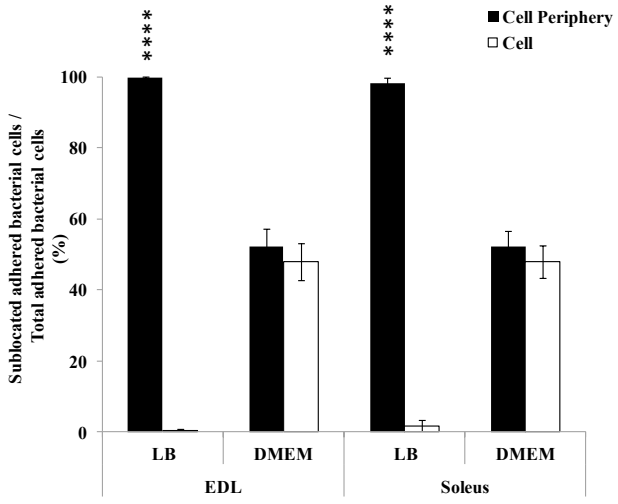

Figure 3: Proportion of *E. coli* 0157:H7 CM-454 adhered to EDL and Soleus muscles as function of their sublocation in the muscle tissue
Bacterial cells sublocated on the muscle cells or at the muscle cell periphery were discriminated. The proportion of each of those bacteria was calculated and based on bacterial cell count from epifluorescence microscopic observations (Figure 2). Bacterial adhesion assays were performed at 25°C using bacterial cells previously grown in DMEM or LB media.

E. coli O157:H7 adhered similarly to the different fiber types

To investigate the hypothesis that the differential composition and proportion of myofibers of types I, IIA, IIX and IIB could influence the adhesion of *E. coli* O157:H7 CM-454 to EDL and Soleus muscles, cross section of muscles were typed and correlated with the adhered bacterial cells (Figure 4A and 4B). While the Soleus muscle is exclusively composed of oxydative fibers (type I and IIA), EDL contains the four types of myofibers (I, IIA, IIX, IIX) but with the glycolitics fibers of type IIX and IIB in a higher proportion (Chagnot *et al* Article 4).

In LB at T0, when considering bacterial cells adhering to the muscle cells and cell periphery, it appeared that the surface coverages were similarly low whatever the types of muscle fiber present in EDL (Figure 4A) or Soleus muscles (Figure 4B). In DMEM and as expected from previously results (Figure 2), the amount of adhered *E. coli* O157:H7 CM-454 was higher than in LB but no difference in the bacterial surface coverages to type I, IIA, IIX and IIB myofibers in EDL (Figure 4A) or type I and IIA myofibers in Soleus could be observed (Figure 4B). However, surface coverage was still significantly higher for Soleus compared to EDL. Altogether, it appeared that whatever the muscles or medium conditions analysed, the study revealed the absence of bacterial tropism respective to the contractile (types I *vs* II) or metabolic (I and IIA *vs* IIX and IIB) types of fibers (Figure 3). This implicates that the metabolic or contractile properties of muscle fibers, and thus the muscle tissues *per se*, did not influence the adhesion of the *E. coli* O157:H7 CM-454 to meat.

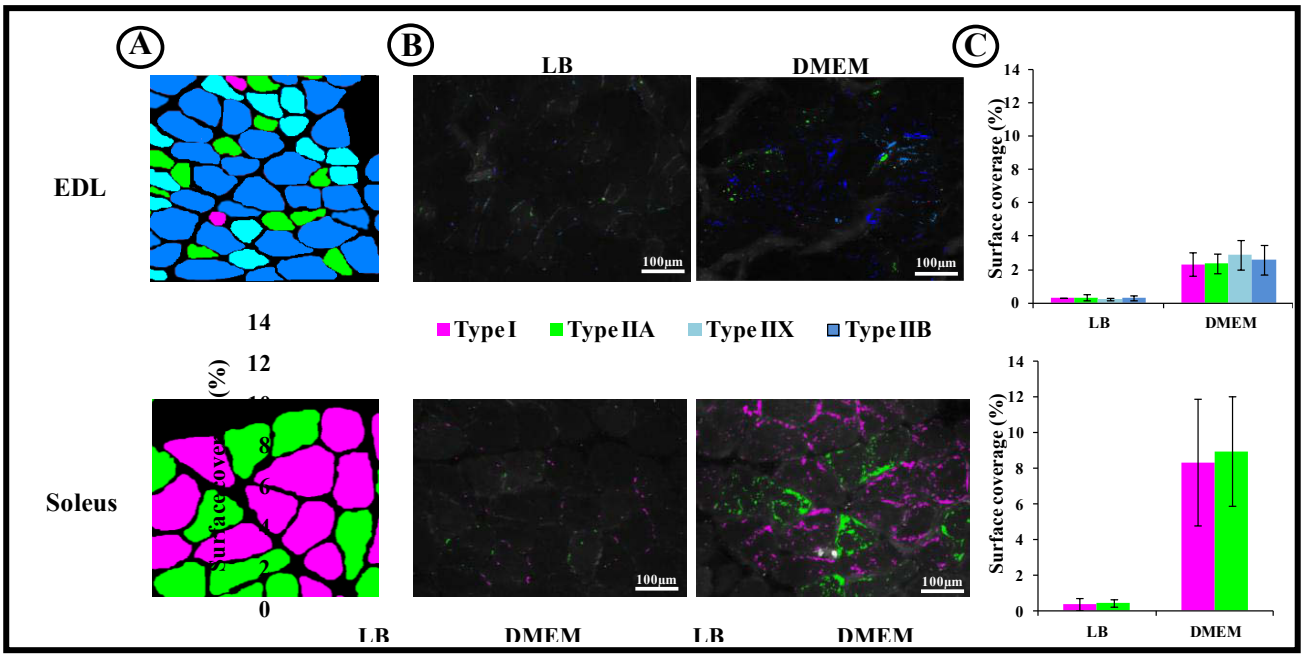

Figure 4: Bacterial adhesion of *E. coli* 0157:H7 CM-454 to to the different ype of myofibers composion EDL and Soleus muscles.

(A) Each muscle fiber type was identified on its MyHC isoform by specific monoclonal antibody. From all immunohistofluorescence images and for EDL and Soleus, a virtual image was reconstructed showing the precise fiber type identification of each cell. (B) On a serial EDL and Soleus cross-sections, level of bacterial adhesionwas assessed by their level of fluorescence intensity *E. coli* 0157:H7 CM-454 was performed at pH7 and 25°C in DMEM or LB media. Bacteria were stained virtually in colour corresponding to the type of muscle. (C)The percentage of area covered by fluorescent *E. coli* 0157:H7 CM-454 was calculated as function of the area of muscle cell.

CONCLUSION

This investigation pinpoints a crucial aspect of *E. coli* O157: H7 adhesion to meat that is the key role of muscular ECM. Whatever the conditions tested with respect to the bacterial physiology, *E. coli* O157:H7 could adhere to the ECM. Moreover and while the fiber types do not affect the attachment of *E. coli* O157:H7, the types of muscle influenced bacterial adhesion and was probably due to some variations in the ECM between the different muscles. Depending on the type of connective tissues in muscle, *i.e.* epymysium, endomysium, perimysium, it is well known the proportion of the different ECM proteins varies. From one muscle type to another, the composition of the ECM can also slightly differ as well as the supramolecular organization of the different components of the ECM but those aspects are not fully uncovered as yet and would require further in-depth investigations (Chagnot *et al.*, 2012). The present study open the way to further characterization of the influence of the ECM from different skeletal muscle tissues on EHEC adhesion as well as as the molecular interactions between bacterial ECM binding proteins and muscle ECM. In the long term, a better understanding of the bacterial tropism for different types of skeletal muscle and from different animal species would allow controlling more efficiently the risk of contamination of meat products by EHEC. This field of research has not attracted a lot of interest so far but is a promising research direction, which should undoubtedly lead to important development not only in the food industry but also in veterinary and/or medical sciences in relation to bacterial infection of muscle tissues for instance.

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DISCUSSION

Les *Escherichia coli* entérohémorragiques (EHEC) sont des bactéries pathogènes à l'origine de toxi-infections alimentaires. L'aliment principalement incriminé lors de ces infections est la viande de bœuf. C'est au cours de l'abattage et de la découpe, que la contamination initiale peut avoir lieu, les bactéries adhèrent essentiellement sur les muscles exposés à la surface de la carcasse. Les déterminants moléculaires impliqués dans ces interactions entre la viande et les EHEC sont méconnus. L'étude des interactions de la souche EHEC O157:H7 EDL933 aux protéines majeures de l'ECM musculaire (collagène I, III et IV, laminine α2, i-fibronectine, élastine) ont mis en évidence plusieurs aspects cruciaux parfois négligés dans la littérature qui ont une grande influence sur l'adhésion des EHEC aux protéines de l'ECM: (i) le pH, (ii) la température et (iii) condition de croissance. Bien que cela suggère que l'affinité d'interaction des protéines MSCRAMM soit modifiée par des paramètres physico-chimiques, cela fournit également de nouvelles orientations de recherche pour un développement de pratiques innovantes et/ou de stratégies de prévention aux points critiques lors de l'abattage des animaux afin de minimiser le risque de contamination par les EHEC O157:H7. Lors du processus d'abattage et de transformation du muscle en viande, les conditions environnementales changent, la température de stockage, le pH et la température des viandes diminuent. Lors de l'abattage la température ambiante et le pH neutre des carcasses correspondent aux conditions optimales d'adhésion pour la bactérie. Le milieu de croissance des bactéries joue également un rôle primordial dans l'expression des protéines de surface potentiellement impliqués dans l'adhésion. Des milieux spécifiques *ex vivo* provenant du contenu intestinal de différentes parties du tube digestif du bovin (rumen, le jéjunum-iléon, caecum) ont été comparé à des milieux de laboratoire classique, dans le but d'imiter l'environnement physiologique de l'intestin des bovins, réservoir principal des EHEC, rencontrés avant l'excrétion et la contamination bactérienne des viandes. *E. coli* O157:H7 EDL933 à la capacité d'adhérer spécifiquement à des composants de l'ECM et non-spécifiquement en fonction des conditions environnementales.

En milieu ruminal, la bactérie adhère spécifiquement aux collagènes fibrillaires (FFCs) I et III, à la laminine-α2, à l'élastine et au collagène IV. L'adhésion aux collagènes I et III a été particulièrement étudiée en raison de leurs localisations dans la partie la plus exposée de l'ECM, la matrice interstitielle; de plus c'est pour ces deux protéines que la biomasse adhérée spécifique est la plus importante dans les différentes conditions étudiées. Ce résultat indique que le ou les facteur(s) moléculaire(s) spécifique(s) pour l'adhésion aux principaux FFCs musculaires sont exprimés dans ces conditions expérimentales. La souche *E. coli* O157:H7 ED933, possède plusieurs déterminants moléculaires de surface connus pour leur rôle dans

l'adhésion et la formation de biofilms comme les pili, le flagelle, l'injectisome ou encore certains autotransporteurs. L'étude de l'expression génétique et protéique ainsi que l'implication fonctionnelle de ces protéines dans le phénotype d'adhésion, compléterait cette étude. Contrairement à nos observations, de précédentes études sur l'adhésion des EHEC aux protéines de l'ECM n'avaient pas révélé d'adhésion aux collagène I et III (Wells *et al.*, 2009, Zulfakar *et al.*, 2012). Ces différences pourraient être en partie liées au protocole expérimental utilisé. Les méthodes impliquant des traitements mécaniques tels que des étapes de centrifugation ou de vortex reduisent significativement l'adhésion en raison de la fragilité des protéines de surface (pili, flagelle…) potentiellement impliquées. Les futures recherches sur l'adhésion bactérienne devront tenir compte du traitement appliqué aux bactéries. En plus de l'adhésion spécifique, la formation de biofilm est également favorisée par la présence de FFCs. Cela peut constituer un risque accru de contamination à EHEC dans les industries alimentaires, en plus de l'adhésion et de la colonisation des carcasses, les EHEC peuvent également se fixer aux plans de travail et aux ustensiles (couteaux de boucher…) souillés par des collagènes résiduels de surface, constituant un vecteur de propagation de la contamination.

D'autre part la souche d'*E. coli* O157:H7 EDL933 a également montré une capacité d'adhésion non-spécifique aux protéines de l'ECM dans les conditions de croissance correspondant aux contenus jejunum-ileum de bovin. L'adhésion non-spécifique est étroitement liée au phénomène d'autoagrégation des bactéries. L'autoagrégation de la souche EDL933 dépend de la nature des milieux de culture, impliquant l'expression différentielle d'un déterminant moléculaire impliqué dans l'agrégation ou d'un déterminant qui pourrait l'entraver, comme des exopolysaccharides ou des pili (Schembri *et al.*, 2001, Ulett *et al.*, 2007, Hasman *et al.*, 1999). La caractérisation phénotypique de mutants de délétion a révélé que le pili F9, les flagelles H7 et l'Ag43 peuvent jouer un rôle dans l'adhésion non spécifique de la bactérie. Cependant, les résultats ont montré que l'Ag43 était le principal facteur responsable de l'adhésion, l'autoagrégation et la formation de biofilm d'*E. coli* O157:H7 EDL933. De façon surprenante, dans les mêmes conditions de culture, la souche *E. coli* O157:H7 Sakai qui a 97% d'identité de séquence génomique avec *E. coli* O157:H7 EDL933, ne peut ni autoagréger, ni adhérer ou former de biofilm. Contrairement à la souche *E. coli* O157:H7 Sakai, *E. coli* O157:H7 EDL933 possède deux copies du gène *cah*, la simple délétion de ces gènes a donné lieu à des phénotypes d'adhésion différents. La duplication du gène semble jouer un rôle important dans l'adhésion, ce qui pourrait s'expliquer par la

régulation des gènes de manière différentielle et/ou qu'une copie du gène conserve sa fonction originale tandis que l'autre copie peut diverger et acquérir de nouvelles fonctions (Torres *et al.*, 2002). L'étude de la régulation de l'expression de l'Ag43 chez les EHEC permettrait de compléter notre étude et de mieux comprendre le mécanisme d'autoagrégation de ces bactéries.

Le profil d'interaction de la souche *E. coli* O157:H7 EDL933 a été comparé aux 7 souches EHEC non-O157 majeures, afin de déterminer si toutes ces souches possèdent également la capacité d'adhérer aux protéines de l'ECM musculaire. L'adhésion et la formation de biofilm des EHEC O157 et non-O157 ont montré des disparités. Ceci implique que les déterminants de surface, impliqués dans l'adhésion, sont différents et/ou exprimés de manière différentielle en fonction des souches. Les facteurs physico-chimique (pH, température et le milieu de croissance) jouant un rôle primordial dans l'adhésion, des recherches supplémentaires sont nécessaires pour caractériser l'influence de ces paramètres clés sur la capacité d'interactions des souches d'*E. coli* non-O157 aux composants de ECM musculaire et d'identifier les déterminants moléculaires impliqués.

Si l'étude de la capacité d'adhésion des *E. coli* O157:H7 aux protéines de l'ECM musculaire à permit d'identifier des déterminants protéiques pouvant jouer un rôle dans l'adhésion, elle doit nécessairement être validée par l'étude des interactions de la bactérie avec la viande. Jusqu'à présent, malgré la conduite de recherches sur la contamination des viandes, l'effet des variations physiologiques et structurales que subissent les tissus musculaires lors de leur transformation en viande, ou encore les variations métaboliques entre types de muscles, sur l'adhésion bactérienne reste mal connue. Dans le cadre de ces recherches, une nouvelle méthode utilisant la microspectroscopie de fluorescence UV couplée à la lumière synchrotron afin de discriminer les types de fibres, a été développée. Cette méthode, basée sur la capacité de fluorescence de certaines molécules dans l'UV, s'est avérée sensible et efficace. En effet, elle permet de discriminer tous les types de fibres, même très proches d'un point de vue métabolique (type I/IIA et IIX/IIB) et contractile (type I et II), grâce aux rapports d'émissions de quatre fluorophores emettant à 302 nm assigné à la tyrosine, à 408, 346 et 325 nm correspondant à trois molécules dont l'identification n'a pas été établie avec certitude mais que l'on pourrait potentiellement assigner au tryptophane (346 nm), NADH (408 nm) et, en raison du large spectre d'emission du tryptophane, le 325 nm pourrait correspondre à une forme modifiée du tryptophane (Pallu *et al.*, 2012, Dufour *et al.*, 2003, Skjervold *et al.*,

2003b, Dufour *et al.*, 1998, Lakowicz, 2006). Cette discrimination implique des variations de compositions cytoplasmique ou physicochimique entres les differents types de fibres.

Dans l'industrie agroalimentaire, les temps de maturation sont particulièrement surveillés afin de garantir la qualité des viandes. Après abattage les muscles subissent des changements structuraux et physiologique important ce qui modifie l'environnement des molécules contenue dans les cellules (Valeur *et al.*, 2001, Lakowicz, 1999) . En se basant sur la sensibilité de fluorescence des molécules à ces changements, nous avons tenté de discriminer les muscles en fonction du temps *postmortem*, en utilisant la microspectroscopie de fluorescence. L'analyse à T24 (24h après abattage) et à T0 (10 min après abattage) a montré une discrimination des temps avec une diminution de l'intensité de fluorescence à 408 nm et à 346 nm, à T24 par rapport à T0, mais également une discrimination en fonction des fibres de même type principalement au niveau de la PC2 à 302 nm (Tyr) et 346 nm. La qualité de la séparation dépend du type de fibre. Le type de fibres IIA est celui dont la différence d'émission est la plus importante entre T24 et T0, ce qui suggère que la discrimination du temps *postmortem* est plus efficace sur des muscles avec une forte proportion de fibres de type IIA. Dans le cadre des recherches sur les contaminations des viandes, la microspectroscopie de fluorescence UV pourrait permettre de visualiser les interactions bactériennes en parallèle des types de fibres et ainsi étudier le tropisme bactérien.

Au cours des dernières années, de nombreuses études microbiologiques se sont intéressées à la contamination des viandes, négligeant, cependant, certains aspects, comme l'étude du tropisme bactérien vis-à-vis du type métabolique et contractile des muscles. Or, afin de mieux comprendre les mécanismes d'intéraction des EHEC avec la viande, il était important d'étudier le rôle de ces variations sur l'adhésion. Dans un premier temps, il s'agissait d'étudier l'adhésion de la souche *E. coli* O157:H7 EDL933 sur des coupes de muscle et d'observer le tropisme bactérien en fonction des 4 différents types pures de fibres musculaires (I, IIA, IIX, IIB). Il s'est avéré que dans les deux conditions de culture étudiées (DMEM et LB) aucun tropisme bactérien n'a été observé. Cependant, les différences moléculaires entre les types de fibres se situent essentiellement au niveau du cytoplasme cellulaire. Or en accord avec nos précédents résultats, en milieu LB, les bactéries adhérent essentiellement à l'ECM. En milieu DMEM, la bactérie adhère sans spécificité de localisation au centre de la cellule ainsi qu'au niveau de l'ECM, cependant, la bactérie forme des agrégats permettant une adhésion non spécifique aux protréines, pouvant expliquer l'absence de tropisme bactérien en fonction des types de fibres.

