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From gene to ecosystem: an integrative study of polysaccharide depolymerases bound to marine viruses

Pierre-Yves Mocaër

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THESE DE DOCTORAT DE SORBONNE UNIVERSITE

Spécialité Microbiologie

Ecole Doctorale « Science de la Nature et de l'Homme : évolution et écologie » (ED227)

Présentée par

M. Pierre-Yves MOCAËR

En vue de l'obtention du grade de
DOCTEUR de SORBONNE UNIVERSITE

From gene to ecosystem: An integrative study of polysaccharide depolymerases bound to marine viruses

Soutenue le 12 décembre 2019, devant le jury composé de :

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« La solitude n'existe pas. Nul n'a jamais été seul pour naître. La solitude est cette ombre que projette la fatigue du lien chez qui ne parvient plus à avancer peuplé de ceux qu'il a aimés, qu'importe ce qui lui a été rendu. »

) Sov Sevcenko Strochnis,
Alain Damasio - *La Horde du Contrevent*.

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LISTE DES ABREVIATIONS

ADN : Acide désoxyribonucléique
ARN : Acide ribonucléique
Å : Angström
ANOVA : Analysis of variance
Ac. : Acide
BLAST : Basic Local Alignment Search Tool
BSA : Bovine serumalbumine
Carin1 : Cobetia mARINa phage 1
CAZY : Carbohydrate Active enZYme
COD : Carbone Organique Dissout
CPS : Capsular PolySaccharide
CryoEM : cryo Electron-Microscopy
DDBJ : DNA Data Bank of Japan
EDTA : Éthylènediaminetétraacétique
EC : Enzyme Class
EEA : Extracellular Enzyme Activity
EM : Electron Microscopy
EMBL : European Molecular Biology Laboratory
EPS : ExoPolySaccharides
GC : Gas-Chromatography
GH : Glycoside Hydrolase
Gt : Giga-tonne
HGT : Horizontal Gene Transfer
IMAC : Immobilized Metal Affinity Chromatography
HMW : High Molecular Weight
kDa : kilo Datlon
KDO : keto-deoxyoctulosonate
LMW : Low Molecular Weight
LPS : LipoPolySaccharide
MAFFT : Multiple Alignment using Fast Fourier Transform
MOD : Matière Organique Dissoute
MOP : Matière Organique Particulaire
MUF : MethylUmbelliFerone
NCBI : National Center for Biotechnology Information
NMDS : Non-metric Multidimensional Scaling
ORF : Open Reading Frame
OTU : Operational Taxonomic Unit
PCR : Polymerase Chain Reaction
PDB : Protein Data Bank
PES : Poly Ether Sulfone
PL : Polysaccharide Lyase

RCC : Roscoff Culture Collection

SAD : Single-wavelength anomalous diffraction

SAXS : Small Angle X-rays Scattering

SDS-PAGE : Sodium Dodecyl Sulfate–PolyAcrylamide Gel Electrophoresis

SEC-RI : Size-Exclusion Chromatography – Refractory Index

SeMet : SelenoMethionine

SM Buffer : Salt-Magnesium Buffer

SOMLIT : Service d'Observation en Milieu LITtoral

TEP : Transparent Exopolymeric Particles

Vigo2 : *Vibrio alginolyticus* phage 2

INTRODUCTION GENERALE

I - LES VIRUS DANS L'ECOSYSTEME MARIN

(D'après Mocaër et Baudoux 2017, Virologie)

I.1 - Introduction

Aujourd'hui, il ne fait plus aucun doute que les virus sont les entités biologiques les plus abondantes de la biosphère. Ces parasites intracellulaires obligatoires sont présents partout où la vie est capable de se développer mais l'océan en constitue le réservoir principal sur la planète. Les virus marins, à l'instar des virus terrestres et humains, sont de minuscules particules, constitués d'un génome, qui peut être soit de l'ADN ou de l'ARN simple ou double brin, protégé dans une coque de protéines (la capside) et parfois d'une enveloppe phospholipidique interne ou externe. Même si leur taille est le plus généralement comprise entre 20 et 150 nm, des virus géants, approximant la taille de bactéries, existent et ont été isolés de façon récurrente dans les écosystèmes marins (Colson et al., 2011; Fischer et al., 2010). Bien que découverts il y a plus de 60 ans (Spencer, 1955), les virus marins n'ont suscité l'intérêt de la communauté scientifique qu'à la fin des années 80 avec la découverte de leur prédominance numérique dans les océans, avec des abondances qui varient généralement entre 10^9 et 10^{11} particules virales par litre (**Figures 1 et 2**) (Bergh et al., 1989; Hara et al., 1991). La prise de conscience que la concentration des virus dépassait généralement celle des procaryotes d'un ordre de grandeur et présentait une forte dynamique spatio-temporelle a fortement contribué à l'essor, dès le début des années 90, de l'écologie des virus marins (**Figures 3**) (Wommack and Colwell, 2000).

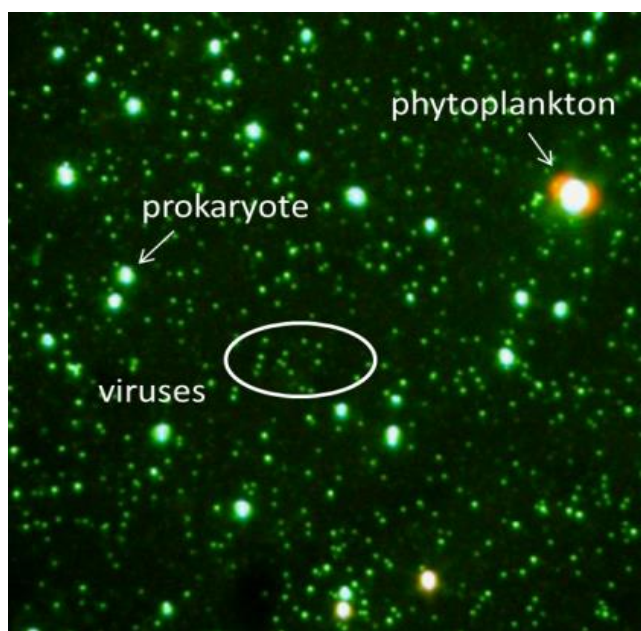


Figure 1 : Visualisation en microscopie à épifluorescence d'un échantillon prélevé d'eau de surface côtière après une coloration au SYBRGreen. Cette image révèle la prédominance numérique des virus par rapport aux autres entités microbiennes, qu'elles soient procaryotiques ou phytoplanktoniques (Patel et al., 2007).