Dans un second temps, dans le but d'étudier le tropisme en fonction du type de fibre et du temps *postmortem*, nous avons testé l'adhésion bactérienne aprés 24h *postmortem*. Une diminution significative de la biomasse adhérée a été observée en milieu LB, impliquant qu'un changement lié à la maturation du muscle agit sur la capacité d'adhésion de la bactérie à l'ECM. Or, il a été montré qu'après quelques heures d'anoxie, les fibres se contractent latéralement, et la taille des espaces extracellulaires augmente, le maillage de l'ECM se relâche et les protéines subissent des modifications structurales (Offer *et al.*, 1988) ce qui pourrait expliquer que la bactérie ne puissent plus adhérer à l'ECM.

Dans un dernier temps, l'etude de la biomasse adhérée de la souche *E. coli* O157:H7 EDL933 sur des muscles non lésés, a révélé un tropisme en faveur du Soleus. La surface des muscles ou épimysium est un tissu non linéaire dont la composition peut varier en fonction des muscles (Gillies *et al.*, 2011), ces variations peuvent impliquer que certains muscles représentent un risque de contamination plus élévé que d'autres. L'étude de la composition et de la structure de l'epimysium de l'EDL et du Soleus pourrait permettre d'identifier les determinants impliqués dans le tropisme bactérien en fonction du muscle.

CONCLUSION

Les EHEC sont des pathogènes portés principalement dans la tube digestif du bovin, et responsables de toxi-infections alimentaires chez l'Homme. De nombreuses épidémies dans le monde sont régulièrement recensées, et les infections à EHEC engendrent des symptômes qui peuvent conduire à des pathologies rares mais graves, le SHU et le PTT qui affectent principalement les jeunes enfants. Aucune thérapie contre les infections à EHEC n'est disponible à ce jour. Malgré l'existence de techniques de prévention et de détection des EHEC, des aliments contaminés se retrouvent régulièrement sur le marché. Il est donc nécessaire de mieux comprendre les mécanismes d'adhésion et de colonisation des EHEC avec la viande afin de proposer de nouvelles stratégies de prévention afin de limiter la contamination des viandes. C'est dans cette optique que s'est inscrit mon travail de thèse. Mes travaux de recherche avaient pour but d'étudier l'adhésion et la colonisation des EHEC, et en particulier du sérotype O157:H7, à la viande à l'échelle moléculaire et cellulaire.

Dans un premier temps, l'étude des intéractions des EHEC avec les proteines de l'ECM, a montré que les bactéries peuvent adhérer de façon spécifique ou non spécifique en fonction des conditions environnementales (pH, température ou milieux de croissance). Néanmoins, des disparités entre les différents EHEC majeurs ont été mises en évidence. La présence des protéines de l'ECM et en particulier du collagène fibrillaire I et III augmente significativement le risque de contamination des viandes par *E. coli* O157:H7. De plus, les conditions de pH des viandes et de température des ateliers correspondant aux premières étapes de l'abattage se sont avérées optimales pour l'adhésion, mettant en évidence un point critique de la contamination. L'identification des facteurs de risque peut orienter la mise au point de nouvelles méthodes et stratégies de décontamination ou de nettoyage plus efficace. Par exemple, limiter la présence de résidus protéiques, tels que le collagène, sur les surfaces de travail pourrait participer à réduire le risque de contamination et de propagation des EHEC. La mise en évidence du rôle majeur du pH et de la température dans le processus d'adhésion et de colonisation est une piste intéressante.

Dans un second temps, l'étude des déterminants impliqués dans l'adhésion de la souche *E. coli* O157:H7 a révélé que la formation d'agrégats était responsable de l'adhésion non spécifique aux protéines de l'ECM. Une étude de génétique fonctionnelle a mis en évidence le rôle majeur de l'Ag43 dans ce phénomène d'autoagregation. L'étude des différents déterminants impliqués dans l'adhésion pourrait à long terme permettre de développer des stratégies préventives de lutte contre les infections, le dévelopement d'un vaccin vétérinaire par exemple, ciblant les protéines responsables de l'adhésion, pourrait limiter le portage des EHEC et ainsi diminuer le risque de contamination des viandes.

Dans un troisième temps, l'étude de l'adhésion d'*E. coli* O157:H7 sur les muscles modèles EDL (glycolitique) et Soleus (oxydatif) n'a pas révélé de tropisme pour un type de fibres musculaires en particulier. Cependant un tropisme en faveur du Soleus par rapport à l'EDL a été observé, ce qui suggére que la contamination par les EHEC dépende du type de muscle. L'identification des déterminants musculaires impliqués dans ce tropisme bactérien, pourrait permettre de répertorier les viandes présentant les plus forts risques de contaminations à EHEC, afin de mieux orienter les contrôles. En plus de l'impact sur l'industrie alimentaire, ces connaissances peuvent également intéresser d'autres domaines. Plus généralement, l'intéraction entre les bactéries et les muscles intéressent également le domaine vétérinaire et/ou médical. Ainsi, les pathologies musculaires impliquant les infections bactériennes telles que les infections à *Staphylococcus aureus* sont courantes, or, une meilleure compréhension des interactions de ces bactéries avec le muscle est un prérequis pour le développement de nouveaux moyens de prévention ou de lutte contre ce type d'infection.

Dans le cadre de mes travaux de recherche sur le tropisme bactérien, une nouvelle technique de caractérisation musculaire utilisant la microspectroscopie de fluorescence UV a été développée. Cette méthode permet de discriminer les muscles et les types cellulaires en fonction de leurs caracteristiques physiologiques. L'utilisation de cette méthode pourrait permettre d'étudier le tropisme bactérien en permettant de visualiser les bactéries à la surface des fibres musculaires, grâce aux propriétés de fluorecence des molécules. Par ailleurs, cette méthode pourrait être adaptée sur des microscopes à fluorescence classique, en laboratoire, afin de réaliser des typages rapides, ne nécessitant aucun marqueur ou coloration. Ces recherches sur l'évolution *postmortem,* démontrent l'intérêt d'exploiter les variations d'autofluorescence des cellules musculaires afin de caractériser rapidement l'effet de l'anoxie prolongée sur les muscles. A plus long terme, la possibilité d'utiliser la spectroscopie Deep UV, dans des domaines comme la médecine, pour diagnostiquer l'effet de l'ischémie musculaire après un accident vasculaire cérébral par exemple serait envisageable. Dans le domaine de l'alimentaire, cette méthode pourrait permettre de caractériser le degré de maturation d'une viande.

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LISTE DES COMMUNICATIONS

Communications orales

Journées de l'école doctorale (2013 France)

- *Mécanismes moléculaires impliqués dans l'interaction de Escherichia coli O157:H7 et protéines de la matrice extracellulaire musculaire*

CHAGNOT Caroline[1,2], ASTRUC Thierry [2], DESVAUX Mickaël [1]
INRA, UR454 Microbiologie[1], UR370 Qualité des produits animaux[2]

10ème Journée des microbiologiste clermontois (2013 France)

- *Adhésion de Escherichia coli O157:H7 à la Matrice Extracellulaire Musculaire: Interactions Moléculaires et Cellulaires*

CHAGNOT Caroline[1,2], DESVAUX Mickaël[1], ASTRUC Thierry[2]
INRA, UR454 Microbiologie[1], UR370 Qualité des produits animaux[2]

Sfu (société française de microscopie) (2013 France) (bourse)

- *Caractérisation des fibres musculaires en microspectroscopie de fluorescence UV couplée au rayonnement synchrotron.*

CHAGNOT Caroline[1,2], VENIEN Annie[2], PEYRIN Frédéric[2], JAMME Frédéric[3,4], REFREGIERS Matthieu[3], DESVAUX Mickaël[2], ASTRUC Thierry [1]

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9th Soleil Users Meeting (2014 France)

- *Synchrotron deep UV fluorescence microspectroscopy: an efficient method for muscle fibers characterization*

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Communication écrites

Journées de l'école doctorale (2012 France)

- *Staphylococcus xylosus et Escherichia coli O157: H7 adhèrent aux muscles et la matrice extracellulaire : implication des pili de type 4 chez les E. coli enterohémorragiques (EHEC)*

CHAGNOT Caroline [1,2], AGUS Allison [1], PEYRIN Frédéric [2], ASTRUC Thierry [2], DESVAUX Mickaël[1]
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Congres FEMS (2013 Allemagne) (bourse jeune chercheur)

- *Bacterial adhesion to meat: microscopic approaches to investigate the interaction of bacteria with muscle cells.*

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- *In vitro* colonization of the muscle extracellular matrix components by *Escherichia coli* O157: H7

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Congres ICOMST (*International Congress of Meat Science and Technology) *(2013 Turquie)

- *Synchrotron deep UV fluorescence microspectroscopy for fibers characterization*

CHAGNOT Caroline[1,2], VENIEN Annie[2], PEYRIN Frédéric[2], JAMME Frédéric[3,4], REFREGIERS Matthieu [3], DESVAUX Mickaël[2], ASTRUC Thierry [1]
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Vulgarisation scientifique

Article de vulgarisation scientifique : www.auvergnesciences.com (2012)

- *Contamination de la viande par Escherichia coli O157: H7 : Quels sont les mécanismes moléculaires impliqués ?*

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ANNEXES

REVUE n°1

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Bacterial adhesion to animal tissues: Protein determinants for recognition of extracellular matrix components

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Microreview

Bacterial adhesion to animal tissues: protein determinants for recognition of extracellular matrix components

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Summary

The extracellular matrix (ECM) is present within all animal tissues and organs. Actually, it surrounds the eukaryotic cells composing the four basic tissue types, i.e. epithelial, muscle, nerve and connective. ECM does not solely refer to connective tissue but composes all tissues where its composition, structure and organization vary from one tissue to another. Constituted of the four main fibrous proteins, i.e. collagen, fibronectin, laminin and elastin, ECM components form a highly structured and functional network via specific interactions. From the basement membrane to interstitial matrix, further heterogeneity exists in the organization of the ECM in various tissues and organs also depending on their physiological state. Back to a molecular level, bacterial proteins represent the most significant part of the microbial surface components recognizing adhesive matrix molecules (MSCRAMM). These cell surface proteins are secreted and localized differently in monoderm and diderm–LPS bacteria. While one collagen-binding domain (CBD) and different fibronectin-binding domains (FBD1 to 8) have been registered in databases, much remains to be learned on specific binding to other ECM proteins via single or supramolecular protein structures. Besides the interaction of bacterial proteins with individual ECM components, this review aims at stressing the importance of fully considering the ECM at supramolecular, cellular, tissue and organ levels. This conceptual view should not be overlooked to rigorously comprehend the physiology of bacterial interaction from commensal to pathogenic species.

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Introduction

The extracellular matrix (ECM) constitutes a protein complex, whose composition and structural organization influence numerous biological processes such as adhesion, migration, proliferation or differentiation of eukaryotic cells (Patti *et al.*, 1994). As a ubiquitous constituent of animal tissues, the ECM can also serve as a substrate for the attachment of colonizing microorganisms, in the case of an infection for instance. Animals are complex organisms with a structured organization. Three levels of organization have been identified: cell, tissue and organ. A group of cells from a same origin and function form a tissue, and the assembly of different tissues leads to the formation of an organ. Four basic tissue types can be differentiated: (i) nervous; (ii) muscle; (iii) epithelial and (iv) connective. The primary role of the ECM is to support and link the cells and tissues, although it is much more than a structuring tissue component. Two main classes of macromolecules constitute the ECM: (i) the fibrous proteins and (ii) the proteoglycans. Most ECM proteins are large and complex, with multiple distinct domains, but are highly conserved among different taxa.

Bacteria secrete proteins that can be expressed at the cell surface to specifically interact with components of the ECM. These proteins are qualified of MSCRAMM (microbial surface components recognizing adhesive matrix molecules). The study of MSCRAMMs necessitates an in-depth understanding and awareness not only of the ECM components but also of the cell biology. While the specific interaction with the ECM components and bacterial cells occurs at a molecular level through protein–

protein interaction, the structure and organization of the different ECM components also have to be considered at molecular, cellular, tissue and even organ level. Such aspects must not be overlooked to rigorously comprehend the bacterial interaction with the ECM in an infected host for instance. Besides an update of the bacterial protein cell surface determinants involved in adhesion to ECM components, the present review aims at providing key information on the cell biology of ECM (composition, structure and organization) in order to bring clarity in an area where the differences at molecular, cellular and tissue levels can be a source of confusion.

Molecular characteristics of ECM and bacterial MSCRAMM proteins

The MSCRAMM proteins localize at the interface between the bacterial cell and its surroundings, i.e. the cell surface. This necessarily implies the translocation of the proteins via protein secretion systems across one (monoderm) or two (diderm) biological membranes depending on the cell envelope architecture (Desvaux, 2006; Desvaux *et al*., 2009). These secreted proteins possess targeting sequences for correct routing and post-translational modifications, e.g. N-terminal signal peptides for protein substrates to the Sec translocon or C-terminal LPXTG domain for covalent anchoring to bacterial cell wall by sortase. Cell surface-exposed proteins localize differentially in monoderm and diderm–LPS (lipopolysaccharide) bacteria (Desvaux *et al*., 2006; Sutcliffe, 2010).

In bacteria, molecular characterization of the protein domains interacting with ECM is essentially available for the main fibrous ECM glycoproteins components, i.e. collagen, fibronectin, laminin and elastin (Table 1). Nonetheless, it must be stressed that other numerous functionally important proteins, such as proteoglycans, are present and exert significant pleiotropic effects. Proteoglycans are categorized into three main families (Schaefer and Schaefer, 2010): (i) SLRP (small leucine-rich proteoglycans); (ii) modular proteoglycans and (iii) cell surface membrane-associated proteoglycans. The glycosaminoglycan (GAG) chains of proteoglycans are composed of repeated disaccharides. These unbranched GAG chains can be discriminated into sulfated and non-sulfated ones. Hyaluronic acid is the only GAG that is not attached to a core protein. The sulfation of GAG chains frequently varies even within the same GAG molecule, and the number and length of GAG chains in a same core protein can also differ dramatically, which results in the highly heterogeneous nature of proteoglycans. Proteoglycans are the space fillers in the ECM that bridge the different components and form a highly hydrated gel-like substance. This substance is responsible for the volume of the ECM and is also resistant to compression.

While ECM is sometimes misleadingly referred to the sole connective tissue, all tissues are composed of an ECM. To rigorously comprehend the bacterial interaction with the fibrous ECM proteins, it is not only important to consider the specific protein–protein interactions occurring at a molecular level but also the supramolecular organization and localization of ECM in tissue.

Supramolecular organization of the ECM components

While fibrous ECM components can be roughly represented as immersed in a gel composed of proteoglycans to form a complex amorphous matrix, specific interactions occur between the different components resulting in a highly structured and functional network (Vakonakis and Campbell, 2007) (Fig. 1). This molecular knitting results in a very complex matrix, whose supramolecular organization is still not fully resolved and understood as yet.

Collagens provide the scaffold for the attachment of other ECM components (Orgel *et al*., 2011). The different types of fibril-forming collagens (FFCs) (Table 1) assembled to form fibres. While beaded filaments-forming collagens (BFFCs) interact with FFCs, FACITs (fibril-associated collagens with interrupted triple helices) associate all along the surface of collagen fibres (Fig. 1B). Multiplexin collagens (MPCs) attach to the fibronectin and to a lesser extent to laminin (Hurskainen *et al*., 2010). Besides, MACITs (membrane-associated collagens with interrupted triple helices) connect the cell and the ECM via a short N-terminal intracellular domain, a plasma membrane anchoring transmembrane domain and a collagenous ectodomain, which binds fibronectin, nidogen and perlecan. These different collagen types anchored fibronectin or laminin, but also interact with cell surface receptors, such as integrins, through which a number of cellular function can be controlled (Leitinger, 2011).

Fibronectin provides a molecular bridge between the collagen scaffold and the other ECM components (Fig. 1). Besides interaction with collagen, the fibronectin modules contain binding sites for the cell surface receptor integrins but also other ECM components, e.g. fibulin, thrombospondin, fibrin or tenascin (Schultz and Wysocki, 2009). Tenascin modulates adhesion to fibronectin and also interacts with proteoglycans, e.g. perlecan, and integrins (Orend *et al*., 2003). Interactions with plasma fibronectin, however, are mediated via glycosylations. Integrins are transmembrane proteins that mediate attachment of cell to the ECM and have a role of signal transduction from the ECM to the cell (Geiger and Yamada, 2011). Besides collagen and fibronectin, integrins also bind laminin. While representing the largest family of cell surface receptors, several other surface cellular receptors than integrins are also involved in binding of ECM components (Heino and Kapyla, 2009).

Table 1. Structural, supramolecular and localization features of the main ECM components and their respective bacterial ECM-binding domains.

ECM components[a]	Type[b]	Structure	Supramolecular organization[c]	Localization[d]	Bacterial ECM-binding domains[e]
FFC	I, II, III, V, XI, XXIV, XXVII	Homo/heterotrimer	FACIT Elastin I-fibronectin Decorin	IM	CBD (IPR008456)
FACIT	IX, XII, XIV, XVI, XIX, XX, XXI, XXII	Homo/heterotrimer	FFC	IM	
MPC	XV, XVIII	Homotrimer	I-fibronectin Laminin	BM	
NFC	IV, VIII, X	Homo/heterotrimer	Laminin Perlecan	IM	
BFFC	VI, VII, XXVI, XXVIII	Heterotrimer	FFC	BM	
MACIT	XIII, XVII, XXIII, XXV	Homotrimer	I-fibronectin Nidogen Perlecan	BM	
S-fibronectin	n/a	Covalent homodimer		PM	FBD1 (IPR011252)
I-fibronectin			Integrin	IM	FBD2 (IPR011266)
			Collagen*	BM	FBD3 (IPR008616) FBD4 (IPR011490) FBD5 (IPR004237) FBD6 (IPR010801) FBD7 (IPR021021) FBD8 (IPR010841)
Laminin	111, 121, 211, 212/222, 213, 221, 3A11, 3A21, 3A32, 3A33, 3B32, 411, 421, 423, 511, 521, 522, 523	Heterotrimer	Integrin Collagen* Proteoglycans Nidogen Perlecan Tenascin	BM	n/a
Elastin	n/a	Multimer	Proteoglycans Collagen* Fibulin Fibrillin	IM	n/a

a. The different subcategories of the main fibrous ECM components, i.e. collagen, fibronectin, laminin and elastin. FFC, fibril-forming collagen; BFFC, beaded filaments-forming collagen; FACIT, fibril-associated collagen with interrupted triple helices; MACIT, membrane-associated collagen with interrupted triple helices; NFC, network-forming collagen; MPC, Multiplexin collagen; S-fibronectin, soluble fibronectin or plasma fibronectin; I-fibronectin, insoluble fibronectin or cellular fibronectin.
b. Different types of collagens and laminin isoforms. For laminin-111, for example, 111 means an $\alpha 1\beta 1\gamma 1$ composition. n/a, not applicable.
c. For more detailed information on the interactions between the different ECM components, refer to the main text. (*) For the different types of collagen it refers to, check out the first part of the table.
d. Localization related to the different specialized forms of ECM in tissues. IM, interstitial matrix; BM, basement membrane; PM, plasma matrix.
e. Characterized domains specifically involved in the adhesion to ECM components. Identification number from InterPro (IPR) database is given in brackets. For the different types of collagen, the specificity of the collagen-binding domain (CBD) remains unclear. A similar comment applies for the specificity of the different types of fibronectin-binding domain (FBD) towards the s-fibronectin. n/a, not available.