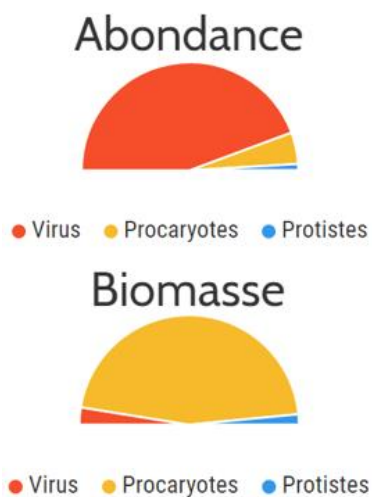


Figure 2 : Ces deux diagrammes révèlent la contribution relative dans l'océan des virus, des procaryotes (organismes dépourvus de noyau, tels que les bactéries et archées) et des protistes (unicellulaires eucaryotes). Les virus sont largement dominants en termes d'abondance (94% des microbes marins sont des virus), mais, étant donné leur taille, ils ne représentent que 5% de la biomasse total dans l'océan (Suttle, 2007).

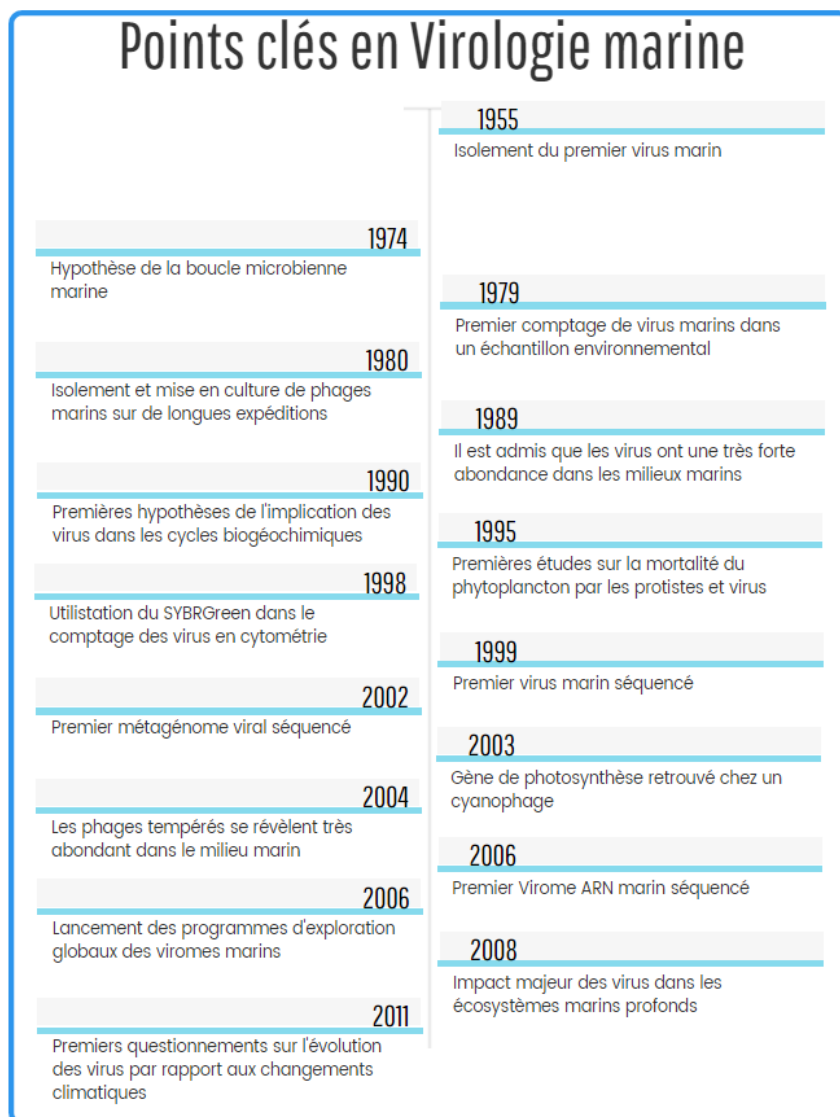


Figure 3 : Frise chronologique retraçant l'histoire de l'écologie virale marine à travers les découvertes importantes. La première étude mentionnant l'isolement d'un virus marin provient de R. Spencer, en 1955 (Spencer, 1955). Il s'agissait alors d'un phage lytique de *Photobacterium phosphoreum*. A cette époque, l'étude des virus marins reposait principalement sur leur propagation chez les poissons, les mollusques, ou leur transmission à l'homme (Beasley et al., 1966; Clem et al., 1965; Hamblet et al., 1969). L'étude des virus marins ne prit de l'ampleur qu'au début des années 90 grâce aux travaux de Bergh et collaborateurs en 1989 (Bergh et al., 1989). Cette équipe norvégienne a en effet mesuré des concentrations virales marines allant de 10^9 à 10^{11} particules par litre. Plus tard, l'utilisation de nouvelles technologies telles que la cytométrie en flux et le séquençage ont permis d'étudier leur diversité, leur dynamique spatiotemporelle, leurs interactions avec les organismes marins ou encore leurs adaptations (Brussaard, 2004; Brussaard et al., 2000; Lang et al., 2004; Marie et al., 1999; Weinbauer, 2004; Wommack and Colwell, 2000). Les premières analyses de viromes (ensemble des génomes de communautés virales) ont été publiées il y a 10 ans (Angly et al., 2006) et ce type d'analyses se développe avec le nombre croissant de base de données globales (VIROME, POV, TaraOcean dataset, iMicrobes). Cette technologie est aujourd'hui la méthode la plus utilisée en écologie virale marine (Bálint et al., 2016; Labonté and Suttle, 2013; Mizuno et al., 2016; Roux et al., 2016; Wommack et al., 2015). (Bergh et al., 1989; Bratbak et al., 1990; Breitbart et al., 2002; Caballero-Morales, 2014; Culley et al., 2006; Danovaro et al., 2011, 2008; Fuhrman and Noble, 1995; Mann et al., 2003; Männistö et al., 1999; Moebus, 1980; Noble and Fuhrman, 1998; Spencer, 1955; Torrella and Morita, 1979; Weinbauer, 2004).

Ces découvertes ont en premier lieu ravivé l'intérêt d'isoler et maintenir des virus en culture afin de caractériser la diversité de ce compartiment biologique et de fournir des systèmes modèles hôte – virus pour les études en laboratoire. Il est aujourd'hui communément admis que les virus sont susceptibles d'infecter n'importe quelle forme de vie. Toutefois, comme la probabilité de rencontre entre les virus et leurs hôtes est densité dépendante, il est probable que les organismes planctoniques unicellulaires (procaryotes et eucaryotes), de par leur abondance dans l'océan, constituent les hôtes préférentiels des virus marins. Actuellement, plusieurs centaines de virus marins sont maintenus dans des collections de cultures privées et publiques (telle que la Roscoff Culture Collection (RCC), <http://roscoff-culture-collection.org/>). Ces virothèques incluent majoritairement des virus (bactériophages ou phages) qui infectent des bactéries marines emblématiques telles que les *Proteobacteria*, *Bacteroidetes* et *Cyanobacteria* (Holmfeldt et al., 2014, 2013; Moebus and Nattkemper, 1983; Sullivan et al., 2009, 2005, 2003). Toutefois, un nombre croissant de virus de microalgues (Brussaard and Martínez, 2008; Short, 2012; Tomaru et al., 2015), d'archaebactéries (Geslin et al., 2003; Gorlas et al., 2012) et de protozoaires (Arslan et al., 2011; Fischer et al., 2010; Garza and Suttle, 1995) ont été isolés au cours des dernières décennies. La caractérisation de ces isolats a révélé une extraordinaire variabilité morphologique, génétique et taxonomique des virus marins. Plus récemment, différents « virus de virus », appelés virophages, ont été isolés de milieux marins et lacustres (Bekliz et al., 2016; Gong et al., 2016; La Scola et al., 2008; Zhou et al., 2015) mais leur rôle écologique reste encore mal connu (W. Zhang et al., 2015; Zhou et al., 2013).

L'impact des virus marins sur leur environnement diffère selon leur stratégie de réplication (cycle lytique, lysogénique, ou chronique). Les virus lytiques (ou virulents), qui induisent la lyse de la cellule hôte, constituent des agents de mortalité importants pour les communautés planctoniques. Les virus sont généralement responsables de 10 à 50% de la mortalité bactérienne dans la colonne d'eau (Suttle, 1994; Weinbauer, 2004; Winter et al., 2004) et jusqu'à 80% dans les systèmes profonds (Danovaro et al., 2008). Ils sont également capables de décimer des efflorescences phytoplanctoniques (Baudoux et al., 2006; Baudoux and Brussaard, 2005; Bratbak et al., 1993; Brussaard et al., 1996; Brussaard, 2004). À la fin des années 90, il est devenu évident que la mortalité induite par les virus lytiques représentait une force directrice majeure non seulement pour la structure mais aussi le fonctionnement des

écosystèmes marins. Les virus lysogéniques (ou tempérés) en s'intégrant sous forme de prophage dans le chromosome hôte semble augmenter le fitness de l'hôte infecté en l'immunisant contre de nouvelles infections et en lui apportant des fonctions nouvelles à l'instar de *Vibrio cholerae* (Faruque and Mekalanos, 2013). En comparaison des virus lytiques et lysogéniques, les virus se répliquant par cycle chronique (ou infection persistante) ont été plus rarement étudiés en milieu marin. Ce type de répllication a été observé de façon récurrente ces dernières années et pourrait constituer une stratégie de survie dans des conditions environnementales néfastes pour l'hôte et le virus (Demory et al., 2017; Lossouarn et al., 2015; Thomas et al., 2011).

Plus récemment, les technologies de séquençage haut-débit appliquées aux communautés virales naturelles ont révélé la remarquable diversité génétique des virus marins. Tout comme pour les communautés bactériennes, seulement quelques génotypes dominant sont représentés (virus de cyanobactéries et du clade bactérien SAR11) pour des centaines de génotypes viraux rares (Angly et al., 2006; Kang et al., 2013; Mizuno et al., 2016; Zhao et al., 2013). L'analyse de ces métagénomés viraux (viromes) a révélé que de 65% à 93% des séquences virales obtenues n'ont aucun homologue dans les banques de données actuelles (Hurwitz and Sullivan, 2013). Cette constatation suggère que les virus marins sont le réservoir d'une diversité moléculaire nouvelle et autant de molécules d'intérêt biotechnologique (Sánchez-Paz et al., 2014). Ces technologies ont en outre permis de mettre en évidence la complexité et l'originalité des interactions entre les virus avec leurs hôtes marins et l'émergence de l'écologie génétique des virus marins. Cette revue propose de dresser l'état des connaissances de l'impact des virus sur l'environnement marin en se focalisant sur différents niveaux d'organisation : du gène à l'écosystème.

I.2 - Les virus marins : ingénieurs métaboliques et manipulateurs génétiques.

Les progrès technologiques de séquençage haut-débit ont permis l'analyse de milliers de génomes viraux ces 15 dernières années et l'analyse de viromes de toutes les régions océaniques majeures. La génomique virale est une discipline indispensable pour appréhender les interactions moléculaires entre les virus et leurs hôtes. Elle a permis un apport de connaissances nouvelles sur les mécanismes par lesquels les virus manipulent leurs hôtes et les implications de ces modifications et innovations génétiques. L'étude des transferts de gènes horizontaux, de la reprogrammation métabolique de l'hôte au cours de l'infection, et l'étude de la diversification génétique par coévolution ont révolutionné notre vision de l'évolution et l'écologie des virus marins.

Le transfert horizontal de gène (Horizontal Gene Transfer ou HGT) est le partage d'informations génétiques entre organismes sans relation parent-descendance. Ce phénomène constitue une force majeure d'évolution chez tous les groupes d'organismes (Soucy et al., 2015). La contribution des virus aux transferts horizontaux de gènes par transduction est connue depuis longtemps chez les procaryotes (Koonin, 2016; Lang et al., 2012). Ce processus induit l'échange d'ADN entre différentes bactéries via des entités virales lorsque du matériel de l'hôte est encapsidé par erreur durant l'assemblage viral. Le virus alors infectieux possède l'ADN de son hôte, qui sera transféré lors de la prochaine infection. L'environnement marin favorise ce processus, dans la mesure où les populations virales sont abondantes et où les interactions avec leurs hôtes sont élevées (McDaniel et al., 2010). Une étude de Jiang & Paul en 1998 (Jiang and Paul, 1998) a estimé le nombre d'évènements de transduction dans la baie de Tampa en Floride à $1,3 \cdot 10^{14}$ par an. L'extrapolation de ces données amène à une moyenne de 10^{28} paires de bases d'ADN transférés par an dans l'océan via la communauté virale (Paul et al., 2002). Ces chiffres doivent néanmoins être pris avec un certain recul : la barrière de spécificité d'hôte est un frein à ces échanges (Popa et al., 2017) : une partie seulement des gènes transférés se fixent dans les populations de leurs nouveaux hôtes, lorsque ceux-ci apportent une fonction avantageuse pour la cellule. De récentes études suggèrent également l'existence d'échanges génétiques entre virus. En effet, plusieurs gènes de virophages ont été retrouvés dans le génome de différents virus marins (Bekliz et al., 2016; Blanc et al., 2015; Santini et al., 2013). Les virophages pourraient également être responsables

de processus évolutifs clés, tel le virophage *Mavirus* qui pourrait être à l'origine du transposon *Maverick/Polinton* (Fischer and Suttle, 2011).