Actually, laminins bind numerous proteoglycans and collagens, especially network-forming collagens (NFCs) (Durbeej, 2010) (Fig. 1). Nidogens interact with fibulin and other numerous ECM proteins including elastin (de Vega *et al.*, 2009). Elastin, which proportionally provides a degree of elasticity to the ECM, further exhibits molecular interaction sites with proteoglycans, fibulin and fibrillin (Hayes *et al.*, 2011). These molecules associate together into microfibrils and associate with tropoelastin to form elastic fibres further associated to other ECM molecules such as full-circle collagens (Fig. 1). This further stresses that ECM supramolecular structures are composite biological amalgamates of co-polymers that differ in their identity and relative abundance (Bruckner, 2010).

Extracellular matrix localization in tissue

Besides this generic supramolecular organization, some specificity arises when considering ECM in various tissues. From one tissue to another and even within one tissue type, depending on its physiological state for instance, the ECM organization varies tremendously. While in ordinary connective tissues ECM components are synthesized by fibroblasts, ECM components can be secreted by the specific cells composing a given tissue. For example, muscle fibres secrete ECM components in the course of myogenesis, collagen IV is expressed by epithelial cells or Schawn cells biosynthesize fibronectin in nervous tissues. The ECM has two basic forms in

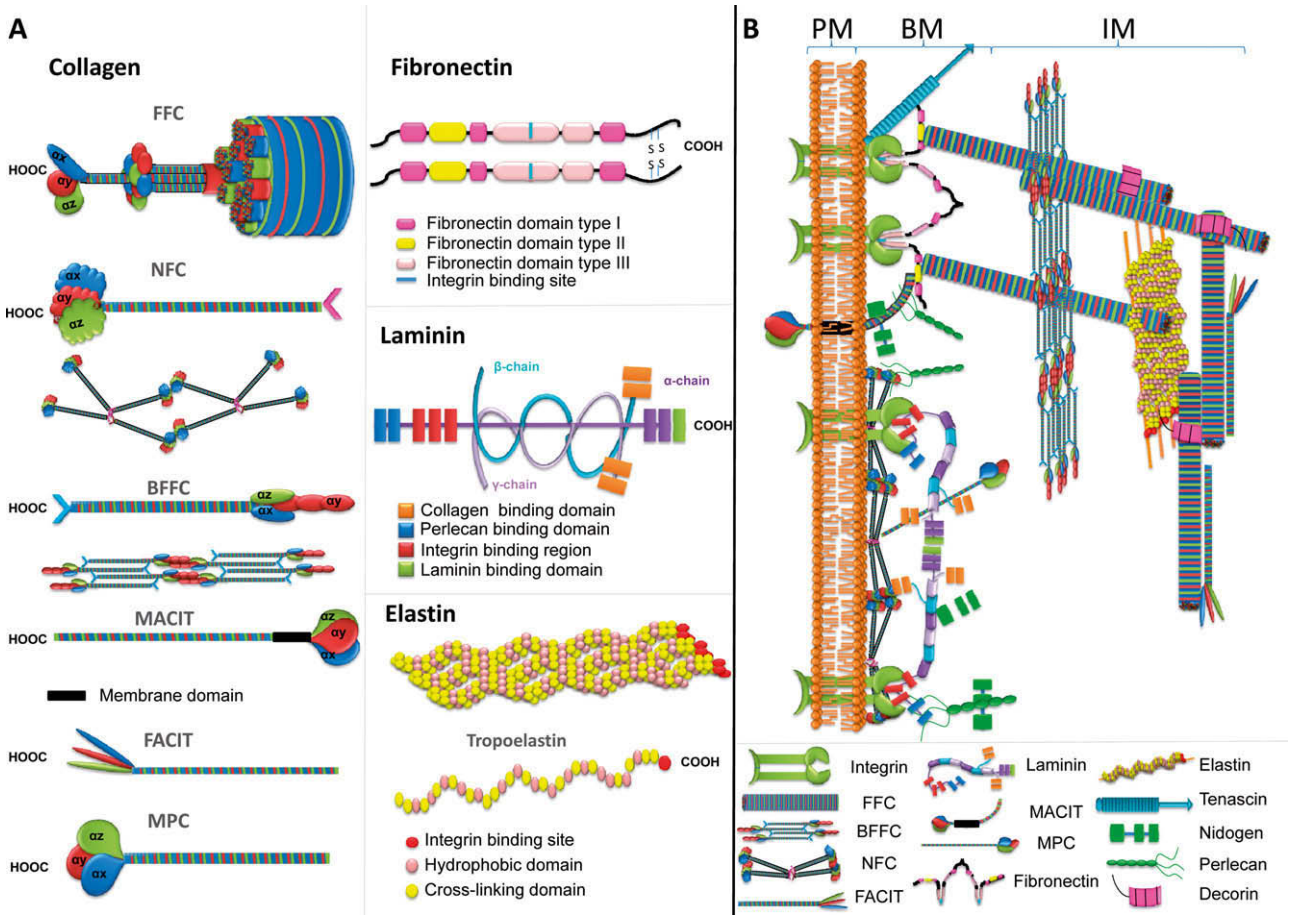

Fig. 1. Schematic representation of the (A) molecular structure and (B) supramolecular organization in tissue of the main ECM components. More detailed information on structure and supramolecular organization of ECM components can be found in the main text and is also summarized in Table 1. FFC, fibril-forming collagen; BFFC, beaded filaments-forming collagen; FACIT, fibril-associated collagen with interrupted triple helices; MACIT, membrane-associated collagen with interrupted triple helices; NFC, network-forming collagen; MPC, multiplexin collagen; PM, plasma membrane; BM, basement membrane; IM, interstitial matrix.

tissue: (i) the basement membrane (BM), also called basal lamina and (ii) the interstitial matrix (IM) (Fig. 1). In some connective tissues such as blood or lymph tissues, the matrix is fluid and called plasma.

The BM is present at the basis of epithelial and connective tissues where it separates the underlying tissue (Yurchenco, 2011). The BM can be further divided into lucida, densa and reticular laminae. Laminins are major components of BM and are not present in the IM (Fig. 1). BM forms a complexed, structured and connected network that further contains proteoglycans (essentially perlecan and nidogen) and collagens, especially the non-fibrillar-forming collagen IV (Table 1). Collagen IV forms a network that constitutes the BM and collagen VII serves as an anchor to it (Amano *et al.*, 2001). The functional diversity of BM arises from the molecular diversity of collagen and laminin isoforms as well as the minor constituents.

The IM composition greatly depends on the tissue and interstitial compartment considered, e.g. it is essentially collagen in connective tissue or fibronectin and vitronectin in blood tissue. In this last, fibulin binds to fibrinogen and incorporates into clots. The most developed part of the ECM is located in the connective tissues. In dense connective tissues, such as cartilage and tendon, almost all the space between the cells is filled by fibrillar collagens, representing 60–85% of the dry weight of these tissues. In articular cartilage, collagen II is the principal component but proteoglycans, e.g. decorin, also contribute to the IM (Fox *et al.*, 2009). This IM composition greatly contributes to the high water content of the IM.

Collagen and collagen-binding proteins

To date, 28 different types of collagens (from I to XXVIII) have been described (Ricard-Blum, 2011) (Table 1). Collagens I to V account for the types the most commonly encountered. Collagens always assemble from three type-specific α chains to form triple-helical protomers

(Fig. 1). The triple helix domains are alternated with non-helical domains depending on the specific type of collagen. The degree of hydrolyxation and glycosylation of hydroxylysine residues depends on the collagen type but also varies with tissues and ageing. Basically, collagens can form diverse supramolecular structures (Table 1), namely fibrils (FFC), networks (NFC), beaded filaments (BFFC), MACITs, FACITs or multiplexins (MPC). Collagens provide scaffolding for the attachment of some other ECM components.

The collagen-binding domain (CBD) was originally identified and characterized in Cna (collagen adhesin) from *Staphylococcus aureus*. Most recent investigation proposed that collagen docking follows a dynamic multistep binding model, called the collagen hug (Zong *et al*., 2005). The CBD (IPR008456) is about 160-amino-acid long with a jelly-roll fold composed of two α helices and two antiparallel β sheets. CBD is often associated with collagen B-type domains (IPR008454 and IPR008970), which do not mediate adhesion to collagen but are required for functional protein conformation by putting the CBD away from the bacteria cell surface (Deivanayagam *et al*., 2000). Of note, CBD belongs to the structural superfamily of bacterial adhesion domain (IPR008966). According to IPR v36.0, the large majority of CBDs are found in proteins from bacteria of the phylum Firmicutes and, at a much lesser extent, in the phylum Actinobacteria and also some diderm–LPS bacteria. As a general trend observed in Cna, CBD enables strong binding to collagen I but there are differences from one collagen-binding protein to another. For instance, binding of Acm (adhesin of collagen from *Enterococcus faecium*) to collagen I is greater than IV (Nallapareddy *et al*., 2003). Cne adheres to native interstitial collagen of types I, II and III but not to collagen IV (van Wieringen *et al*., 2010). CbpA (collagen-binding protein A) binds quite similarly to collagens I–IV and XI and to a lesser extent to collagens V and IX (Pietrocola *et al*., 2007).

Besides CBD, some other domains involved in collagen binding have been described but no pattern/profile is as yet available in databases. Streptococcocal M proteins mediate high-affinity interaction with collagen IV via an N-terminal PARF (peptide associated with rheumatic fever) motif (Reissmann *et al*., 2012). The N-terminal region of CbsA (collagen-binding S-layer protein A) binds to collagens I and IV (Antikainen *et al*., 2002). In diderm–LPS bacteria, the trimeric autotransporter YadA (*Yersinia* adhesin A) forms a lollipop-shaped structure, whose conformation is absolutely required for binding to collagens I, II, III, IV, V and XI. The collagen-binding activity resides within the central and C-terminal portion of the head domain and the interaction involves NSVAIG–S repeats (Tahir *et al*., 2000). For several reported collagen-binding proteins (CgBPs), the domain responsible for specific adhesion to collagens still remains to be determined, e.g. p26 (protein of 26 kDa) (Howard *et al*., 2000), the M-like protein FoG (fibrinogen-binding protein of G streptococci) (Nitsche *et al*., 2006), Lsa63 (leptospiral surface adhesin of 63 kDa) (Vieira *et al*., 2010) or EhaB (enterohemorrhagic *Escherichia coli* autotransporter B) (Wells *et al*., 2009).

Fibronectin and fibronectin-binding proteins

Fibronectins are generally dimers covalently linked by a pair of disulfide bonds near their carboxyl termini (Pankov and Yamada, 2002) (Fig. 1). Fibronectins have a modular architecture composed of a combination of three different types of homologous domains, i.e. types I (FnI; IPR000083), II (FnII; IPR000562) and III (FnIII; IPR003961). These repeating modules are all primarily composed of antiparallel β strand. FnI is the most conserved region of fibronectin among different taxa. Modules $FnI_{1–5}$ constitutes a heparin-binding domain (HBD) and $FnI_{6–9}$ a gelatin/collagen-binding domain (GBD) (Erat *et al*., 2009). FnII is involved in the binding to collagen. Contrary to the two other domains, FnIII cannot form inter- or intramolecular disulfide bonds but constitutes the largest part of the fibronectin. The presence/absence of these extra domains A (EDA) and B (EDB) results in two variations of fibronectins: (i) insoluble fibronectin (i-fibronectin), also called cellular fibronectin, contains variable proportion of EDA and EDB and (ii) the soluble form (s-fibronectin), also called plasma fibronectin, lacks these alternative spliced type III domains and is present in the ECM of some connective tissues, e.g. the plasma matrix in blood or lymph tissues. S-fibronectin can shift to fibrils upon binding to integrins. In any case, the fibronectins have rod-like structure and, with many molecular binding sites, play a key role in cell adhesion (Pankov and Yamada, 2002).

Contrary to collagen binding, eight different bacterial domains involved in adhesion to fibronectin are registered in databases. To ease the description, a classification of the fibronectin-binding domains (FBDs) from types 1 to 8 is proposed.

FBD1 (IPR011252) has a β-sandwich topology as resolute in the first fibronectin γ-chain-binding *S. aureus* adhesin, ClfA (clumping factor A) (Deivanayagam *et al*., 2002). As determined in SdrG (serine-aspartate repeat-containing protein G), this domain binds to its ligand with a dynamic 'dock, lock and latch' mechanism (Ponnuraj *et al*., 2003). FBD1 is generally found in tandem with FBD2 (IPR011266), as in the two major fibronectin adhesins of *S. aureus*, FnBPA (fibronectin-binding protein A) and FnBPB. FBD1 is essentially found within species of the phyla Firmicutes and Actinobacteria. FBD2 is exclusively found among bacteria of the phylum Firmicutes with

the vast majority belonging to the genera *Staphylococcus* and *Streptococcus*.

FBD3 (IPR008616) was originally characterized in FbpA (fibronectin-binding protein A) from *Streptococcus gordonii* (Christie *et al*., 2002), an orthologue to Fbp54 (fibronectin-binding protein of 54 kDa) and PavA (pneumococcal adhesion and virulence protein A) form. While FBD3 is also involved in binding to fibrinogen, it interacts with fibronectin at the HBD. Interestingly, the ability to bind soluble fibronectin would vary from one fibronectin-binding protein (FnBP) to another; e.g. PavA cannot but Fbp54 can bind the soluble form (Henderson *et al*., 2011). With 1160 species, FBD3 is the most largely distributed FBD in bacteria. It is mostly found within the phylum Firmicutes including the class Clostridia (Desvaux *et al*., 2005), but it is also present in the phyla Proteobacteria (classes ε- and δ-proteobacteria), Cyanobacteria or Spirochaetes.

FBD4, the FIVAR (found in various architectures) repeat (IPR011490), forms α-helical bundles allowing specific interaction with fibronectin. In EcmbP (ECM-binding protein), it was demonstrated to interact with FnIII module (Christner *et al*., 2010). FBD4 is essentially present in the phylum Firmicutes. Besides Actinobacteria, it is also identified in the phyla Thermotogae and Bacteroidetes.

FBD5 (IPR004237) is certainly the domain having attracted the most interest (Henderson *et al*., 2011). It is exclusively found in the phylum Firmicutes, essentially the genera *Staphylococcus* and *Streptococcus*. Each FBD5 repeat is an array that binds to FnI modules in HBD and where a change of conformation occurs upon interaction following a tandem β-zipper model (Bingham *et al*., 2008). Of note, this domain can be found in conjunction with FBD1 and FBD2, e.g. in FnBPA and FnBPB.

FBD6 (IPR010801) was originally identified in Fap (fibronectin attachment protein) from *Mycobacterium*, and is quite restricted to this genus. FBD6 binds to fibronectin HBD. FBD7, or SSURE (streptococcal surface repeat) domain (IPR021021), was first uncovered in SP0082 from *Streptococcus pneumonia* (Bumbaca *et al*., 2004). FBD7 is exclusively found in bacterial species of the phylum Firmicutes, essentially genus *Streptococcus*. FBD8 (IPR010841) was originally identified in *Listeria monocytogenes* Lmo0721, i.e. FbpB (fibronectin binding protein B) (Gilot and Content, 2002). FBD8 is essentially present in phylum Firmicutes and in a couple of species of the phyla Proteobacteria, Bacteroidetes and Chloroflexi. Structural information in terms of protein folding and site-specific recognition of fibronectin is still awaited for FBD7 and FBD8.

Interestingly enough, several FnBPs are also CgBPs, e.g. RspA (*Rhusiopathiae* surface protein A) harbours both a FBD1 and a CBD and also binds to polystyrene (Shimoji *et al*., 2003). Of note, the involvement of MAP (MHC class II analogous protein) domain (IPR005298) in specific binding to fibronectin remains to be ascertained. Besides FBDs, some other domains involved in fibronectin binding have been described but no pattern/profile is as yet available in databases (Henderson *et al*., 2011), e.g. EWYYQ motif in antigen 85B, FRLS motif in CadF (*Campylobacter* adhesin to fibronectin), glutamine-rich motifs in Hlp3 (HMW3-like protein) and PlpA (pneumoniae-like protein A), terminal immunoglobulin (Ig)-like repeats (IPR003343) in LigA (*Leptospira* immunoglobulin-like protein A) and LigB, or the motif within the 46–205 residues of Bbk32 (*Borrelia burgdorferi* protein encoded by locus *bbk32*). In some YadA proteins, an N-terminal motif (termed the uptake domain) in the head region can be present and mediate tight binding to fibronectin (Heise and Dersch, 2006).

Additional uncharacterized regions are also known to interact with fibronectin (Henderson *et al*., 2011), e.g. C-terminus of LipL32 (leptospira lipoprotein of 32 kDa), central region of Cha (cell surface high molecular weight adhesin of *Granulicatella adiacens*) or N-terminal of RevA (reverse strand encoding protein A). In some other FnBPs, structure–function analyses are still awaited, e.g. UafB (uroadherance factor B) (King *et al*., 2011).

Laminin and laminin-binding proteins

Laminins are heterotrimers composed of three distinct subunits, called α, β and γ, linked together by disulfide bonds (Durbeej, 2010). Chain variations of the subunits, as well as alternatively spliced variants, have been uncovered with five α, three β and three γ chains. The nomenclature of laminins is based on the number of three different subunits composing them; e.g. laminin-111 has an α1β1γ1 composition. To date, 18 distinct laminin isoforms based on different arrangements of the subunits have been identified. This large supramolecular structure (400–900 kDa) can form either cruciform, Y-shaped or rod-shaped heterotrimeric structures. Within the ECM, laminins form a network with a web-like structure. The laminin network assembled through polymerization, in which the primary interactions lead to a trimer formation by the binding of three different laminins. By binding to several other proteins, laminins are key components leading to a highly cross-linked ECM.

Contrary to collagen and especially fibronectin, no laminin-binding domain is currently registered in databases. Nonetheless, several protein regions are known to confer specific adhesion to laminin. The CgBP CbsA can also bind to laminin via its N-terminal region (Antikainen *et al*., 2002). With the absence of the uptake domain, YadA gains adhesion potential to collagen but also laminin (Heise and Dersch, 2006). While structure–function

analysis remains to be performed, laminin binding has been reported for several additional proteins, e.g. EhaB (enterohemorrhagic *E. coli* autotransporteur A) (Wells *et al*., 2009), Tp0136 (*Treponema pallidum* protein encoded by locus *tp0136*) (Brinkman *et al*., 2008), OmpL47 (outer membrane protein of *Leptospira* of 47 kDa) (Pinne *et al*., 2010) or Lsa24 (leptospiral surface adhesin of 24 kDa), Lsa27 and Lsa63 (Vieira *et al*., 2010).

Elastin and elastin-binding proteins

The assembly of monomeric precursor tropoelastin results in the formation of polymeric elastin (Muiznieks *et al*., 2010). Tropoelastin alternates hydrophobic and cross-linking domains. Hydrophobic domain is essentially composed of G, V, P and A residues, which are arranged into motifs responsible for structural disorder and are directly involved into a self-association process called coaservation. The hydrophobic domains play a crucial role in the elastic property. The cross-linking domains are predominantly composed of lysine, which permits the covalent association of tropoelastin monomers as a result of lysyl oxidase activity. This is the association tropoelastins by weak and covalent interactions, via hydrophobic and cross-linking domains, respectively, that result in the formation of elastin.

Similarly to laminin, bacterial protein domains responsible for specific binding to elastin have not attracted a lot of attention, which is in mark contrast with fibronectin. In the membrane protein EbpS (elastin-binding protein of *S. aureus*), the motif TNSHQD is the minimum sequences required for recognition and binds to the N-terminal 30 kDa region of elastin (Park *et al*., 1999). In FnBPA, the FBD1-2 region was shown to display adhesion properties against elastin in addition to fibronectin (Keane *et al*., 2007). In LigB, the Ig-like repeats are involved in charge–charge interactions with elastin (Lin *et al*., 2009). While structure–function analysis is awaited, OmpL37 demonstrates a pronounced specificity for elastin (Pinne *et al*., 2010).

Bacterial cell surface supramolecular protein structures interacting with ECM components

In diderm–LPS bacteria, several protein secretion systems are involved in assembly of pili, some of them have been demonstrated to interact with ECM components (Desvaux *et al*., 2009). Besides pili, H6 and H7 flagella have been demonstrated to mediate specific adhesion to collagen, laminin and fibronectin (Erdem *et al*., 2007).

In diderm–LPS bacteria, the type 4 pilus HCP binds specifically laminin and fibronectin but not collagen (Ledesma *et al*., 2010). P-fimbriae have been demonstrated to bind fibronectin (Westerlund *et al*., 1991) as well as type 1 fimbriae, whose ability to bind fibronectin HBD and GBD is strain-dependent and results of sequence heterogeneity in fimbrilin FimH (Sokurenko *et al*., 1994). In FimA, the binding motif was identified (Murakami *et al*., 1996). In AafA (aggregative adherent fimbrilin A) binding was demonstrated to occur at the glycosylated group (Farfan *et al*., 2008). Lpf (long polar fimbriae) have been reported to adhere to PEYER patch and intestinal cells by attachment to fibronectin, but also collagen IV and laminin (Farfan *et al*., 2011). The DraE (Dr haemagglutinin) member of Afa (afimbrial adhesin) family forms fimbriae and binds collagen IV (Korotkova *et al*., 2006). Curli allows specific interactions with laminin and fibronectin (Zogaj *et al*., 2003).

In monoderm bacteria, Ebp (endocarditis- and biofilm-associated pilus) contributes to adhesion to collagen and fibrinogen (Nallapareddy *et al*., 2011). Mtp (*Mycobacterium tuberculosis* pili) bind to laminin (Alteri *et al*., 2007). The major adhesin subunit of the protofilament, RrgA (RlrA-regulated gene A), shows pronounced binding against collagen I, laminin, fibronectin and fibrinogen (Hilleringmann *et al*., 2008).

Conclusion and perspectives

While ECM shares some general features, its composition, structure and organization greatly varies from one tissue to another. The proportion and organization of the different tissue strongly differ from one organ to another. Besides, the ageing also impacts on the composition of the ECM in the tissues. This high heterogeneity from molecular, cellular, tissue and organ levels is often overlooked and should be carefully considered when interpreting and investigating the physiology of bacterial interaction with ECM components.

FnBPs have clearly attracted the most interest leading to the identification of eight different FBDs. The need for further investigations is quite pronounced for CgBPs with only one CBD identified and especially laminin- or elastin-binding domain with no pattern/profile as yet described. Bacterial adhesion to some other ECM components has been reported such as fibrinogen, plasminogen, vitronectin or tenascin as well as some proteoglycans such as decorin or nidogen. It must be stressed that several primarily cytoplasmic proteins have now been demonstrated to bind some ECM components and a specific ECM-binding site has even been identified, e.g. in the malate synthase of *M. tuberculosis* to laminin or in enolase from *S. pneumonia* to the plasmin(ogen).

From the phylogenetic distribution, it clearly appears that ECM-binding proteins are not restricted to pathogenic species, which suggests a physiological and ecological role beyond the sole infection of a host. Adhesion and

colonization of host tissue are a crucial step not only in the course of an infection but also in the interaction of commensal bacteria in their ecological niche. The stability and ubiquity of ECM in animal tissues have provided a structural support of choice with which numerous bacterial species must have evolved. Regarding the other numerous ECM components, plentiful of investigations lay ahead to identify and characterize the ECM-binding proteins responsible for adherence. This is a promise for exciting new development in the field of bacterial adhesion to ECM in the very near future.

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REVUE n°2

Caroline Chagnot, Mohamed A.Zorgani, Thierry Astruc and Mickaël Desvaux

Proteinaceous determinants of surface colonization in bacteria:Bacterial adhesion and biofilm formation from a protein secretion perspective

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Proteinaceous determinants of surface colonization in bacteria: bacterial adhesion and biofilm formation from a protein secretion perspective

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Bacterial colonization of biotic or abiotic surfaces results from two quite distinct physiological processes, namely bacterial adhesion and biofilm formation. Broadly speaking, a biofilm is defined as the sessile development of microbial cells. Biofilm formation arises following bacterial adhesion but not all single bacterial cells adhering reversibly or irreversibly engage inexorably into a sessile mode of growth. Among molecular determinants promoting bacterial colonization, surface proteins are the most functionally diverse active components. To be present on the bacterial cell surface, though, a protein must be secreted in the first place. Considering the close association of secreted proteins with their cognate secretion systems, the secretome (which refers both to the secretion systems and their protein substrates) is a key concept to apprehend the protein secretion and related physiological functions. The protein secretion systems are here considered in light of the differences in the cell-envelope architecture between diderm-LPS (archetypal Gram-negative), monoderm (archetypal Gram-positive) and diderm-mycolate (archetypal acid-fast) bacteria. Besides, their cognate secreted proteins engaged in the bacterial colonization process are regarded from single protein to supramolecular protein structure as well as the non-classical protein secretion. This state-of-the-art on the complement of the secretome (the secretion systems and their cognate effectors) involved in the surface colonization process in diderm-LPS and monoderm bacteria paves the way for future research directions in the field.

Keywords: secretome, adhesin, pili/fimbriae/curli, cell surface, aggregation, secreted protein, MSCRAMM, protein secretion system

INTRODUCTION

Relative to the bacterial cell, three major classes of interactions can be distinguished: (i) the symbiotic relationships with others biological entities from eukaryotic cells, bacterial cells to viruses (bacteriophages), e.g., mutualism, amensalism, competition, etc..., (ii) the sensing of products or stimuli (cell-cell communication, mechano-physico-chemico sensitivity), and (iii) the direct contacts with surfaces or interfaces. Bacterial colonization corresponds to the presence of microorganisms in a particular environment. Colonization of biotic or abiotic surfaces results from two quite distinct microbiological processes, namely bacterial adhesion or biofilm formation. Initial bacterial adhesion can either be reversible or irreversible. Broadly speaking, a biofilm is defined as the sessile development of microbial cells. The engagement into a sessile mode of growth arises following irreversible bacterial adhesion (Zhao et al., 2013); though, not all single adhered bacterial cells necessarily engage into sessile development. Considering the differences in the molecular physiology of the bacterial cells, the distinction between single adhered and biofilm cells is of importance (Buncic et al., 2013). While initial attachment depends on the physiological state of the cell prior to adhesion, significant changes in gene/protein expression occur upon irreversible adhesion and further sessile cell division to colonize the site of adhesion. In all cases, the bacterial cell envelope plays a critical role in the primary interactions between the bacterial cell and its environment.

Numerous factors promote bacterial cell adhesion to surface and interfaces (An and Friedman, 1998; Shirtliff et al., 2002). They can be categorized into (i) general physico-chemical surface properties, (ii) the exopolymeric matrix, and (iii) the cell surface biochemical components. The physico-chemistry of the interactions (such as van der Waals attraction, gravitational force, electrostatic charge, or hydrophobic interaction) (Gottenbos et al., 2000) have been theorized into models by the thermodynamic (Morra and Cassinelli, 1996) and the DLVO (Derjaguin-Landau-Verwey-Overbeek) theories, as well as its extended version (Jucker et al., 1998; Hermansson, 1999). While those models can help explaining some experimental observations, neither of them can fully describe bacterial adhesion as they fail in taking into account a fundamental properties of a biological system that is its adaptability and variability (Katsikogianni and Missirlis, 2004). Indeed, in the course of adhesion and/or biofilm formation, bacterial

cells change their physiology at different regulatory levels, e.g., gene/protein expression. This can induce modifications of cell morphology, general surface properties and/or express specific determinants for adhesion. Among those, the exopolymers can be prominent components synthetized in the course of bacterial adhesion and biofilm formation where they play the role of molecular glue (Flemming and Wingender, 2010). A large variety of exopolymers can be involved in bacterial colonization process namely various exopolysaccharides (EPS), e.g., alginate, cellulose, or poly-*N*-acetylglucosamine (PNAG) (Ryder et al., 2007; Vu et al., 2009; Bazaka et al., 2011), extracellular DNA (eDNA) and polyglutamate (Candela and Fouet, 2006). In some cases, those exoplymers are closely associated with the bacterial cell envelope, such as some polyglutamate covalently linked to peptidoglycan (Candela et al., 2012). Some other components of the cell envelope can participate in biofilm formation such as lipopolysaccharides (LPS) (Nakao et al., 2012), (lipo)teichoic acids (Gross et al., 2001; Fabretti et al., 2006), or mycolic acids (Ojha et al., 2005; Zambrano and Kolter, 2005). Among cell envelope components, proteins are undoubtedly the most functionally diverse active components. To be present on the bacterial cell surface, though, a protein must be secreted in the first place (or released by non-active translocation process, e.g., cell lysis or membrane budding).

Protein secretion is a key event for the presence of effectors at the interface between the bacterial cell and its immediate environment (Henderson et al., 2004). Those effectors can be displayed on the bacterial cell surface following anchoring to the cell envelope, released into the extracellular milieu or even beyond, i.e., within a host cell. Secreted proteins feature the lifestyle of a bacterium, its interaction within the ecosystems, microbiota, and ecological niches; for instance, virulence factors in pathogenic bacteria or degradative enzymes in saprophytic bacteria. As such, protein secretion is a key player in bacterial cell physiology and interactions with their environment. To understand protein secretion systems in bacteria, it is crucial to consider the cell envelope architecture. In recent years, it clearly appeared the grouping of bacteria into Gram-positive and Gram-negative bacteria was not satisfactory but ambiguous to describe and categorize accurately the protein secretion systems. Considering the inherent ambiguities of the Gram-negative and Gram-positive terminology, which can refer to three different and sometimes completely unrelated aspects (i.e., Gram staining, cell envelope architecture, and taxonomic grouping) (Desvaux et al., 2009b), the description of monoderm and diderm bacteria is much more appropriate in the field of protein secretion, at least. Indeed, monoderm bacteria (monodermata) refers specifically to species exhibiting only one biological membrane, i.e., the cytoplasmic membrane (CM), whereas diderm bacteria (didermata) refers specifically to species exhibiting two biological membranes, i.e., a CM, then also called inner membrane (IM) and an outer membrane (OM). Diderm bacteria can be further discriminated into (i) diderm-LPS bacterial cells, which possess an archetypal and assymetrical OM containing lipopolysaccharide (LPS) on the external side, (ii) simple-diderm bacterial cells, which possess an OM lacking LPS, and (iii) diderm-mycolate bacterial cells, where an outer lipid layer resembling an OM is composed of mycolic acid molecules arranged in a highly ordered form (Brennan and Nikaido, 1995; Sutcliffe, 2010; Gupta, 2011).

Considering the close association of secreted proteins with their cognate secretion systems, the secretome is a key concept to apprehend the protein secretion and related physiological functions. The secretome refers both to the secretion/translocation systems and the protein substrates of these transport systems (Tjalsma et al., 2000; Antelmann et al., 2001, 2006; Van Dijl et al., 2001; Economou, 2002; Sarvas et al., 2004; Buist et al., 2006; Desvaux et al., 2009b). The secretome concept provides an integrated and global view of protein secretion by considering protein routing, transport mechanisms, post-translational modifications, and protein subcellular location. Confusion between the secretome and the secreted/extracellular proteins is a common misunderstanding promulgated in part of the scientific literature but it must be stressed again the secretome is neither the exoproteome (extracellular proteome) nor an "omics" approach *per se* (Desvaux et al., 2009b). The exoproteome refers specifically to the subset of proteins present in the supernatant (which are not necessarily secreted). Secreted proteins are not necessarily free soluble extracellular proteins (exoproteins) since they can have different final subcellular locations (membranes, cell wall, extracellular milieu) or be subunits of supramolecular protein complexes (e.g., flagellum, pilus, cellulosome). Secreted proteins can even have multiple final subcellular locations, which are described following the gene ontology (GO) for "Cellular component" (**Figure 1**). All extracytoplasmic proteins are not systematically secreted since some exoproteins can be released upon molecular events that are not active translocation process and thus not secretion *per se*, e.g., cell lysis (autolysis, allolysis, bacteriophage lysis), GTA (gene transfer agent) or membrane budding (vesicles). The translocation corresponds to the active transport across a biological membrane (Desvaux et al., 2009b); the secretion refers to active transport from the interior to the exterior of the cell and the export to active transport across the CM (Economou et al., 2006). While in monoderm bacteria secretion and export are synonymous, in diderm bacteria the secretion is completed only upon translocation across the OM. For the sake of clarity, these key definitions in the field of protein secretion are reminded in **Table 1**.

With these different concepts in hand, it becomes clear a comprehensive understanding of protein determinants involved in bacterial adhesion and/or biofilm formation necessitates a consideration of the cell envelope architecture, i.e., respective to the diderm-LPS, monoderm and diderm-mycolate bacteria trichotomy, as well as their respective protein secretion systems.

THE PROTEIN SECRETION SYSTEMS IN DIDERM-LPS, MONODERM AND DIDERM-MYCOLATE BACTERIA

In diderm-LPS bacteria, nine protein secretion systems have been unravelled so far. For better or worse, these systems have been numerically classified from the Type I (T1SS) to Type IX secretion systems (T9SS) (Desvaux et al., 2009b; McBride and Zhu, 2013) (**Figure 2**). The T1SS refers to a three-component complex, i.e., a pore-forming OM protein (OMP) of the TolC family, a membrane fusion protein (MFP), and an IM ATP-binding cassette (ABC) exporter (Delepelaire, 2004; Holland et al., 2005;

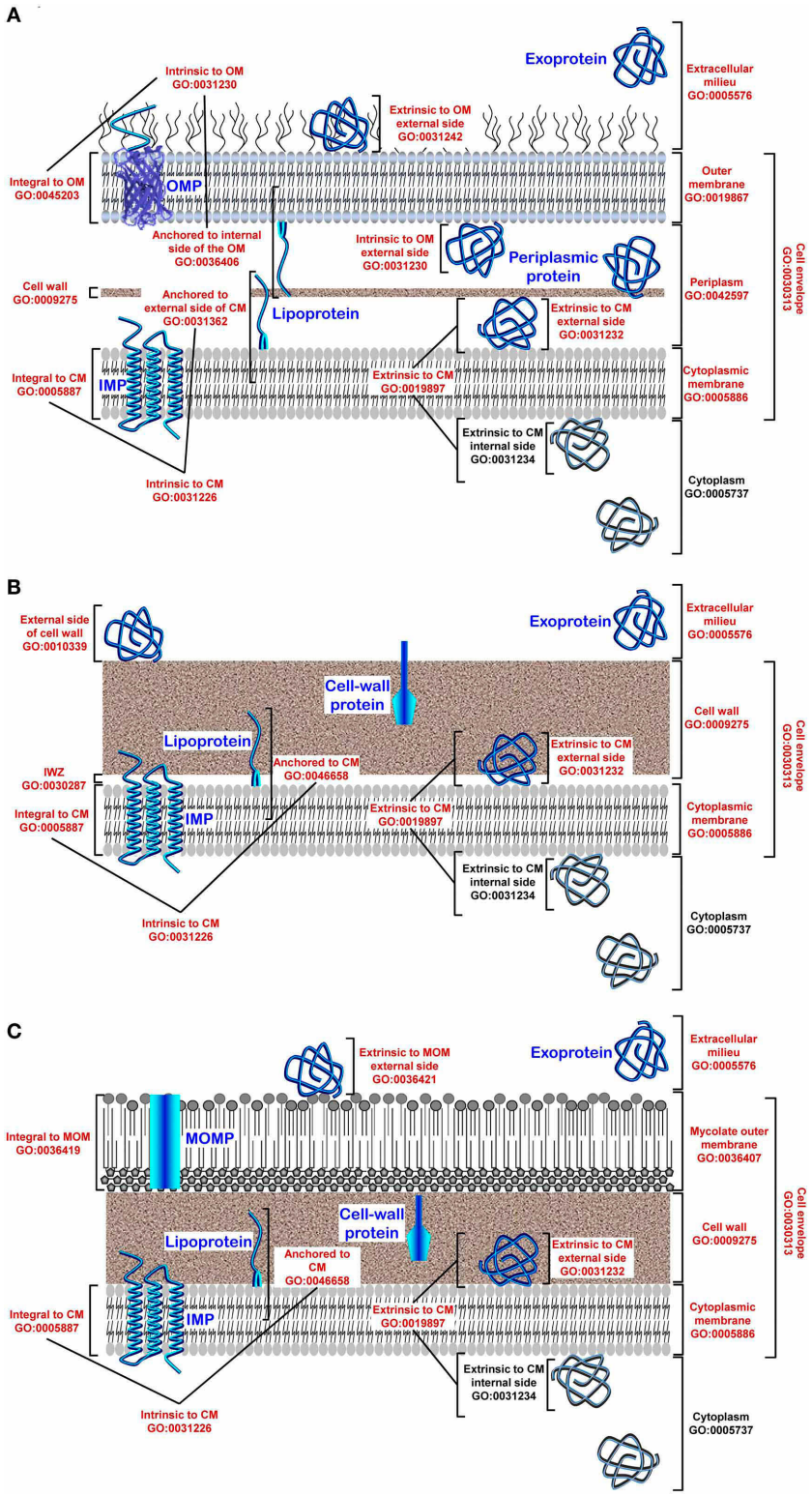

FIGURE 1 | Gene Ontology (GO) for cellular components and protein subcellular location in bacteria. (A) In diderm-LPS bacteria, five clearly defined compartments are considered (i) the cytoplasm (CP; GO:0005737), (ii) the cytoplasmic membrane (CM; GO:0005886), (iii) the periplasm (PP; GO:0042597), (iv) the outer membrane (OM; GO:0019867), and (v) the extracellular milieu (EM; GO:0005576). The cell envelope (GO:0010339) is constituted of the OM and CM, also called inner membrane (IM), as well as a thin cell wall (CW) located in-between. The situation at the CM can be discriminated even further between locations either intrinsic (GO:0031226) or extrinsic (GO:0019897) to the CM. The former refers to gene products with covalently attached moieties embedded in the CM, which splits into