S'il est bien établi que les virus, quelle que soit leur origine, ont la capacité d'acquérir et de transférer des gènes par HGT, l'analyse des génomes et viromes marins semble toutefois indiquer que les virus d'origine marine ont acquis une panoplie de gènes bien différente de leurs congénères terrestres. Ces gènes, aussi appelés gènes auxiliaires de métabolisme, codent souvent pour des fonctions clés, souvent limitantes, du métabolisme de l'hôte. Ils sont par exemple impliqués dans la biosynthèse de pigments photosynthétiques (*ho1*, *pebS*, *cpeT*, *peyA*), dans le métabolisme des nucléotides (*nrdA*, *nrdB*, *cobS*, *purH*, *purL*, *pyrE*), du carbone (*talC*, *gnd*, *zwf*), du phosphate (*phoA*, *phoH*, *pstS*) ou encore dans les modifications traductionnelles ou post traductionnelles et la réponse au stress (*mazG*) (Breitbart, 2011; Hurwitz and U'Ren, 2016; Millard et al., 2009; Rohwer et al., 2000; Sullivan et al., 2010, 2005; Thompson, 2010; Weigele et al., 2007). Le gène le plus étudié, *psbA*, est retrouvé chez un grand nombre de virus de cyanobactéries (aussi appelés cyanophages) et code pour la protéine D1 impliquée dans le transport des électrons du photosystème II. Chez les cyanobactéries, le taux de renouvellement de la protéine D1 est très rapide, ce qui en fait une étape limitante de la photosynthèse. Au cours de l'infection virale, l'expression du gène *psbA* de la cyanobactérie est drastiquement réduite, tandis que celle dérivant du cyanophage augmente significativement (Clokier and Mann, 2006; Lindell et al., 2005). Ces études suggèrent que l'expression du gène *psbA* d'origine virale permet de maintenir la photosynthèse durant le cycle d'infection, et donc un niveau énergétique suffisant pour la production de nouveaux virions. L'expression de ces gènes au cours du cycle d'infection permettrait de renforcer ou de reprogrammer le métabolisme de l'hôte infecté, favorisant ainsi la propagation des virus et leur survie dans l'océan (Rosenwasser et al., 2016). A l'instar des cyanophages, les virus inféodés aux systèmes marins profonds possèdent des gènes impliqués dans l'oxydation du sulfure indispensable aux bactéries se développant dans les conditions extrêmes des cheminées hydrothermales profondes (Anantharaman et al., 2014). Chez le virus géant EhV qui infecte la microalgue eucaryote *Emiliana huxleyi*, la voie métabolique quasi-complète de biosynthèse des sphingolipides est retrouvée (Monier et al., 2009; Wilson et al., 2005). Elle permettrait une répllication et un assemblage optimal des particules virales (Rosenwasser et al., 2014). L'analyse des gènes métaboliques auxiliaires

suggèrent donc que les virus marins ont développé un grand nombre de stratégies pour exploiter et manipuler le métabolisme de leur hôte. Ils fournissent des connaissances nouvelles aussi bien sur la complexité que sur l'originalité des mécanismes fonctionnels de ces virus.

La coévolution entre les virus et leurs hôtes constitue un autre processus de diversification génétique chez les deux partenaires. Le processus de coévolution dérive de la coexistence hôte-virus. Pour s'extirper de l'étreinte virale, les hôtes sont contraints à évoluer et se diversifier, et les virus évoluent à leur tour pour déjouer ces mécanismes de résistance. Il est aujourd'hui clairement établi que par cette perpétuelle course aux armements les virus manipulent leur environnement par pression de sélection (Pal et al., 2007) et constituent une force directrice majeure dans l'évolution moléculaire de leurs hôtes (Buckling and Rainey, 2002; Paterson et al., 2010; Weitz et al., 2005). Malgré ces implications importantes, les mécanismes de résistance induits par l'infection virale sont peu explorés dans le milieu marin. La plupart des études à ce sujet ont été conduites sur le système modèle *Synechococcus* – cyanophages. Les cyanobactéries et leurs cyanophages coexistent à des concentrations élevées dans les océans. L'acquisition de résistance virale par mutations et diversifications génétiques se font très rapidement au sein des *Synechococcus*. Moins de 170 générations (soit 1 à 2 mois) sont nécessaires pour obtenir des différences phénotypiques au sein d'une même communauté (Stoddard et al., 2007). Ces mutations se produisent majoritairement dans les voies de biosynthèse des lipopolysaccharides bactériens. Ces derniers sont connus pour être impliqués dans l'attachement des phages aux bactéries, dont *Synechococcus*. La modification de la chaîne de résidus polysaccharidiques de l'antigène O crée un obstacle à l'attachement du cyanophage, entraînant un échec de l'infection. Il en résulte une communauté cyanobactérienne résistante et dynamique, constituée d'une multitude de phénotypes distincts (Marston et al., 2012). Différents mécanismes de résistance originaux ont aussi été mis en évidence chez des microalgues eucaryotes. Chez la picoalgue verte *Ostreococcus tauri*, des modifications génétiques importantes ont été observées sur un chromosome qui semble spécialisé dans la défense antivirale. Ce chromosome regroupe un nombre important de gènes liés au métabolisme des glucides et il a été observé des schémas d'expressions changeant lors d'infections virales (Thomas et al., 2011; Yau et al., 2016). Une autre stratégie, celle dite du « Chat de Cheshire », a été observée chez la microalgue *Emiliana huxleyi* pour résister aux

attaques virales (Frada et al., 2008). Cette microalgue, sous sa forme diploïde (les cellules sont alors couvertes d'écailles calcaires) est vulnérable aux attaques virale mais le passage au stade haploïde (non calcifié) la rend résistante aux infections (ou invisible aux virus, comme le Chat du Cheshire). A ce jour, aucun virus infectant la forme haploïde d'*Emiliana huxleyi* n'a pu être isolé.