(Continued)

FIGURE 1 | Continued
locations (i) integral to CM (GO:0005887) where some part of the peptide sequence spans all or part of the CM, i.e., the integral membrane proteins (IMPs), and (ii) anchored to the external side of CM (GO: 0031362) corresponding to proteins tethered to the CM by non-polypeptidic covalently attached anchor, i.e., the lipoproteins. The latter refers to proteins extrinsic to the CM, i.e., neither anchored by covalent bonds to any moiety nor directly embedded in the CM. Peripheral proteins are loosely bound to the surface components of the CM on the internal (GO:0031234) or external side (GO:0031232). Some proteins localized at the OM can also be subunits of supramolecular protein complexes (GO:0043234). The situation at the OM can be discriminated even further between locations intrinsic (GO:003230) or extrinsic (GO:0031242) to the OM. The former refers to gene products with a covalently attached moiety embedded in the OM, which splits into locations (i) integral to OM (GO:00045203) where some part of the peptide sequence spans all or part of the OM, i.e., the outer membrane proteins (OMPs), and (ii) anchored to internal side of OM (GO:0036406) corresponding to proteins tethered to the OM by non-polypeptidic covalently attached anchor, i.e., some lipoproteins. **(B)** In monoderm bacteria, four clearly defined compartments are considered (i) the cytoplasm (CP; GO:0005737), (ii) the cytoplasmic membrane (CM; GO:0005886), (iii) the cell wall (CW; GO:0009275), and (iv) the extracellular milieu (EM; GO:0005576). An inner wall zone (IWZ) (Matias and Beveridge, 2005) has been identified (GO:0030287); importantly, it should not be considered *sensu stricto* as a periplasm since the CW is porous and therefore it is not bordered (bounded) contrary to the situation in diderm-LPS bacteria where the periplasmic space is strictly delimited by two biological membranes. The CM and CW constitute the cell envelope (GO:0010339). The situation at the CM is similar to what is described for diderm-LPS bacteria. Some proteins localized at the CM or CW can also be subunits of protein complex (GO:0043234) or be extrinsic to the CW (GO:0010339). **(C)** In diderm-mycolate bacteria, five clearly defined compartments are considered (i) the cytoplasm (CP; GO:0005737), (ii) the cytoplasmic membrane (CM; GO:0005886), (iii) the cell wall (CW; GO:0009275), (iv) the mycolate outer membrane (MOM) or mycomembrane (GO:0036407), and (v) the extracellular milieu (EM; GO:0005576). The cell envelope (GO:0010339) is constituted of the MOM, CW, and CM; a pseudo-periplasm might exist but remains to be evidenced. The situation at the CM is similar to what is described for diderm-LPS or diderm bacteria. The situation at the MOM (GO:0036407) can be discriminated further between location integral to the mycomembrane (GO:0036419), i.e., the MOM proteins (MOMPs), or extrinsic to the MOM (GO:0036420). Location at the cell surface (GO:0009986) refers (i) in diderm-LPS, to the OM and/or external side of the OM and is intended to proteins exposed externally (GO:0031242) or intrinsic to the OM (GO:0031230) (similar rermarks apply to diderm-mycolate bacteria and the MOM), and (ii) in monoderm bacteria, to the CW and/or external side of the CM and is intended to proteins exposed externally or attached to the CW (GO:0009275) or the CM, i.e., integrated (GO:0005887), anchored (GO:0046658) or loosely bound (GO:0031232). As the CW is not a permeability barrier in monoderm bacteria but porous, the surface proteins do not necessarily have domains protruding from the confine of the cell envelope to interact with the external environment. Altogether with the subset of proteins localized extracellularly (GO:0005576), i.e., the exoproteome, these gene products (GO numbers in red) correspond to the extracytoplasmic proteins, i.e., the extracytoproteome (proteins depicted in blue).

Lee et al., 2012); a common misunderstanding is to make the T1SS synonymous to an ABC transporter (Desvaux et al., 2009b). The T2SS, also called the secreton-depend pathway (SDP) is a protein complex composed of around a dozen of proteins bridging the IM and OM to allow secretion of proteins translocated in the first place by the Sec or Tat export system (Sandkvist, 2001; Voulhoux et al., 2001; Cianciotto, 2005; Douzi et al., 2012; McLaughlin et al., 2012). It can be stressed again that referring to the general secretory pathway (GSP) or to the main terminal branch (MTB) for this system is nowadays obsolete and misleading (Desvaux et al., 2004b); as referred to in Pfam (Bateman et al., 2004), the naming of the different T2SS subunits as T2SE for instance is much more preferable than GspE (Peabody et al., 2003; Desvaux et al., 2004b). The T3SS is a highly complex molecular machine composed of at least 20 proteins and also one of the most extensively investigated protein secretion system (Ghosh, 2004; Cornelis, 2006, 2010; Minamino et al., 2008; Büttner, 2012). The T4SS is composed of around a dozen of proteins subunits forming a protein-conducting channel spanning the entire bacterial cell envelope (Christie et al., 2005; Waksman and Fronzes, 2010; Zechner et al., 2012); there is still much misunderstanding and confusion in part of the scientific literature about the T4SS and T2SS, as well as Type 4 pili (T4P), but it must be stressed again they can be clearly phylogenetically differentiated (Nunn, 1999; Planet et al., 2001; Mattick, 2002; Peabody et al., 2003; Hazes and Frost, 2008). T5SS refers to proteins depending on the Sec machinery for IM transit and then transported across the OM *via* a translocation unit formed by a β-barrel to complete secretion (Henderson et al., 2004; Leo et al., 2012); the BAM (β-barrel assembly machinery) complex as well as several periplasmic chaperones (namely SurA, Skp, DegP, and FkpA) are taking part to the secretion process across the OM (Desvaux et al., 2004a; Knowles et al., 2009; Ruiz-Perez et al., 2010; Rossiter et al., 2011b; Leyton et al., 2012). The T6SS is a composite system of at least 13 protein subunits from various hypothetical phylogenetic origins, with essentially two subassemblies, i.e., one dynamic structure related the contractile bacteriophage tail-like structure and one cell-envelope-spanning membrane-associated complex (Cascales and Cambillau, 2012; Silverman et al., 2012). The T7SS corresponds to the chaperone-usher pathway (CUP) used for pilus assembly (Desvaux et al., 2009b; Waksman and Hultgren, 2009; Busch and Waksman, 2012); as further explained below, this system for diderm-LPS bacteria must not be mistaken with the diderm-mycolate bacterial "Type VII secretion system," which is in fact the ESX (ESAT-6 system). The T8SS corresponds to the extracellular nucleation-precipitation pathway (ENP) (Barnhart and Chapman, 2006; Desvaux et al., 2009b; Blanco et al., 2012; Dueholm et al., 2012). The T9SS corresponds to the Por (porphyrin accumulation on the cell surface) secretion system (Sato et al., 2010, 2013; Shoji et al., 2011; McBride and Zhu, 2013). In diderm-LPS bacteria, the complement of the secretome potentially involved in bacterial colonization process gathers some secreted proteins and their associated secretion systems, which can be either the T1SS, T2SS, T3SS, T4SS, T5SS, T7SS, T8SS, or T9SS (**Figure 2**). In general, the secreted proteins involved in bacterial colonization are either cell-surface exposed single proteins or subunits of cell-surface supramolecular complexes, such as pili or flagella. In the rest of the manuscript, pili will be used as a generic term synonymous with fimbriae or curli (which are just some particular types of pili).

There are no counterparts to the molecular machineries required for transport across the OM of diderm bacteria in monoderm bacteria as this membrane is not present in the latter organisms. Consequently, the use of the numerical classification

Table 1 | Some key definitions in the field of bacterial protein secretion.

Terminology	Definition	Note[a]	References
Monoderm bacteria	Bacterial species exhibiting only one biological membrane, i.e. the CM.	Corresponds to the archetypal Gram-positive bacteria (i.e., with a CW) but also includes the *Mycoplasma* (devoided of a CW) since they also possess only one biological membrane. Monoderm bacteria are also called monodermata.	Shatalkin, 2004; Desvaux et al., 2009b; Sutcliffe, 2010; Gupta, 2011
Diderm-LPS bacteria	Bacterial species exhibiting two biological membranes and where the assymetrical OM contains lipopolysaccharide (LPS) on the external side.	Corresponds to the archetypal Gram-negative bacteria. Some diderm bacteria lack LPS in their OM and are called simple-diderm bacteria. Diderm bacteria are also called didermata.	Shatalkin, 2004; Desvaux et al., 2009b; Sutcliffe, 2010; Gupta, 2011
Diderm-mycolate bacteria	Bacterial species exhibiting two biological membranes and where an outer lipid layer called MOM is composed of mycolic acid molecules arranged in a highly ordered form.	Corresponds to the archetypal acid-fast bacteria, e.g., *Mycobacterium* and *Corynebacterium*.	Shatalkin, 2004; Desvaux et al., 2009b; Sutcliffe, 2010; Gupta, 2011
Secretome	Concept for an integrated and global view of the protein secretion by considering protein routing, transport mechanisms, post-translational modifications, and protein subcellular location.	The secretome considers both secreted proteins and proteins constituting the secretion machinery (and associated maturation pathways). This original definition of the secretome has been somehow usurped, misused, and misunderstood by some authors in the literature. The secretome can be investigated by different "omics" approaches (i.e., proteogenomics, transcriptomics, proteomics, and meta-omics counterparts) but is not a proteome *per se*. The secretome is not the exoproteome, which is the most commonly investigated but only one of the complement of the secretome. Other complements of the secretome can be (i) the protein secretion systems, (ii) the cell-surface proteins (including single and supramolecular protein structure), or (iii) the lipoproteome, etc... Since it is not secretion *per se*, the secretome do not cover protein release upon molecular events that are not active translocation process, e.g., cell lysis (autolysis, allolysis, bacteriophage lysis), or membrane budding (vesicles).	Tjalsma et al., 2000; Antelmann et al., 2001, 2006; Van Dijl et al., 2001; Economou, 2002; Sarvas et al., 2004; Buist et al., 2006; Desvaux et al., 2009b
Secretion	Active transport from the interior to the exterior of the cell.	Applies to protein entirely outside of the outer-most lipid bilayer, including exoproteins, surface proteins, and cell-surface appendages (e.g., pili and flagella, cellulosomes). In diderm bacteria, secretion is mediated by specific translocon for transport across the OM (or MOM in diderm-mycolate bacteria) and cannot be defined by the translocons located at IM (CM). *Sensu stricto*, it does not cover molecular events that are not active translocation process and thus not secretion *per se*, e.g., cell lysis (autolysis, allolysis, bacteriophage lysis) or membrane budding (vesicles).	Desvaux et al., 2004b, 2009b; Economou et al., 2006
Export	Active transport across the CM	In monoderm bacteria, export and secretion are synonymous but not in diderm bacteria. In diderm bacteria, the Sec and Tat translocon directs proteins to the CM (IM) or periplasm but cannot ensure their secretion *sensu stricto*.	Desvaux et al., 2004b, 2009b; Economou et al., 2006

(Continued)

Table 1 | Continued

Terminology	Definition	Note[a]	References
Translocation	Active transport across a biological membrane.	Relative to the monoderm/diderm-LPS/diderm-mycolate bacteria trichotomy, it can occur at the CM (IM), OM, and/or MOM. Translocation event results in a change of subcellular location.	Economou et al., 2006; Desvaux et al., 2009b
Extracytoplasmic protein	Protein found outside the cytoplasm.	Corresponds to a protein either located elsewhere than the cytoplasm, e.g., intrinsic to a membrane (IMP, OMP, MOMP, lipoproteins), extrinsic to a membrane but on the side away from the cytoplasm, within the periplasm, associated to CW, at the cell surface or into the extracellular milieu. In other words, every protein except a cytoprotein. This subset of proteins corresponds to the extracytoproteome.	Dinh et al., 1994; Skorko-Glonek and Sobiecka-Szkatula, 2008
Exoprotein	Protein present in the extracellular milieu, i.e., an extracellular protein.	Corresponds to free soluble protein find in the extracellular milieu or within a host cell. Some exoproteins are not necessarily secreted *sensu stricto*, since it can occur by not active translocation process e.g., cell lysis (autolysis, allolysis, bacteriophage lysis), or membrane budding (vesicles).	Pugsley and Francetic, 1998; Desvaux et al., 2009b
Cytoprotein	Protein present in the cytoplasm, i.e., a cytoplasmic protein.	Corresponds to a cytosoluble protein, protein extrinsic to the CM located on the cytoplasmic side or a protein subunit of a CM protein complex but which subunit is entirely outside of the CM.	
Exoproteome	The subset of proteins present in the extracellular milieu (the exoproteins), i.e., the extracellular proteome.	It corresponds to one of the complements of the secretome.	Tjalsma, 2007; Desvaux et al., 2009b
Surface proteome	The subset of proteins present on the bacterial cell surface (the cell-surface proteins).	It corresponds to one of the complements of the secretome. The terminology "surface proteome" should be preferred to "surfaceome" (or "surfacome") because it stresses it focuses on the protein content and not all the components on the bacterial cell surface (LPS, teichoic acids, exopolysaccharides, polyglutamate, etc. . .) as misleadingly suggested by the term "surfaceome."	Cullen et al., 2005; Desvaux et al., 2006a, 2009b; Dreisbach et al., 2011; Voigt et al., 2012

[a] *IM, inner membrane; CM, cytoplasmic membrane; CW, cell wall; OM, outer membrane; MOM: mycolate outer membrane; IMP, integral membrane protein; OMP, outer membrane protein; MOMP, mycolate outer membrane protein.*

for systems dedicated to protein secretion in diderm-LPS bacteria (i.e., protein transport from inside to outside the cell across the IM and OM) does not make any sense and cannot be applied to monoderm bacteria. However, this does not prohibit phylogenetic relationships between the protein translocation systems in monoderm and diderm-LPS bacteria. Indeed, they both possess a cytoplasmic membrane (also called IM in didermata) with some common protein transport systems allowing secretion in monoderm bacteria and export in diderm-LPS bacteria, respectively. The protein secretion system present in monoderm bacteria are (i) the Sec (secretion), (ii) the Tat (twin-arginine translocation), (iii) ABC protein exporter, (iv) the FPE (fimbrilin-protein exporter), (v) the holin (hole forming), (vi) the Tra (transfer), misleadingly called the "Type IV-like secretion system" in monoderm bacteria), (vii) the FEA (flagella export apparatus), and (viii) the Wss (WXG100 secretion system) (Desvaux et al., 2009b; Desvaux, 2012) (**Figure 3**). From the current knowledge in the field, the complement of the secretome potentially involved in the colonization process in monoderm bacteria gathers the Sec, FPE and FEA as well as some of their respective substrates (**Figure 3**). These systems secrete either cell-surface exposed single proteins or subunits of cell-surface supramolecular complexes, such as pili, cellulosome, or flagella (Renier et al., 2012).

In diderm-mycolate (archetypal acid-fast) bacteria, the term "Type VII secretion system" has also been coined to describe a Wss-like machinery (Economou et al., 2006; Abdallah et al., 2007; Schneewind and Missiakas, 2012). It must be stressed, this "Type VII secretion system" is restricted to diderm-mycolate bacteria

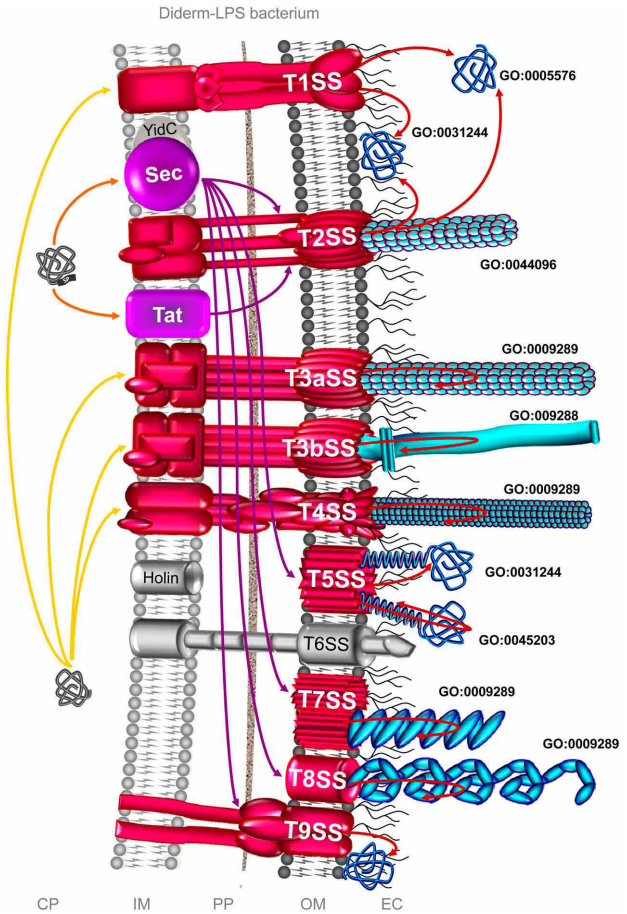

FIGURE 2 | The complement of the secretome involved in colonization process in diderm-LPS bacteria. Among the 9 distinct secretion systems through which a secreted protein can be translocated across the OM in diderm-LPS bacteria, the T1SS, T2SS, T3SS, T4SS, T5SS, T7SS, T8SS, and T9SS can be involved in colonization process (depicted in red). Translocation machineries depicted in violet are protein export pathway participating to the protein transport of effectors involved in colonization process. The T1SS can secrete adhesins, which the release into the extracellular milieu (GO:0005576) and/or the association to the external side of the OM (GO:0031244) remain to be clarified. Besides the secretion of adhesion factors, the T2SS is involved in the formation of Type 4 pilus (GO:0044096), i.e., the T2SS subfamily c (T2cSS). The subfamily a of the T3SS (T3aSS) can be involved in the formation of pilus structure (GO:0009289), i.e., either the injectisome or the Hrp (hypersensitive response and pathogenicity) pilus, whereas the subfamily b of the T3SS (T3bSS) is involved in flagellum assembly (GO:009288). The T4SS is involved in the formation of pili (GO:0009289), either pilus T (T4aSS) or pilus F (T4bSS). The T5SS is involved in the secretion of adhesion either integral (GO:0045203) or extrinsic (GO:0031244) to the OM. The T7SS (CUP; chaperone-usher pathway) is involved in the formation of Type 1 pilus, and the T8SS (ENP; extracellular nucleation-precipitation pathway) in the formation of pilus of the type curli. The T9SS (Por secretion system) can secrete adhesins involved in gliding motility. In addition, some surface proteins could use systems as yet uncovered, the so-called non-classical (NC) secretion. Only branches corresponding to the complement of the secretome involved in bacterial colonization are colored. Extracytoplasmic proteins, i.e., single proteins and supramolecular protein structures, potentially involved in surface colonization are depicted in blue. Orange and yellow arrows indicate the routes for proteins targeted to the CM possessing or lacking an N-terminal SP, respectively. Violet arrows indicate the routes for exported proteins and red arrows for secreted proteins. CP, cytoplasm; IM, inner membrane; PP, periplasm; OM, outer membrane; EC, extracellular milieu; SP, signal peptide.