I.3 - Les virus marins sont des agents de contrôle des communautés planctoniques

De par leur nature parasitaire obligatoire, les virus détournent les mécanismes de leur hôte pour se répliquer. Dans le cas des virus virulents, i.e. qui se répliquent par cycle lytique, l'infection va conduire à la lyse de la cellule infectée et donc à la mort de l'organisme lorsque celui-ci est, comme le plancton, unicellulaire. Depuis la découverte de leur prédominance dans les océans, l'impact des virus sur la mortalité des communautés planctoniques a fait l'objet d'une attention particulière. De nombreuses approches telles que la détermination du nombre de cellules infectées par microscopie électronique à transmission (Hara et al., 1991), le suivi de la production virale (Weinbauer, 2004; Wilhelm and Suttle, 2006), la mesure des pertes virales (Fuhrman and Noble, 1995) ou la technique de dilution modifiée (Evans et al., 2003) ont été développées et déployées dans des environnements contrastés (voir la très complète revue de littérature de Weinbauer de 2004 (Weinbauer, 2004)). Bien que basées sur des principes et hypothèses différentes, toutes ces techniques indiquent que les virus marins constituent des agents de mortalité importants pour les communautés bactériennes et phytoplanctoniques. Ils sont responsables d'une part important de la mortalité des communautés bactériennes (10 et 50% de la mortalité totale à la surface des océans) avec des taux de lyse virale généralement plus importants dans les régions côtières que dans les régions hauturières (Danovaro et al., 2011). L'impact des virus sur les communautés bactériennes augmente avec la profondeur de la colonne d'eau pour atteindre jusqu'à 80% de la mortalité procaryotique dans les environnements marins profonds (Danovaro et al., 2008).

Les virus ont également un impact spectaculaire sur la régulation des efflorescences (ou blooms) phytoplanctoniques qui se développent au large des côtes. Durant les blooms saisonniers de microalgues telles que *Emiliana huxleyi* (Bratbak et al., 1993; Brussaard et al.,

1996; Martinez Martinez et al., 2007), *Phaeocystis globosa* (Baudoux et al., 2006; Brussaard et al., 2005) ou *Heterosigma akashiwo* (Nagasaki et al., 1994; Tarutani et al., 2000), les taux de mortalité par lyse virale égalent voire supplantent la mortalité par broutage (prédation protiste), traditionnellement reconnu comme le facteur de perte majeur. A l'instar des communautés procaryotiques, la mortalité par lyse virale des communautés phytoplanctoniques varie dans le temps et l'espace. Une étude récente rapporte des taux de lyse phytoplanctoniques globalement plus importants aux basses latitudes en comparaison des hautes latitudes (Mojica et al., 2016). Les raisons expliquant ces variations ne sont à l'heure actuelle pas élucidées. Sans aucun doute, les communautés virales et microbiennes inféodées à ces milieux contrastés sont différentes. Toutefois, il est possible que les stratégies de répllication virale diffèrent dans ces environnements. Différentes études tendent en effet à montrer une plus forte proportion de virus tempérés (se répliquant par lysogénie) dans des environnements où l'abondance et l'activité bactérienne est faible, et inversement, une majorité de virus virulents (se répliquant par cycle lytique) dans des eaux enrichies en nutriments où les niveaux de production bactériennes sont plus importants (Brum et al., 2016; Paul, 2008; Payet and Suttle, 2013). D'autres études suggèrent également que l'environnement thermique pourrait jouer un rôle clé non seulement pour la régulation des taux de lyse (Demory et al., 2017; Tomaru et al., 2015) mais aussi dans les transitions de stratégie de répllication (Demory et al., 2017) voir même dans le développement de résistance à l'infection (Demory et al., 2017; Kendrick et al., 2014; Tomaru et al., 2015).

Le caractère spécifique (les virus marins franchissent rarement la barrière d'espèce) et densité-dépendant des infections virales en font un facteur important de structuration des communautés planctoniques. Les exemples les plus caractéristiques de ce phénomène proviennent de l'étude des virus du phytoplancton. L'hypothèse dite du « Killing the Winner » (Thingstad, 2000; Thingstad and Lignell, 1997) prédit que les virus régulent préférentiellement les espèces les plus abondantes, i.e. les plus compétitives pour une ressource. La répression des espèces dominantes lors d'efflorescence phytoplanctoniques permet l'utilisation des ressources et le développement d'espèces moins compétitives et donc une diversification de la communauté (**Figure 4A**). A l'échelle de la population, les virus conduisent également à augmenter la diversité intra-spécifique. Par exemple, les procaryotes photosynthétiques du genre *Synechococcus* sont souvent observés en abondance (excédant 10^3 cellules par

millilitre) même en présence d'une forte concentration de cyanophages infectieux (Lu et al., 2001; Suttle and Chan, 1994; Waterbury and Valois, 1993). Comme mentionné précédemment, les populations de *Synechococcus* ont su développer des résistances (modification des propriétés de surface membranaire par exemple) que les virus peuvent déjouer engendrant ainsi un état d'équilibre entre les abondances cyanobactériennes et virales (Marston et al., 2012; Stoddard et al., 2007). Cette dynamique va induire des modifications continues des génomes (mutation de résistance, acquisition de nouveaux gènes...), favorisant la diversité au sein des populations hôtes et virales. Dans ce cas, les fluctuations d'abondance des cyanobactéries sont majoritairement contrôlées par la variabilité saisonnière des paramètres environnementaux (lumière, température, nutriments) (**Figure 4B**).

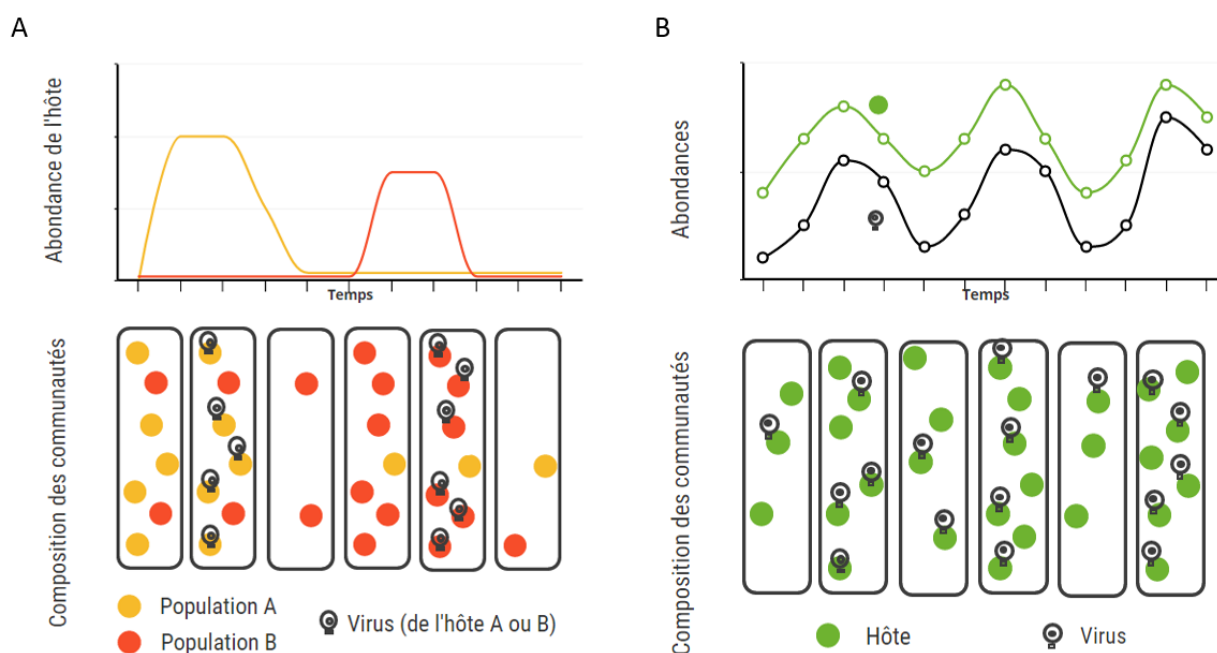


Figure 4 : Schématisation du contrôle des communautés microbiennes orchestré par les virus marins. En A, les efflorescences/proliférations d'espèces planctoniques sont stoppées par les populations virales. Cela permet à long terme un maintien de la structure des communautés et une diversité inter-espèces importante. Ce cas se retrouve généralement chez les systèmes virus-microalgues (Bratbak et al., 1993; Nagasaki et al., 1994). En B, la population de l'hôte est maintenue par pression virale mais reste présente en concentration élevée dans l'environnement. Cette coexistence est permise par l'alternance entre développement de résistances chez l'hôte et adaptation du virus, ce qui favorise la diversité intra-espèce. Les fluctuations de concentrations sont majoritairement dues aux différences de conditions environnementales sur l'année (i.e. nutriments disponibles, température, ensoleillement). Ce cas se retrouve habituellement avec les bactéries hétérotrophes et les cyanobactéries (Waterbury and Valois, 1993; Weinbauer, 2004).

Très peu étudiés jusqu'à présent, les virophages semblent aussi avoir un impact écologique non négligeable sur la structure des communautés planctoniques. Une étude de Yau et al. en 2011 suggère que ce compartiment modifie les dynamiques écologiques, positivement pour l'algue-hôte, négativement pour son virus associé, augmentant la fréquence des blooms printaniers (Yau et al., 2011). Au laboratoire, des dynamiques similaires sont observées entre le protozoaire marin *Cafeteria roenbergensis*, le virus géant CroV et son virophage *Mavirus*. Ici, le génome de *Mavirus* est intégré à plusieurs endroits au sein du génome de *Cafeteria roenbergensis*, mais ne sera exprimé que lors d'une surinfection par le virus CroV. La lyse de l'hôte *Cafeteria roenbergensis* libèrera alors une grande quantité de *Mavirus*, qui inhibera la réplication et la prolifération de CroV chez *Cafeteria roenbergensis* (Fischer and Hackl, 2016).

L'action des virus marins sur la structure des communautés planctoniques semble être une réponse au paradoxe du plancton (Plankton paradox) soulevé par Hutchinson et collaborateurs en 1961 (Hutchinson, 1961). En effet, pourquoi dans cet environnement aquatique aux ressources relativement homogènes, retrouve-t-on des assemblages divers d'espèces phytoplanctoniques et bactériennes ? La compétition pour des ressources similaires prédit théoriquement l'existence d'un nombre restreint d'espèces dominantes (i. e. environnement macroscopiques). Or, dans le milieu naturel marin, un nombre important d'espèces planctoniques coexistent et rivalisent pour les mêmes ressources, sans séquestration des ressources disponibles par une seule espèce compétitrice. Les virus, de par (i) leur dynamique spatio-temporelle, (ii) leur distribution globale et leur dispersion facilitée, (iii) leur mode d'action, et (iv) le caractère transitoire des pics d'abondances viraux hôte-spécifique, pourraient expliquer, du moins en partie, ce paradoxe (Wommack and Colwell, 2000). En outre, l'implication des virus dans la structuration et la diversité des communautés planctoniques a été vérifiée par modélisation (Weitz et al., 2015). La coexistence est possible sans virus, mais l'ajout d'une composante virale aux modèles dynamiques multitrophiques favorise l'émergence de communautés planctoniques complexes.

I.4 - L'impact des virus dans l'écosystème marin global

Les océans jouent un rôle fondamental dans le cycle du carbone global et la régulation du climat. La moitié du dioxygène atmosphérique est produit dans les océans et c'est tout autant de CO₂ qui est absorbé par le phytoplancton grâce à la production primaire (Field, 1998; Hemsley et al., 2015). Via ce processus, le phytoplancton transforme le CO₂ en biomasse vivante qui pourra être transférée aux échelons supérieurs du réseau trophique (chaîne alimentaire classique) mais aussi en matière organique dissoute (MOD) ou particulaire (MOP) dont le devenir conditionne le fonctionnement des écosystèmes marins. La MOD initie la boucle microbienne (Azam, 1998), et sera assimilée à la surface des océans par les procaryotes. Ceux-ci la transforment, d'une part, en biomasse, disponible pour les brouteurs, et, d'autre part, en nutriments, disponibles pour le phytoplancton. Une partie de la MOD est quant à elle réfractaire aux transformations microbiennes. Elle sédimente de la zone photique vers les couches océaniques profondes et contribue à la séquestration du carbone. Le transfert de carbone dans les fonds océanique, aussi appelé la pompe biologique, est aujourd'hui la voie majeure de diminution des concentrations atmosphériques en CO₂ (Herndl and Reinthaler, 2013; Jiao and Zheng, 2011). L'hypothèse originale de la boucle microbienne n'inclut pas l'impact des virus sur les différents compartiments microbiens (**Figure 5**). La prise de conscience des forts taux de lyse virale enregistrés dans les communautés phytoplanctoniques et procaryotes ont toutefois amené à réviser cette hypothèse. En effet, les virus en infectant et en tuant par lyse cellulaire leurs hôtes microbiens détournent la biomasse vivante destinée aux brouteurs et la transforment en MOD qui devient disponible pour les procaryotes. Cette dérivation de matière organique ou « shunt viral » (Fuhrman, 1999; Wilhelm and Suttle, 2006) induit le recyclage de près de 150 gigatonnes de carbone par an provenant de la lyse des organismes planctoniques, soit l'un des flux de matière les plus importants dans l'océan (Suttle, 2007, 2005).