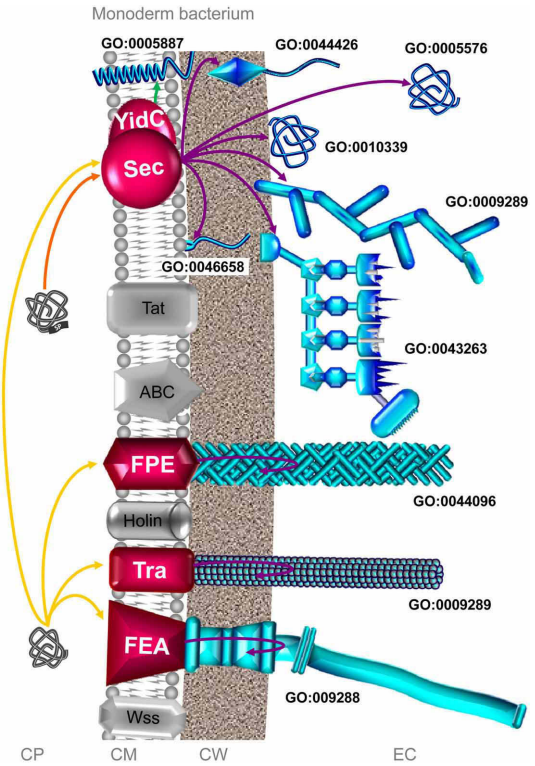

FIGURE 3 | The complement of the secretome involved in colonization process in monoderm bacteria. Among the 8 distinct secretion systems through which a secreted protein can be translocated across the CM, the Sec, FPE, Tra, and FEA pathways can potentially be involved in colonization process in monoderm bacteria (depicted in red). The Sec pathway covers (i) integration of membrane protein (GO:0005887) *via* YidC, (ii) the anchoring to CM (GO:0046658) *via* the lipoprotein maturation pathway, (iii) the anchoring to the CW (GO:0044426) in a covalent or non-covalent manner, (iv) the association on the external side of the CW (GO:0010339), (v) the formation of cell surface supramolecular structure, namely pilus (GO:0009289) and cellulosome (GO:0043263), and (vi) protein secretion in the extracellular milieu (GO:0005576). It is worth noting that some proteins with no N-terminal SP can be translocated *via* Sec in a SecA2-dependent manner in monodermata (Rigel and Braunstein, 2008; Renier et al., 2013). The FPE is involved the formation of Type 4 pilus (GO:0044096). The Tra system (misleadingly called "Type IV-like secretion system" in monoderm bacteria) is involved in the formation of conjugative pili (GO:0009289). The FEA is involved in the secretion and assembly of the flagellum protein subunits (GO:009288). In addition, some surface proteins could use systems as yet uncovered, the so-called non-classical (NC) secretion. Extracytoplasmic proteins, i.e., single proteins and supramolecular protein structures, potentially involved in surface colonization are depicted in blue. Only branches corresponding to the complement of the secretome involved in bacterial colonization are colored. Orange and yellow arrows indicate the routes for proteins targeted to the CM possessing or lacking an N-terminal SP. Violet arrows indicate the routes for exported/secreted proteins (export and secretion are synonymous in monoderm bacteria). Green arrow indicates proteins integrated into the CM. CP: cytoplasm; CM: cytoplasmic membrane; CW: cell wall; EC, extracellular milieu; SP: signal peptide.

only since this system is absolutely not found in any archetypal diderm-LPS bacteria and, consequently, does not fit with standard numerical classification of protein secretion systems specifically designated for diderm-LPS (archetypal Gram-negative) bacteria (Salmond and Reeves, 1993; Sutcliffe, 2010). This "Type VII secretion system" further stands alone in diderm-mycolate

bacteria since there are neither T1SS, T2SS, T3SS, T4SS, T5SS, nor T6SS but only Sec and Tat systems (which are not included in the standard numerical classification) (Digiuseppe Champion and Cox, 2007) (**Figure 4**). The molecular machinery described for this diderm-mycolate bacterial "Type VII secretion system" is an export pathway (protein transport across the cytoplasmic membrane) just as the Sec and Tat systems are (Economou et al., 2006; Desvaux et al., 2009a; Houben et al., 2012). In fact, no translocon in the mycolate outer membrane (MOM), which would truly enable protein secretion and thus form a complete secretion pathway, has been uncovered as yet in diderm-mycolate bacteria [the secretion of proteins exported in the first instance by the Wss, Sec, and Tat could then be completed by the very same MOM translocon, or different MOM translocons specific to each of these export systems (Desvaux et al., 2009a)] (Niederweis, 2003; Converse and Cox, 2005; Ize and Palmer, 2006; Digiuseppe Champion and Cox, 2007; Song et al., 2008; Desvaux et al., 2009a; Niederweis et al., 2010; Stoop et al., 2012; Freudl, 2013; Van Der Woude et al., 2013). This "Type VII secretion system" nomenclature in diderm-mycolate bacteria is clearly not compatible with the numerical classification basically designed to describe OM translocation systems in diderm-LPS bacteria (Salmond and Reeves, 1993; Economou et al., 2006; Desvaux et al., 2009a). All-in-all, using the "Type VII secretion system" denomination for the phylogenetically related secretion systems in monoderm bacteria (i.e., the Wss) is very much confusing since it does not align with the other secretion systems present such as Sec and Tat (which do not withstand the numerical classification terminology) (Desvaux et al., 2004b, 2009a). When designating this "Type VII secretion system" it is then highly advisable to clearly specify it relates to diderm-mycolate bacteria only to prevent any confusion with the unrelated T7SS in diderm-LPS bacteria. Ultimately, its use should be refrained in favor of the "ESX (ESAT-6 system)" designation in diderm-mycolate bacteria (archetypal acid-fast bacteria) and/or the generic "WXG100 secretion system (Wss)" designation especially relevant to monodermata (archetypal Gram-positive bacteria). At the moment only protein export systems have been reported in diderm-mycolate bacteria (Digiuseppe Champion and Cox, 2007; Feltcher et al., 2010; Ligon et al., 2012) but *sensu stricto* no protein secretion system has been identified (**Figure 4**). As for the T1SS to T9SS in diderm-LPS bacteria, only the identification of translocon components at the MOM would truly permit to define a protein secretion system (Desvaux et al., 2009a), which at worse could be the unique terminal branch for all the three export systems (Sec, Tat, and ESX) in diderm-mycolate bacteria (**Figure 4**).

For effectors involved in bacterial colonization, this review will especially focus on protein secretion systems in diderm-LPS (archetypal Gram-negative) and monoderm (archetypal Gram-positive) bacteria since none has been characterized as yet in diderm-mycolate (archetypal acid-fast) bacteria.

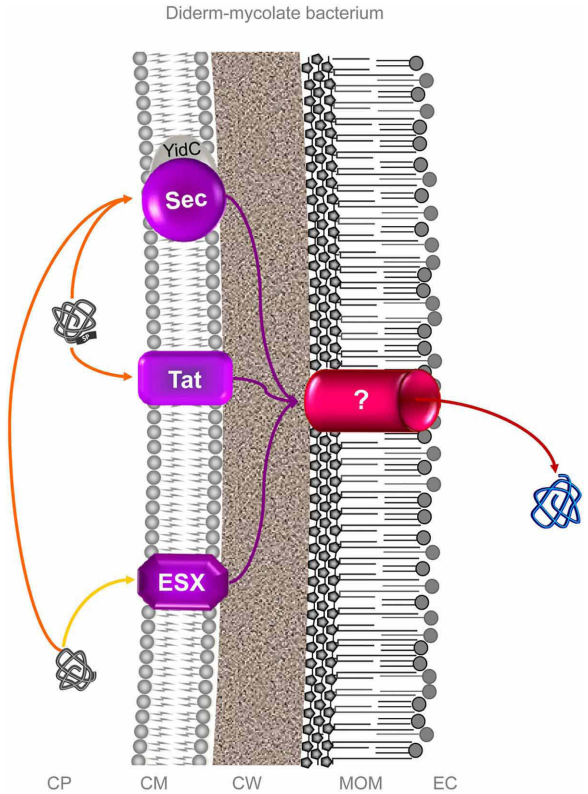

FIGURE 4 | Protein secretion in diderm-mycolate bacteria. In diderm-mycolate bacteria, three protein export systems are currently recognized, the Sec, Tat and ESX (ESAT-6 system) (Digiuseppe Champion and Cox, 2007; Feltcher et al., 2010; Ligon et al., 2012). So far, none have been clearly reported and characterized as involved in surface colonization process. As in monodermata, some proteins with no N-terminal SP can be translocated *via* Sec in a SecA2-dependent manner (Feltcher and Braunstein, 2012). While it is clear some exported proteins (i.e., first translocated across the CM by these export systems) are further secreted into the extracellular milieu (i.e., translocated across the MOM), no mycomembrane machinery allowing the translocation across the MOM have been identified to date (Niederweis et al., 2010; Houben et al., 2012; Ligon et al., 2012; Freudl, 2013; Van Der Woude et al., 2013). In other no words (**Table 1**), no protein secretion system has been *sensu stricto* identified as yet in diderm-mycolate bacteria. It is still an enigma whether one MOM translocon or specific translocons for each of the three protein export systems are present or if the protein secretion is completed in a one-step or two-steps process (Desvaux et al., 2009a). This (or those) MOM translocon would truly correspond to a protein secretion system. For these different reasons and others, numbering the ESX (as "Type VII secretion system") in diderm-mycolate bacteria is clearly premature and misleading (Desvaux et al., 2009a,b). Orange and yellow arrows indicate the routes of proteins targeted to the CM possessing or lacking an N-terminal SP. Violet arrows indicate the routes for exported proteins and red arrows for secreted proteins. CP, cytoplasm; CM, cytoplasmic membrane; CW, cell wall; MOM, mycolate outer membrane (or mycomembrane); EC, extracellular milieu; SP, signal peptide.

SECRETED PROTEINS INVOLVED IN SURFACE COLONIZATION IN DIDERM-LPS BACTERIA

Out of the 9 distinct protein secretion systems through which a secreted protein can be translocated across the OM, only the T6SS has never been reported so far to be involved in bacterial adhesion and/or biofilm formation in diderm-LPS bacteria (**Figure 2**). The T2SS, T3SS, T4SS, and T5SS are further divided into different subtypes.

ADHESINS SECRETED VIA THE T1SS

The T1SS has been demonstrated to allow the secretion of large bacterial adhesins belonging to the Bap family (Delepelaire, 2004; Lasa and Penadés, 2006; Latasa et al., 2006). In *Salmonella enterica*, SiiE (*Salmonella* intestinal infection E) and BapA (Biofilm-associated protein A) have been characterized. SiiE is a very large adhesin of 600 kDa of *Salmonella enteric* required for adhesion to epithelial cells. SiiE would essentially exist as an exoprotein that may only be loosely associated the OM but upon host cell contact it is retained on the bacterial cell surface (Wong et al., 1998; Morgan et al., 2004; Gerlach et al., 2007). The ability of SiiE to bind Ca^{2+} ions would confer a rigid rod-like habitus that is required for reach out beyond the LPS and initiates bacterial adhesion to polarized host cells (Wagner et al., 2011; Griessl et al., 2013). BapA is a 386 kDa protein allowing homotypic adhesion during *Salmonella* biofilm formation and is involved in pathogenesis, especially internalization and invasion of intestinal epithelium (Latasa et al., 2005; Jonas et al., 2007; Biswas et al., 2011; Suez et al., 2013). BapA is secreted extracellularly where it remains in loose association with the bacterial cell surface. In *Pseudomonas fluorescens*, LapA (large adhesion protein A) is the largest T1SS-dependent adhesin uncovered so far, with a molecular weight estimated at 888 kDa (Hinsa et al., 2003; Hinsa and O'toole, 2006). LapA enables irreversible adhesion and biofilm formation on abiotic surfaces but also adhesion to corn seeds in *Pseudomonas putida* (Mus20 or Mus24; mutant unattached to seeds) (Espinosa-Urgel et al., 2000; Huber et al., 2002; Hinsa et al., 2003; Ivanov et al., 2012; Zhang et al., 2013). LapA is found both in the extracellular milieu and in a loose association with the bacterial cell surface but not in the OM (Hinsa et al., 2003). In *Burkholderia cepacia*, the loss of Bap resulted in decreased surface hydrophobicity and in colony conversion to a rough morphotype (Huber et al., 2002). Additional adhesins of the Bap family, presumably secreted *via* a T1SS as supported by the genomic context, have been characterized in diderm-LPS bacteria, namely VPA1445 (*Vibrio parahaemolyticus* locus 1445) (Enos-Berlage et al., 2005) and YeeJ (systematic nomenclature) in *Escherichia coli* (Roux et al., 2005). In all cases, the mediation of adhesion *via* an extracellular protein is difficult to conceptualize and still demands experimental investigations for validation (Gerlach and Hensel, 2007).

TYPE 4 PILUS (T4P) AND COLONIZATION FACTOR *VIA* THE T2SS

Based on phylogenetic analysis, T2SSs were further subdivided into the subfamilies T2aSS and T2bSS, corresponding, respectively, to Xcp (*Xanthomonas campestris* general secretion pathway) and Hxc (homolog to Xcp) systems (Filloux, 2004; Michel and Voulhoux, 2009; Durand et al., 2011). At the same period, it was proposed to include the Type 4 piliation (T4P) system (Desvaux et al., 2009b) within the T2SS since the T4P is homologous to the Xcp and falls into discrete phylogenetic cluster (Planet et al., 2001; Peabody et al., 2003; Tomich et al., 2007; Ayers et al., 2010). To make the nomenclature coherent, we propose to classify those systems as T2aSS for the classical SDP extensively investigated in *Pseudomonas aeruginosa*, i.e., Xcp type system (Voulhoux et al., 2001), T2bSS for the Hxc type system (Durand et al., 2011), and T2cSS for the T4P system (Mattick, 2002). The Type 4 pili promote initial bacterial attachment and are of importance for the early stages of biofilm development, i.e., microcolony formation (Hahn, 1997; Giltner et al., 2006; Burrows, 2012). The Type 4 pili are subdivided into two main classes assembled by the T2cSS (Strom and Lory, 1993; Kachlany et al., 2001; Pelicic, 2008), i.e., (i) the T4a pilins, and (ii) the T4b pilins (Strom and Lory, 1993; Skerker and Shapiro, 2000; Craig et al., 2004; Craig and Li, 2008; Giltner et al., 2012). Besides adherence, the T4aP are responsible for a number of related processes associated with bacterial motility, including twitching, swarming, crawling, walking, and slingshoting, which participate to biofilm development (Desvaux et al., 2005a; Burrows, 2012; Conrad, 2012).

The colonization factor GbpA (N-acetylglucosamine-binding protein A) from *Vibrio cholerae* is secreted in a T2SS-dependent manner in the extracellular milieu (Kirn et al., 2005). This protein is required for efficient environmental colonization by organisms such as zooplankton, and also intestinal colonization, especially of human epithelial cells, by binding to a sugar present on both surfaces (Stauder et al., 2012). GbpA has a modular architecture with chitin-binding and mucin-binding domains enabling attachment to different host surfaces and other domains binding to the bacterial cell surface (Wong et al., 2012). Despite its secretion into the extracellular milieu, GbpA thus has a bridging function between *V. cholerae* and its host allowing efficient colonization of chitinous exoskeletons of arthropods or the intestinal epithelium.

T3SS: INJECTISOME, HRP (HYPERSENSITIVE RESPONSE AND PATHOGENICITY) PILUS AND FLAGELLUM

The T3SSs can be subdivided into (i) the non-flagellar T3SS, i.e., the T3aSS, involved in the assembly of the injectisome or Hrp (hypersensitive response and pathogenicity) pilus, and (ii) the flagellar T3SS, i.e., T3bSS, responsible for assembly of the flagellum (Tampakaki et al., 2004; Journet et al., 2005; Pallen et al., 2005; Desvaux et al., 2006b).

While acting primarily as a molecular syringe for injecting protein effectors directly into the cytosol of a host cell (Cornelis, 2006, 2010), the injectisome (T3aSS) mediates intimate bacterial adhesion and colonization of cells such as gut epithelial cells (Garmendia et al., 2005). In enterohemorrhagic *Escherichia coli* (EHEC), it was further demonstrated to play a role in the adhesion to lettuce leaves with a marked tropism for the stomata, and thus to contribute to the transmission of this food-borne pathogen to humans (Shaw et al., 2008; Berger et al., 2010). While the injectisome is found in animal-pathogenic bacteria, the Hrp pilus (T3aSS) is found in plant-pathogenic bacteria (Tang et al., 2006; Tampakaki et al., 2010). By injecting T3SS effectors into the host cell, the Hrp pilus allows the phytopathogens to subdue the vegetable-cell response (Zhou and Chai, 2008) but also participate to plant colonization through initial bacterial adhesion (Darsonval et al., 2008; Correa et al., 2012).

The flagella (T3bSS) are involved in cell motility through swimming and/or swarming, where it can contribute to bacterial colonization by bringing the cell in contact with biotic or abiotic surfaces by chemiotaxis for instance, or by translocating the bacterial cells over a surface, respectively (Pratt and Kolter, 1998; Harshey, 2003; Verstraeten et al., 2008). Besides, the flagella mediate bacterial adhesion directly to biotic and abiotic surfaces, e.g.,

to plasticware, human mucus and mucin, epithelial cells, enterocytes, vegetables (Grant et al., 1993; Ramphal et al., 1996; Lillehoj et al., 2002; Kirov et al., 2004; Berger et al., 2009b; Mahajan et al., 2009; Shaw et al., 2011; Tran et al., 2011; Bucior et al., 2012; Troge et al., 2012). Interestingly in *Salmonella enterica*, adhesion to salad leaves mediated by the flagella was shown to be strain-specific (Berger et al., 2009a). *Salmonella enterica* can further internalize into vegetable leaves through flagella-chemotaxis via open stomata (Kroupitski et al., 2009a). Although the internalization is variable in leafy vegetables and fresh herbs (Golberg et al., 2011; Kroupitski et al., 2011), the flagella is clearly an important determinant for colonization of some vegetables and can thus contribute to the transmission of this food-borne pathogen to humans even after washing (Berger et al., 2009a; Kroupitski et al., 2009a,b).