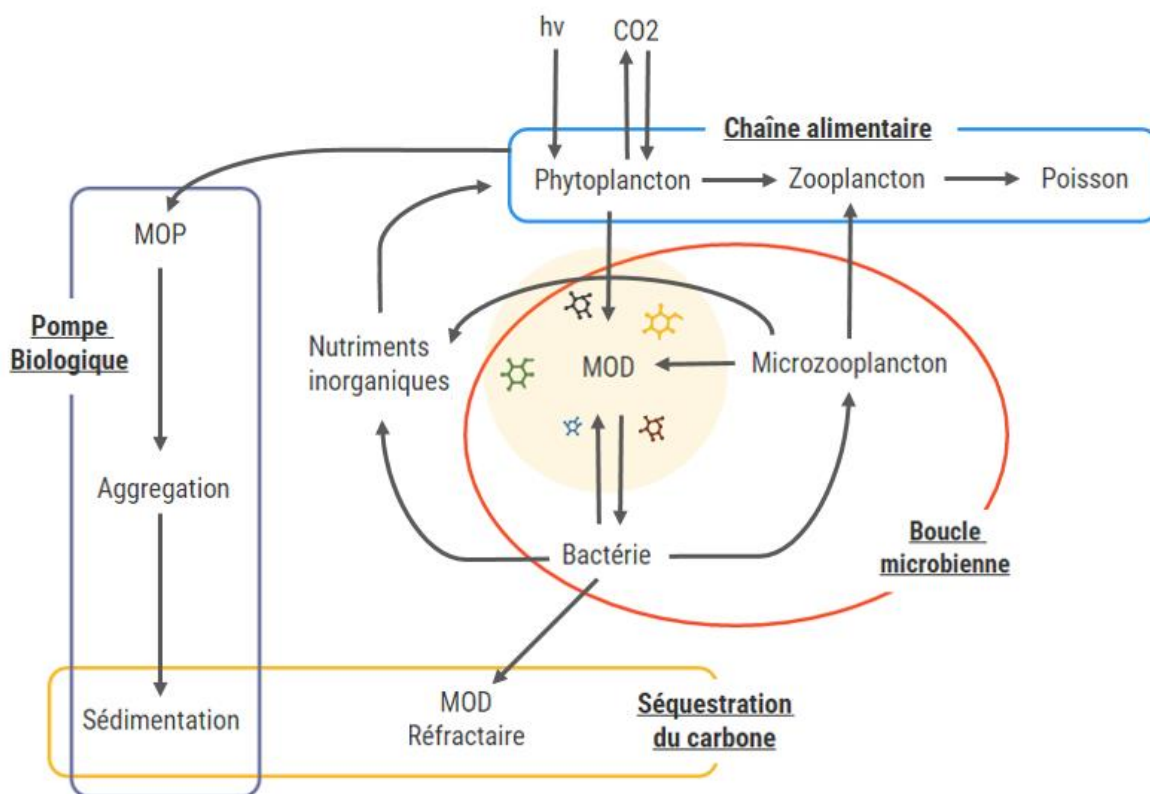


Figure 5 : Schématisation des interactions entre microorganismes dans l'environnement marin telles qu'établies sans les communautés virales. La matière organique dissoute (MOD) générée par les producteurs primaires et les microorganismes marins (lyse cellulaire, excréation) se retrouve ; Soit redirigée vers les échelons trophiques supérieurs via la boucle microbienne puis la chaîne alimentaire ; Soit séquestrée dans les océans et fonds marins grâce à la pompe biologique et indirectement la formation de matière organique réfractaire (Azam, 1998).

Il apparaît alors crucial de caractériser les produits de la lyse virale, d'estimer leur biodisponibilité et de mieux appréhender l'impact du « shunt viral » sur le fonctionnement de la boucle microbienne. Bien que les implications biogéochimiques des virus aient fait l'objet d'un certain nombre d'études, leur influence sur la pompe biologique est toujours largement débattue. L'étude de différents systèmes modèles hôte – virus indiquent que le lysat viral est constitué majoritairement de matière organique dissoute labile et donc directement assimilable par les procaryotes hétérotrophes (Lønborg et al., 2013; Middelboe et al., 1996; Middelboe and Jørgensen, 2006). La lyse virale résulterait en une diminution de la respiration du microzooplancton et une augmentation nette de la respiration bactérienne, forçant ainsi la boucle microbienne vers une voie régénérative qui ralentirait la pompe biologique (Brussaard et al., 2008; Fuhrman, 1992, 1999; Suttle, 2005; Wilhelm and Suttle, 2006).

Inversement, d'autres études indiquent que les virus pourraient accélérer la séquestration du carbone en induisant, d'une part, le relargage d'éléments traces qui stimulent la production primaire (Gobler et al., 2008; Poorvin et al., 2004) et, d'autre part, la formation de particules susceptibles de s'agréger et de sédimenter (Lønborg et al., 2013; Mari et al., 2005; Uitz et al., 2010). De la même façon, si la lyse virale conduit majoritairement à la libération de matière organique labile, qui restera des heures ou des jours/semaines dans l'environnement, elle génère également une fraction moindre de matière organique réfractaire, qui subsistera des milliers d'années dans l'océan, car non assimilable. Si l'on considère que 10^{23} infections virales ont lieu chaque seconde dans l'océan (Suttle, 2007), une accumulation progressive de matière réfractaire pourrait augmenter la séquestration du carbone atmosphérique dans les océans (Jiao and Zheng, 2011) (**Figure 6**).