PILI ASSEMBLED BY THE T4SS

The T4SSs are broadly subdivided into (i) T4aSS corresponding to the prototypical VirB/D4 complex extensively investigated in *Agrobacterium tumefaciens*, and (ii) T4bSS corresponding to the prototypical F-conjugal transfer system of the self-transmissible IncI plasmid composed of the widely conserved Tra (transfer) proteins also present in monoderm bacteria (Christie and Vogel, 2000; Sexton and Vogel, 2002; Lawley et al., 2003; Harris and Silverman, 2004; Christie et al., 2005; Hazes and Frost, 2008; Voth et al., 2012). The T4SS allows transport of proteins into prokaryotic or eukaryotic cells. This system is also involved and ancestrally related to bacterial conjugation with the transport of DNA as nucleo-protein complex (Lawley et al., 2003). Rather than focusing on protein secretion system only, the nomenclature is quite confusing at the moment since it mixes up homologic (phylogenetic) and analogic (functional) based-classifications on aspects related to DNA transport as well as the systems present in monoderm and diderm-LPS bacteria (Alvarez-Martinez and Christie, 2009; Wallden et al., 2010); in addition, some phylogenetic relatedness between the T2SS assembling Type 4 pili (T4P) and T4SS assembling conjugative pili of Type 2 (Ottow, 1975) accentuates the complexity of the situation (Hazes and Frost, 2008). As it has been done previously for other protein secretion systems (Henderson et al., 2000; Desvaux et al., 2004b, 2006b, 2009b), this stresses the need to clarify the classification within the T4SS respective to the current terminology and ontology issues in the field of bacterial protein secretion, which should be rather based on homology and phylogeny rather than analogy (Desvaux et al., 2006b, 2009b). Regarding bacterial colonization, the F episome (natural conjugative plasmid) was demonstrated to induce the formation of a thick biofilm in *E. coli* and that conjugative pilus synthesis from the *tra* operon of the F plasmid was required for biofilm formation (Ghigo, 2001). In fact, the bacterial cells harboring the F episome had increased adhesion ability thanks to the expression of conjugative pili. It appeared these plasmids can be further transferred by conjugation from these donor cells to recipient cells. Then, the increasing proportion of transconjugant cells within the biofilm further improved the colonization ability of the bacterial population. While the positive influence of conjugative plasmid on biofilm formation was reported in other species (Bahl et al., 2007; Burmolle et al., 2008), it can also have adverse effects in complex multi-species biofilms and would depend on the composition of the bacterial community (Roder et al., 2013).

Phylogenetic analyses revealed the tight-adherence (Tad) system originally uncovered in *Aggregatibacter* (formerly *Actinobacillus*) *actinomycetemcomitans* was actually related to the T4SS and would constitute a major subfamily (Tomich et al., 2007). The Tad system is involved in the piliation of the Flp (fimbrial low-molecular-weight protein). Those pili are composed of Type 4 pilin of the subfamily b (Giltner et al., 2012). Flp pili are highly adhesive and essential for biofilm formation (Kachlany et al., 2001). Rather than binding to specific receptor(s), the bacterial–bacterial and bacterial–host interactions lead to strong attachment. Bacterial aggregation is mediated by the association of pilus fibers, which leads to pilus bundling. This system is widespread among diderm-LPS bacterial species, including the genera *Haemophilus, Pasteurella, Pseudomonas, Yersinia,* or *Caulobacter* (Kachlany et al., 2000, 2001; Bernard et al., 2009).

CELL-SURFACE ADHESINS OF THE T5SS

T5SSs are subdivided into five subtypes (Henderson et al., 2000, 2004; Desvaux et al., 2003, 2004a; Henderson and Desvaux, 2004; Salacha et al., 2010; Leo et al., 2012), (i) the T5aSS corresponding to the classical autotransporter or autotransporter of Type 1 (AT-1), (ii) the T5bSS corresponding to the two-partner secretion (TPS), (iii) the T5cSS corresponding to the trimeric autotransporter or autotransporter of Type 2 (AT-2), (iv) the T5dSS corresponding to hybrid autotransporter between AT-1 and TPS, or autotransporter of Type 3 (AT-3), and (v) the T5eSS corresponding to invasin/intimin family of inverted autotransporter or autotransporter of Type 4 (AT-4). No function in bacterial colonization has been reported as yet for the hybrid autotransporters (T5dSS) (Salacha et al., 2010).

Adhesins of the classical autotransporter pathway (T5aSS)

Among the classical autotransporters (T5aSS), the SAATs (Self-associating autotransporters) actively participate in biofilm development and encompasse the Ag43 (antigen 43), AIDA (adhesin involved in diffuse adherence,) and TibA (enterotoxigenic invasion locus b protein A) (Klemm et al., 2006; Van Der Woude and Henderson, 2008). The SAATs promote bacterial autoaggregation and interaction with other SAAT partners, for instance between AIDA and Ag43, leading to the formation of mixed bacterial cell aggregates (Sherlock et al., 2004). Self-recognition is sensitive to environmental conditions, such as pH or the presence of bile salt (Klemm et al., 2004; Sherlock et al., 2004, 2005; Girard et al., 2010) and intercellular aggregation can be abolished by pili or exopolymers, which compromise SAATs interaction (Klemm et al., 2006). SAATs further mediate and enhance biofilm formation (Klemm et al., 2006). Ag43, however, would not contribute significantly to intestinal colonization (Parham et al., 2005; De Luna et al., 2008). Besides SAATs, several other AT-1s act as adhesins (Henderson et al., 2004; Girard and Mourez, 2006). YfaL, YpjA, and YcgV (*E. coli* systematic nomenclature) are involved in initial bacterial adhesion and biofilm formation (Roux et al., 2005). BrkA (*Bordetella* resistance to killing protein A) (Ewanowich et al., 1989; Fernandez

and Weiss, 1994) and Pertactin (Leininger et al., 1991) promote adhesion to different mammalian cells. Aae (*A. actinomycetemcomitans* epithelial cell binding) (Rose et al., 2003; Fine et al., 2005), App (adhesion and penetration protein) (Serruto et al., 2003), CapA (*Campylobacter* adhesion protein A) (Ashgar et al., 2007), EspP (extracellular serine protease P) (Dziva et al., 2007; Puttamreddy et al., 2010), McaP (*Moraxella catarrhalis* adherence protein) (Timpe et al., 2003; Lipski et al., 2007), PmpD (Polymorphic membrane protein D) (Wehrl et al., 2004), rOmpB (Rickettsia outer membrane protein B) (Uchiyama et al., 2006), and Sab (STEC autotransporter mediating biofilm formation) (Herold et al., 2009), enable adherence to epithelial cells; for some of them it was further demonstrated they participated to intestinal colonization and/or biofilm formation, namely CapA, EspP and Sab. Hap (*Haemophilus* autotransporter protein) further mediates microcolony formation as well as binding to fibronectin, laminin, and collagen (Fink et al., 2003), whereas MisL (membrane insertion and secretion protein L) and ShdA (shedding protein A) bind to collagen and fibronectin (Kingsley et al., 2000, 2002, 2004; Dorsey et al., 2005). EhaA (EHEC autotransporter A) (Wells et al., 2008), EhaB (Wells et al., 2009), and EhaJ (Easton et al., 2011) are MSCRAMM (microbial surface components recognizing adhesive matrix molecules) proteins binding differentially to various extracellular matrix (ECM) proteins and contributing to biofilm formation (Chagnot et al., 2012, 2013). Tsh (temperature-sensitive haemagglutinin) also binds to ECM proteins and further adheres to red blood cells and hemoglobin (Kostakioti and Stathopoulos, 2004). AlpA (adherence-associated lipoprotein A) (Odenbreit et al., 1999), BabA (blood group antigen–binding adhesin) (Ilver et al., 1998), and SabA (sialic acid–binding adhesin) (Mahdavi et al., 2002) are involved in adhesion to human gastric epithelia by the fucosylated Lewis b histo-blood group antigen for BabA and by sialyl-dimeric-Lewis × glycosphingolipid for SabA.

Of major importance in the colonization process, the expression of many autotransporters (such as Ag43) is subjected to phase variation (Henderson et al., 1999; Van Der Woude and Henderson, 2008; Rossiter et al., 2011a). In addition, proteolytic processing of the passenger domain of the autotransporter on the bacterial cell surface can have a regulatory function on bacterial adhesion (Leyton et al., 2012). In fact, the passenger domain of AT-1s can either remain attached or cleaved off the translocation unit upon OM translocation. This proteolytic cleavage can arise following different scenarios (Leyton et al., 2012) but basically the cleavage can either be intramolecular and autocatalytic or intermolecular resulting in the release of passenger domain into the extracellular milieu, for example the SPATEs (serine protease autotransporters of *Enterobacteriaceae*). Some processed AT-1s, though, remain strongly associated with their cognate translocation unit, e.g., the adhesins Hap, pertactin, AIDA, or Ag43. It also appeared, the protein function is not modified when the cleavage is abolished (Charbonneau et al., 2009), which questions the purpose or benefit of this processing. For Hap, however, it was demonstrated the cleavage influenced the adherence to epithelial cells as well as adhesion to ECM proteins (Fink et al., 2002, 2003). Interestingly, NalP (*Neisseria* autotransporter lipoprotein), which is subjected to phase-variable expression by slipped-strand mispairing (Saunders et al., 2000) and modifies the cleavage patterns of other surface-associated proteins (including some other autotransporters and surface-exposed lipoproteins) (Van Ulsen et al., 2003, 2006; Roussel-Jazede et al., 2010; Serruto et al., 2010), affects in turn adhesion and biofilm formation (Arenas et al., 2013). Similar proteins are found in other bacterial pathogens.

Adhesins of the TPS pathway (T5bSS)

In the TPS (T5bSS), the passenger domain or exoprotein (TpsA) is translated separately from its cognate translocation unit (TpsB) (Jacob-Dubuisson et al., 2004). The exoprotein FHA (filamentous haemagglutinin) is a multifaceted adhesin and major attachment factor of *Bordetella* spp required for colonization of the lower respiratory tract (Locht et al., 1993). While part of the exoprotein is found to be surface-associated, another part is released into the extracellular milieu. Contrary to our previous understanding, it appeared that the C-terminus of FHA is oriented away from the bacterial cell surface and is required for adherence to epithelial cells (Mazar and Cotter, 2006). The exoproteins HMW1 (high-molecular-weight protein 1) and HMW2 of *Haemophilus influenza* are crucial colonization factors involved in bacterial adherence to a variety of respiratory epithelial cell types (St Geme et al., 1993; St Geme and Yeo, 2009). HrpA (hemagglutinin/hemolysin-related protein A) from *Neisseria meningitidis* and MhaB1 (*M. catarrhalis* FhaB-like protein 1) from *Moraxella catarrhalis* mediate binding to human epithelial cells (Schmitt et al., 2007). In the phytopathogen *Xanthomonas axonopodis*, XacFhaB (*X. axonopodis* pv. *citri* filamentous haemagglutinin-like protein B) is required for leaf tissue colonization, especially adhesion and biofilm formation (Gottig et al., 2009). In *Pseudomonas putida*, HlpA (haemolysin-like protein A) would play a direct role in the bacterial cell-root surface interaction (Molina et al., 2006). HecA (hemolysin-like *E. chrysanthemi* protein A) from *Erwinia chrysanthemi* contributes to the attachment and cell aggregation on leaves (Rojas et al., 2002). Interestingly, the exoprotein EtpA (ETEC two-partner secretion protein A) mediates bacterial adhesion by bridging the flagella with the host cells (Roy et al., 2009).

Adhesins of the trimeric autotransporter pathway (T5cSS)

A cardinal feature of the T5SS is a functional OM translocation unit, e.g., located at the C-terminus in monomeric classical autotransporter (T5aSS) or the TspB for the T5bSS. In the T5cSS, the C-terminal translocation unit is formed upon trimerization and serves as an OM anchor for cell-surface exposure of the passenger domains (Cotter et al., 2005; Lyskowski et al., 2011). YadA (*Yersinia* adhesin A) is the prototypical member of this T5SS subfamily (Hoiczyk et al., 2000; El Tahir and Skurnik, 2001; Eitel and Dersch, 2002; Nummelin et al., 2004; Leo et al., 2010). The high-molecular weight trimer formed by the passenger domains protrudes from the OM and consists of three domains, the stalk, necks and head (Linke et al., 2006; Hartmann et al., 2012). Differences in the size of the protruding AT-2s result from the length of their stalks (Linke et al., 2006). The head is involved in binding to some ECM proteins, including collagen, and makes YadA a major adhesion factor. All trimeric

autotransporters (T5cSS) characterized to date are involved in bacterial adhesion (Linke et al., 2006). Besides YadA, several other AT-2s have been characterized to date in this expanding protein family (Cotter et al., 2005), e.g., Hia (*Haemophilus influenzae* adhesin) (Hoiczyk et al., 2000; St Geme and Cutter, 2000), UspA (ubiquitous surface protein A) of *Moraxella catharralis* (Conners et al., 2008), Hag (hemagglutinin) (Pearson et al., 2002; Bullard et al., 2007), NadA (*Neisseria* adhesin A) (Comanducci et al., 2002), BadA (*Bartonella henselae* adhesin) (Riess et al., 2004), NcaA (necessary for collagen adhesion A) (Fulcher et al., 2006), NhhA (*Neisseriahia* Hsf homologue protein A) (Scarselli et al., 2006), DsrA (*ducreyi* serum resistance A) (Leduc et al., 2008), EhaG (EHEC adhesin G) (Valle et al., 2008), SadA (*Salmonella* adhesin A) (Raghunathan et al., 2011), or UpaG (UPEC adhesin G) (Totsika et al., 2012). Each of these trimeric autotransporters are quite multifunctional and in terms of bacterial colonization they can be involved in autoagglutination, hemagglutination, ECM-binding, and/or adhesion to epithelial cells (El Tahir and Skurnik, 2001; Linke et al., 2006).

Intimins/invasins of the inverted autotransporter pathway (T5eSS)

Intimins/invasins (T5eSS) belong to a large and novel subfamily of the T5SS (Tsai et al., 2010; Leo et al., 2012; Oberhettinger et al., 2012). Compared to the classical autotransporter, the secretion mechanism is inverted in the sense, the translocation unit is located at the N-terminal instead of the C-terminal end of the monomeric autotransporter. The intimin/invasin family regroups adhesins that mediate bacterial adhesion and/or invasion of their host cells. In *Yersinia* spp., invasin binds to mammalian cell receptors of the integrin family (Isberg et al., 2000; Palumbo and Wang, 2006). In enteropathogenic *E. coli*, intimin mediates intimate adherence between the bacteria and the host cells by interacting with Tir (translocated intimin receptor) (Frankel and Phillips, 2008). Tir is a T3aSS-secreted protein, which integrates into the plasma membrane of the host cell (Devinney et al., 1999); upon binding to intestinal epithelial cells, the intimin-Tir interaction leads to the formation of actin pedestals beneath bound bacteria (Campellone and Leong, 2003; Brady et al., 2011). Interestingly, intimin can further promote intestinal colonization in a Tir-independent way (Mallick et al., 2012).

PILI ASSEMBLED BY THE T7SS

In diderm-LPS bacteria, the T7SS corresponds to the chaperone-usher pathway (CUP) (Desvaux et al., 2009b); as explained above it has nothing to do with the "Type VII secretion system" of diderm-mycolate (archetypal acid-fast) bacteria, i.e., the ESX. The T7SS is involved in the OM secretion and assembly of pili, which have been named very differently from one bacterial species to another (Sauer et al., 2004; Zav'yalov et al., 2010; Busch and Waksman, 2012; Thanassi et al., 2012), e.g., Afa/Dr (diffuse adherence fibrillar adhesin/Dr blood group antigen), AF/R1 (adhesive fimbriae on RDEC-1), AafD (aggregative adherence fimbriae), P pili, Type 3 fimbriae, Lpf (long polar fimbriae) or CS (coli surface) pili. More rationally, those pili can be categorized into (i) monoadhesive pili (represent the majority of cases), which display either thick rigid or thin flexible morphology and present a single adhesive domain at the tip, and (ii) polyadhesive pili, which display non-pilar, amorphous or capsule-like morphology and present two independent binding sites specific to different host-cell receptors for each of the subunits composing the organelle (Zav'yalov et al., 2010). Monoadhesive pili are also named FGS (F1-G1 short loop short) chaperone-assembled monoadhesin (e.g., Lpf) (Hung et al., 1996), and polyadhesive pili can either be FGL (F1-G1 long loop) chaperone-assembled polyadhesin (e.g., Afa/Dr) or FGS chaperone-assembled polyadhesin (e.g., AF/R1) (Zavialov et al., 2007). Of note, the so-called "alternate chaperone-usher pathway" (Soto and Hultgren, 1999) is obsolete and now included within the T7SS (Nuccio and Baumler, 2007; Poole et al., 2007; Zav'yalov et al., 2010; Thanassi et al., 2012). Pili secreted and assembled by the T7SS are involved bacterial adhesion, interbacterial interactions, aggregation, thereby promoting biofilm formation (Zav'yalov et al., 2010; Thanassi et al., 2012). Those pili can also initiate contact with host-cell receptors and mediate colonization of host cell surfaces. Interestingly, the adhesion of FimH pili (subfamily 2.5 FGS chaperone-assembled monoadhesins-5-1) is modulated by the shear force (Nilsson et al., 2006; Yakovenko et al., 2008; Le Trong et al., 2010).

PILI ASSEMBLED BY THE T8SS

The T8SS corresponds to the extracellular nucleation-precipitation (ENP) pathway (Desvaux et al., 2009b) involved in the OM secretion and assembly of thin and aggregative pili called curli (Hammar et al., 1996; Barnhart and Chapman, 2006; Dueholm et al., 2012; Hammer et al., 2012). Curli are functional amyloid fibers (Epstein and Chapman, 2008). They are involved in cell aggregation, bacterial adhesion and the formation of mature biofilms (Fronzes et al., 2008). They can actually change bacterial surface properties thereby enhancing adherence and attachment to surfaces. In the course of sessile development they constitute a significant part of the proteinaceous component of the biofilm matrix (Blanco et al., 2012). Curli also mediate host cell–bacteria interactions during infection (Wurpel et al., 2013).

ADHESINS SECRETED VIA THE T9SS

The T9SS corresponds to the Por (porphyrin accumulation on the cell surface) secretion system (Sato et al., 2010, 2013; Shoji et al., 2011; McBride and Zhu, 2013). This secretion system has been essentially investigated in *Porphyromonas gingivalis* and *Flavobacterium johnsoniae* and seems restricted to members of the phylum *Bacteroidetes* (McBride and Zhu, 2013). Respective to surface colonization process, the T9SS is required for the secretion of cell-surface motility adhesins, namely SprB (colony-spreadingprotein B) and RemA (redundant motility protein A), but also some hemin-binding proteins (Nelson et al., 2007; Shoji et al., 2011; Shrivastava et al., 2012; Sato et al., 2013). The cell-surface adhesin SprB allows attachment to the substratum but is also required for efficient gliding as it is propelled along a closed helical loop track, generating rotation and translation of the bacterial cell (Nelson et al., 2007; Nakane et al., 2013a,b). While SprB is involved in movement over agar, RemA is involved in movement over surfaces coated with *F. johnsoniae* polysaccharide (Shrivastava et al., 2012). Gliding involves the rapid movement of the semiredundant motility adhesins SprB and RemA along

the cell surface (Shrivastava et al., 2013). T9SS-secreted proteins exhibit C-terminal domains (CTDs) considered essential for attachment to the bacterial cell surface by an A-LPS anchor containing anionic polysaccharide repeating units (Kondo et al., 2010; Shoji et al., 2011; Slakeski et al., 2011; Shrivastava et al., 2013). CTD region function as a recognition signal for the T9SS and glycosylation occurs after removal of the CTD region (Shoji et al., 2011; Glew et al., 2012).

SECRETED PROTEINS INVOLVED IN SURFACE COLONIZATION IN MONODERM BACTERIA

In monoderm bacteria, no protein secreted *via* the Tat, ABC exporter, holin, or Wss pathways has been reported as yet to be involved in bacterial adhesion and/or biofilm formation (**Figure 3**) but some proteins secreted by Sec, FPE, Tra and FEA have.

SEC-SECRETED PROTEINS INVOLVED IN BACTERIAL COLONIZATION

Once translocated by Sec, secreted proteins can have radically different locations in monoderm bacteria as they can either be (i) integrated or anchored to the CM, i.e., IMPs (inner membrane proteins) and lipoproteins, respectively, (ii) associated with the CW, i.e., parietal proteins (CW-proteins), (iii) subunits of pili, or (iv) released into the extracellular milieu and beyond (e.g., into a host cell) (Desvaux et al., 2006a, 2009b; Renier et al., 2012) (**Figures 1**, **3**). Sec-secreted CW-proteins can either be anchored (i) covalently to the CW by a sortase if they exhibit an LPXTG domain (Ton-That et al., 2004; Schneewind and Missiakas, 2012), or (ii) non-covalently if they exhibit a CWBD (cell-wall binding domain), i.e., SLHD (S-layer homology domain), CWBD1 (CWBD of Type 1), CWBD2, LysM (lysin motif), WXL or GW motifs (Desvaux et al., 2006a). While no protein located at the CM has been reported to be involved in bacterial colonization in monodermata, several proteins located at the CW or forming cell-surface supramolecular complexes were reported to participate to this process.

Colonization factors located at the cell wall

In monoderm bacteria, proteins of the Bap family exhibit a C-terminal LPXTG domain enabling their covalent anchoring to the CW by sortases (Lasa and Penadés, 2006; Latasa et al., 2006). While Bap is involved in biofilm formation in *S. aureus* (Cucarella et al., 2001), the *bap* gene has never been found in *S. aureus* human isolates but only in isolates associated with ruminant mastitis (Lasa and Penadés, 2006). Strains encoding *bap* show lower adherence to some ECM proteins (fibrinogen and fibronectin) but also epithelial cell cultures, suggesting Bap might act as an anti-attachment factor preventing initial attachment to host tissues (Cucarella et al., 2002). At the same time, Bap facilitates the colonization of host tissues and the establishment of persistent infections by *S. aureus* (Cucarella et al., 2004; Valle et al., 2012). The interaction of Bap with Gp96/GRP94/Hsp90 provokes a significant reduction of epithelial cell invasion by interfering with the fibronectin binding protein invasion pathway. While Bap is often considered as a key determinant for biofilm formation (Lasa, 2006), a recent investigation revealed that there is no direct correlation with biofilm formation and that a single gene or subset of genes cannot be utilized as a biofilm indicator for morphology in *S. aureus* (Tang et al., 2013). Interestingly, Bap is subjected to phase variation and the switch between Bap ON/Bap OFF states might regulate sessile development (Henderson et al., 1999; Tormo et al., 2007). In *Enterococcus faecalis*, Esp (enterococcal surface protein) also leads to a significant increase in biofilm formation (Tendolkar et al., 2004) and contributes to persistence in the host (Sava et al., 2010). In *S. epidermidis* and *Listeria monocytogenes*, however, Bhp (Bap homologue protein) and BapL, respectively, are not clearly involved in biofilm formation (Tormo et al., 2005; Lasa and Penadés, 2006; Jordan et al., 2008; Renier et al., 2011).

As recently reviewed (Chagnot et al., 2012), different MSCRAMM surface proteins have been investigated in monoderm bacteria (Vengadesan and Narayana, 2011). Among the main ECM fibrillar proteins, namely collagen, fibronectin, laminin and elastin, eight fibronectin-binding domains (FBD1 to FBD8) and only one collagen-binding domain (CBD) have been characterized to date and much remains to be learned about specific binding to other ECM components (Chagnot et al., 2012). Very interestingly, domains involved in binding to ECM proteins such as fibronectin and collagen could also bind polystyrene as demonstrated with RspA (*Rhusiopathiae* surface protein A) (Shimoji et al., 2003).

In *B. subtilis*, the S-layer protein BslA (*Bacillus* S-layer protein A; formerly YuaB) is important for pellicle biofilms (Ostrowski et al., 2011; Vlamakis et al., 2013). BslA has amphiphilic properties and forms a hydrophobic layer on the surface of the biofilm which can contribute to colonization of the air–surface interface (Kovacs and Kuipers, 2011; Kobayashi and Iwano, 2012).

Pili: Type 3 (T3P) and amyloid fibers

A little bit more than a decade ago, pili were considered to be absent from monoderm bacteria. The first pathway recognized in pilus biogenesis in monodermata involves polymerization of LPXTG-pilins by different types of transpeptidase sortases, (Shimoji et al., 2003; Ton-That and Schneewind, 2003; Ton-That et al., 2004; Telford et al., 2006; Kang and Baker, 2012). Those pili of Type 3 (T3P) are involved in adhesion, biofilm formation and host colonization (Swierczynski and Ton-That, 2006; Danne and Dramsi, 2012). LipA (light-inducible pilin A) from *Arthrobacter photogonimos* was proposed as the major pilin of an alternative pilus formation pathway in monoderm bacteria (Yang and Hoober, 1998, 1999; Ton-That and Schneewind, 2004; Swierczynski and Ton-That, 2006).

In *B. subtilis*, pili formation of the amyloid-fiber type has been recently demonstrated to be essential in colony and pellicle biofilms but not for surface-adhered biofilms (Romero et al., 2010; Vlamakis et al., 2013). The CW-protein TapA (TasA anchoring and assembly protein A; formerly YqxM) allows the CW anchoring and assembly of TasA (translocation-dependent antimicrobial spore component A) into long amyloid fibers (Stover and Driks, 1999; Romero et al., 2011), these proteins are not required in biofilms. In *Peptostreptococcus micros*, auto-aggregative pili are formed at least from a CWBD1 protein called FibA (fibril-like structure subunit A) (Kremer et al., 1999).

Cellulosome

Cellulosomes are supramolecular protein complexes found on the cell surface of some cellulolytic anaerobic monoderm bacteria, such as the thermophilic *Clostridium thermocellum* or mesophilic *C. cellulolyticum*, and dedicated to specific adhesion, colonization and degradation of insoluble lignocellulosic substrates (Bayer et al., 2004, 2008; Gilbert, 2007; Fontes and Gilbert, 2010). It is also responsible for regulating the entering carbon metabolic flux in the bacterial cell, which in turn can influence the bacterial colonization of the insoluble lignocellulosic substratum (Guedon et al., 1999, 2000; Payot et al., 1999; Desvaux et al., 2001; Desvaux and Petitdemange, 2001, 2002; Desvaux, 2004). The cellulosome is composed of a non-catalytic protein called scaffoldin, which exhibits domains called cohesin of Type I (Coh I) domains allowing the specific binding of a dockerin domain of Type I (Doc) borne by cellulosomal enzymes. Actually, a single Doc I can simultaneously bind two Coh I and thus allow the linking of two scaffoldins resulting in the formation of polycellulosomes (Carvalho et al., 2003). In *C. thermocellum*, a cellulosome has a size ranging from 2.0 to 6.5 MDa depending on the bacterial strain (Béguin and Lemaire, 1996) and forms polycellulosomal protuberances of up to 100 MDa (Shoham et al., 1999). The cellulosomal enzymes and/or the scaffoldin contain carbohydrate-binding modules (CBMs) involved in the tight binding to a carbohydrate-polymer such as cellulose (Shoseyov et al., 2006; Guillen et al., 2010). The scaffoldin can also exhibit a Doc II domain that can interact with Coh II present in SLHD cell-wall proteins, thus permitting cell-surface display of the cellulosome. In *C. cellulolyticum* for instance, the molecular mechanisms for attachment of the cellulosome to the bacterial cell surface remains unknown (Desvaux, 2005a,b). The architecture of the cellulosome can be even more complex with the assembly of scaffoldins on other scaffoldins like in *Acetivibrio cellulolyticus* where one supramolecular complex can potentially bind 84 different cellulosomal enzymes (Rincon et al., 2003; Xu et al., 2003, 2004). However, the molecular mechanisms permitting the coordinated assembly of the different protein subunits on the bacterial cell-surface remain unclear (Desvaux, 2005b, 2006).

TYPE 4 PILUS (T4P) ASSEMBLED BY THE FPE

The Com (Competence development) pathway involves both the bacterial competence-related DNA transformation transporter and the FPE system (Dubnau, 1997, 1999; Dubnau and Provvedi, 2000; Chen and Dubnau, 2004). In *B. subtilis*, the FPE system is involved in the formation of a pseudopilus from Type 4 prepilins (Chen and Dubnau, 2004; Chen et al., 2006) but do not form a complete Type 4 pilus (T4P). While this was considered as a paradigm for monoderm bacteria, it was recently demonstrated the FPE could form complete Type 4 pilus in *Streptococcus pneumonia* (Laurenceau et al., 2013). While this T4P was only considered in the context of DNA transformation, its implication in bacterial colonization as reported for T4P in diderm-LPS bacteria is an intriguing possibility that would require further investigations in other species such as *L. monocytogenes* (Rabinovich et al., 2012). GP25 (glycoprotein of 25kDa) from *Ruminococcus albus* was characterized as the major protein subunit of T4P present at the bacterial cell surface (Pegden et al., 1998; Mosoni and Gaillard-Martinie, 2001; Rakotoarivonina et al., 2002, 2005). Those pili could be involved in cellulose colonization and cellulose degradation. T4P involved in biofilm formation and gliding motility was also uncovered in *Clostridium perfringens* (Varga et al., 2006; Mendez et al., 2008; Varga et al., 2008).

Protein components of the FPE machinery are homologous to some proteins found in T2SS, T4SS, and T4P assembly apparatus in diderm-LPS bacteria and have been collectively called PSTC (Pilus/Secretion/Twitching motility/Competence) (Fussenegger et al., 1997; Dubnau, 1999; Peabody et al., 2003). In *Bifidobacterium breve,* a functional Tad system involved in the formation of T4bP has recently been uncovered (O'connell Motherway et al., 2011). These pili are essential for intestinal colonization of the host. Besides the genus *Actinobacteria*, a Tad system was also identified in *Clostridia*, namely *Cl. acetobutylicum* (Desvaux et al., 2005b). Again, there is much confusion between the pili assembly by FPE, Tad and the misleading called "Type IV-like secretion system" in monoderm bacteria corresponding more adequately to the Tra system. This would necessitate in-depth phylogenetic and functional-genetic analyses to clarify the situation.

FLAGELLUM ASSEMBLED BY THE FEA

In monoderm bacteria, the flagellar subunits are secreted and assembled by the FEA but the involvement and exact contribution of flagella in biofilm formation remain difficult to establish. Taking *L. monocytogenes* as a case study, it is clear that the regulation of flagella expression is quite complex with the intervention of several transcription regulators but basically that flagella are not expressed at temperatures higher than 30°C and bacterial cells are then non-motile (Renier et al., 2011). Regarding listerial colonization, it was shown flagella were not essential for biofilm development but facilitated initial attachment to surfaces (Vatanyoopaisarn et al., 2000) and were even crucial for initial adhesion (Tresse et al., 2006). During infection, though, the flagella would act as mediators of motility rather than adhesins to enhance the adhesion of *L. monocytogenes* to targeted host cells (O'neil and Marquis, 2006). By contrast with early findings, it was further shown the role of the flagella as mediators of motility rather than adhesins was critical for initial surface attachment and subsequent biofilm formation (Lemon et al., 2007). In dynamic culture conditions for sessile development (i.e., using flow cells), the loss of the flagella resulted in the formation of a very dense biofilm (hyperbiofilm) (Todhanakasem and Young, 2008). These conflicting findings could arise from the use of different *L. monocytogenes* strains as some of them can be more or less laboratory-adapted (domesticated) and have different regulations of flagella expression (Grundling et al., 2004). It could also result from the use of different culture conditions, such as the growth media (e.g., complex undefined or chemically defined), temperature (e.g., related to infection or environmental conditions), support (e.g., plasticware, stainless steel or glass) and/or culture systems (e.g., static or dynamic conditions) (Renier et al., 2011).

In *Bacillus cereus*, flagella motility (i) is necessary for biofilm formation at air-liquid interface as it allows bacteria to reach it, (ii) promotes recruitment of planktonic cells within the biofilm by allowing motile bacteria to invade the whole biofilm, and (iii)

allows spreading of the biofilm on surfaces (Houry et al., 2010). In *B. subtilis*, however, motility and biofilm formation are quite clearly coordinately and oppositely controlled (Kearns et al., 2005; Newman et al., 2013; Vlamakis et al., 2013). It was further demonstrated a protein clutch arrested flagellar motilily by disengaging motor force-generating elements in cells engaged in sessile development (Blair et al., 2008). Nonetheless, and while the number of motile cells decreases in the course of sessile development, a subpopulation of motile cells remains in mature biofilms (Vlamakis et al., 2008) and a biofilm exclusively composed of unflagellated cells is delayed in forming pellicle biofilms (Kobayashi, 2007) and defective in the formation of surface-adhered biofilms (Chagneau and Saier, 2004). Interestingly, motile *Bacillus thuringiensis* can tunnel deep within a biofilm structure (Houry et al., 2012). Those swimming cells create transient pores that increase irrigation within the biofilm. This can either improve biofilm bacterial fitness by increasing nutrient flow in the matrix or facilitating the penetration of toxic substances detrimental for biofilm formation.

NON-CLASSICAL PROTEIN SECRETION AND BACTERIAL COLONIZATION

Analysis of the protein content of the extracellular milieu and/or the cell surface, revealed the presence of unexpected proteins primarily predicted or known to be localized in the cytoplasm, with no N-terminal signal peptide (SP), and no dedicated protein secretion system. While early investigations suggested it could result from bacterial cell lysis, it later appeared some of those proteins were actually secreted by previously unrecognized pathways, e.g., some SecA2-dependent secreted proteins (Desvaux and Hébraud, 2006, 2009; Rigel and Braunstein, 2008; Renier et al., 2013). Non-classical (NC) protein secretion refers to the uncharacterized mechanisms responsible for secretion of those proteins (prior to characterization such apparent secretion could be the result of phenomenon unrelated to secretion *per se*, such as membrane budding, allolysis, phage-mediated lysis, GTA, etc. . .) (Bendtsen et al., 2005; Bendtsen and Wooldridge, 2009). Another unexpected finding was the involvement of some of those proteins in an additional function completely different form the primary one. Indeed, the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) is a well-known and key glycolytic enzyme but when present on the bacterial cell surface it exhibits very significant binding activity against plasmin(ogen), fibrinogen, and human cells (C1q component of the classical component pathway) (Bergmann et al., 2004; Jin et al., 2005; Egea et al., 2007; Matta et al., 2010; Tunio et al., 2010; Terrasse et al., 2012). Several of those so-called moonlighting proteins have been identified in bacteria and play a significant role in bacterial colonization, especially in an infection context (Jeffery, 2003, 2009; Huberts and Van Der Klei, 2010; Copley, 2012; Henderson and Martin, 2013). Most enzymes of the EMP (Embden-Meyerhof-Parnas) pathway can moonlight and a role as an adhesin is quite common, e.g., for enolase, aldolase, or phosphoglucomutase (PGM) (Henderson and Martin, 2011, 2013). However, the molecular mechanisms responsible for their bacterial cell-surface localization remain unknown.

CONCLUSIONS AND PERSPECTIVES

While extremely useful, the secretome concept has not been fully exploited and is still in its infancy. In the context of bacterial colonization, it allows a rational approach for comprehending the secreted proteins directly involved in bacterial adhesion and/or biofilm formation. Considering the protein routing and secretion mechanisms embrace the idea that some of those adhesins and/or adhesion supramolecular complexes share common post-translational and post-translocational maturation mechanisms that may lead to the identification of novel targets for therapeutic strategies. While the majority of investigations are focused on colonization factors in the context of infection, their involvement in the interactions of commensal bacterial, as well as bacteria in an environmental context, are also important areas and should not be overlooked. Bacterial colonization is not the prerogative of pathogens and there is much to gain from the study of colonization of host species by commensal bacteria, as well as environmentally orientated studies. Clearly, there are numerous proteins and supramolecular structures involved in bacterial adhesion and biofilm formation but their functional characterization still demands extensive investigations, e.g., alternative pili in monoderm bacteria or collagen-/laminin-/elastin-binding domains. The understanding of the global and coordinated regulation of the secretome in the course of colonization at the single cell, homogenous population, and multi-species microbial community levels is a major challenge in different ecological niches. Phase variation for instance is a well-known phenomenon especially for factors involved in colonization process but is rarely considered and is poorly understood at a global scale. In the last couple of years, new secretion system such as the T6SS or T9SS have been unexpectedly uncovered and there is much to bet that other protein secretion system(s) are yet to be discovered. Besides, the exact contribution of NC secretion and other protein trafficking mechanisms in bacterial colonization (e.g., allolysis, phage-mediated lysis, membrane budding or GTA) remains to be determined. Regarding the different secretion systems present in a single bacterial cell and numerous systems within microbiota as well as their associated secreted proteins involved in colonization, they promise to be the subject of a lot of research work ahead as well as exciting new developments in the field.

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