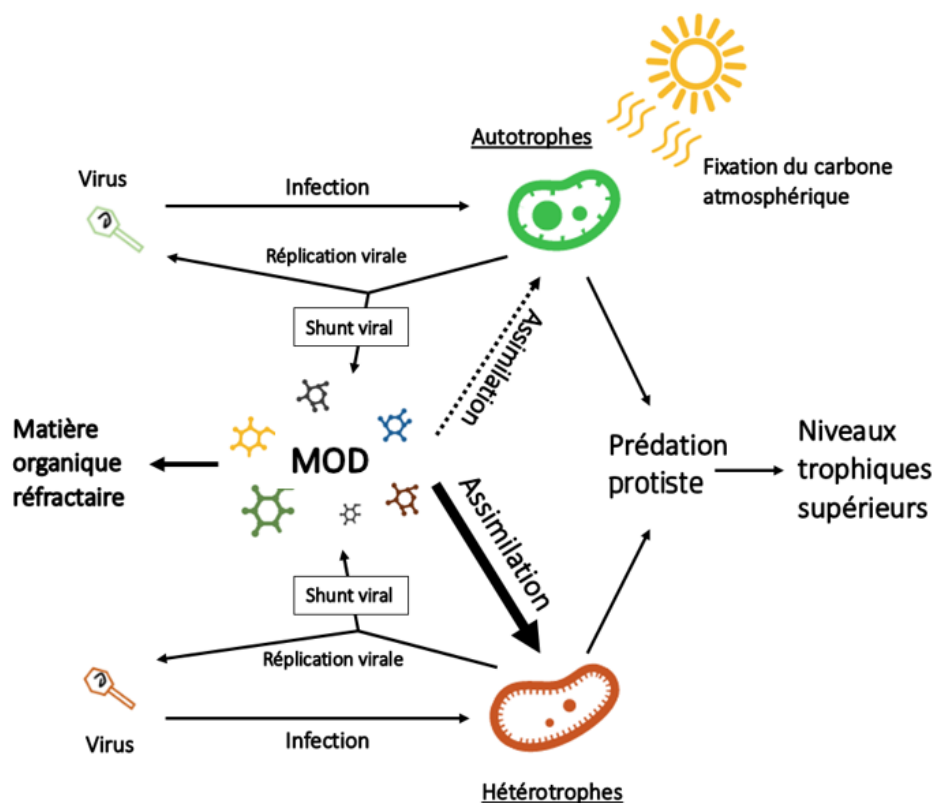


Figure 6 : Schématisation des implications biologiques et biogéochimiques des virus marins. Les producteurs primaires fixant le carbone atmosphérique pour leur développement vont être la cible des virus dès leurs proliférations, ce qui aura pour effet de libérer de la matière organique dissoute (MOD) dans l'environnement de façon abondante (shunt viral). Cette matière organique sera utilisée par d'autres microorganismes dont majoritairement les bactéries hétérotrophes, ce qui permettra le développement de cette communauté et des niveaux trophiques supérieurs. Une grande partie de la communauté bactérienne subira l'action lytique des virus, ce qui reconstituera de la MOD (shunt viral). Une partie minoritaire de cette matière organique, réfractaire, sera séquestré dans les océans durant des milliers d'années (Adapté de Weitz et Wilhelm, 2012).

Il reste difficile d'évaluer l'influence des virus sur les écosystèmes dans leur globalité. Un nombre considérable d'études sont disponibles sur les interactions des virus marins avec leurs hôtes, mais il en existe aujourd'hui trop peu qui se sont focalisées sur un impact à grande échelle. En outre, l'utilisation de méthodes différentes et la variabilité des modèles biologiques utilisés rendent difficile l'interprétation des résultats obtenus au laboratoire et sur le terrain (Weitz and Wilhelm, 2012). En conséquence, bien que l'importance des virus soit reconnue dans la communauté scientifique internationale (Guidi et al., 2016a; Puxty et al., 2016; Weitz et al., 2015; Wommack et al., 2015), ce compartiment est omis des modèles mathématiques visant à prédire les conséquences du changement climatique (Barton et al., 2010; Follows et al., 2007). L'impact du changement climatique sur l'écologie des virus marins est également très peu documenté. Dans une étude menée en Atlantique Nord, il apparaît que la température et la salinité influencent l'importance relative de la lyse virale et du broutage du phytoplancton. Plus précisément, la lyse virale serait plus importante dans les eaux de surfaces aux températures plus élevées, ce qui aurait pour effet de diminuer les populations phytoplanctoniques. Cette diminution du nombre d'organismes photosynthétiques pourrait alors avoir un impact sur les transferts de matière, et finalement sur la séquestration du CO₂ atmosphérique par l'océan Atlantique Nord (Mojica et al., 2016). À la lumière de ces observations, il est aujourd'hui indispensable d'homogénéiser les méthodes d'étude afin de mieux prédire la réponse des virus aux effets du changement climatique global.

I.5 – Conclusion

De la manipulation génétique de leurs hôtes à la modification des cycles biogéochimiques, les virus marins jouent un rôle essentiel dans leur environnement et ne peuvent plus être écartés des modèles écologiques globaux. Leur action ne se limite pas au contrôle des communautés microbiennes par lyse virale, ils sont également garants de la diversité biologique et un facteur important d'évolution génétique. Il est aujourd'hui indispensable de continuer à caractériser ce compartiment encore trop mal connu. La plupart de nos connaissances sur les virus marins se limitent en effet aux virus à ADN double brin tandis que la diversité et les fonctions des virus à ADN simple brin ou des virus à ARN restent largement inexplorées ; Ils pourraient néanmoins représenter près de la moitié de la communauté virale (Steward et al., 2013). Par ailleurs, l'étude des interactions virus-hôte peut offrir des potentiels étonnants, que ce soit au niveau de la manipulation moléculaire (i.e. CRISPR-Cas9 pour le plus célèbre) ou au niveau écologique. Des recherches liées à la conservation des écosystèmes coralliens sont par exemple en cours : l'utilisation d'un cocktail de phage (thérapie phagique) pourrait favoriser la survie du corail infecté par des bactéries pathogènes (Cohen et al., 2013a; Efrony et al., 2009). Pendant longtemps, les virus ont été considérés comme de véritables fléaux pour l'environnement dû aux mortalités de masse qu'ils induisaient. Sir Peter Medawar, prix Nobel de physiologie et de médecine en 1960, les décrivait à cette époque comme des « pieces of bad news wrapped up in proteins ». L'essor de l'écologie virale au cours des dernières décennies a cependant profondément modifié notre vision de ces parasites. Désormais, les virus sont considérés comme une force directrice majeure de l'évolution et du fonctionnement des écosystèmes océaniques.

II - LES POLYSACCHARIDE DEPOLYMERASES VIRALE : ENZYMES CLE DES INTERACTIONS HOTE-VIRUS

Les polysaccharide dépolymérase sont des enzymes spécialisées dans la dégradation de polysaccharides. Ces derniers sont des biopolymères synthétisés par tous les organismes vivants et participent à de nombreux rôles biologiques. Ces substrats vont dans un premier temps être définis, puis les polysaccharides dépolymérase virales seront plus précisément décrites.

II.1 - Les polysaccharides bactériens

Définition et généralités

Les polysaccharides sont définis comme des enchaînements de résidus monosaccharidiques (ou oses) liés entre eux par des liaisons glycosidiques. Les chaînes polysaccharidiques sont généralement constituées de plus de 25 unités osidiques liées. Dans le cas où la chaîne polysaccharidique est moins longue, on parle d'oligosaccharide. Il existe deux classes majeures de polysaccharides. Les homopolysaccharides sont définis par l'enchaînement d'une unique espèce monosaccharidique. La seconde classe regroupe les hétéropolysaccharides dont l'unité de répétition comporte au moins deux monosaccharides différents. Un grand nombre de monosaccharides ont été reportés (> 100), la plupart étant des hexoses (6 carbones, ex : glucose) ou des pentoses (5 carbones, ex : ribose). Les sucres à 7 carbones ou plus sont plus rares (ex : acide ulosonique). Ces sucres peuvent être neutres (ex : galactose) ou chargés (ex : acide mannuronique). Les oses sont généralement sous leur forme furanose (cycle à 4 carbones) ou pyranose (cycle à 5 carbones). Les polysaccharides peuvent être linéaires, ou présenter des ramifications, qui divergent suivant leur position (régulière, aléatoire, groupée, etc.) et leur complexité (branchement sur branchement). Les polysaccharides peuvent être substitués par des groupements organiques et inorganiques (ex : acétate, lactate, pyruvate, etc., ou phosphate et sulfate). Il existe donc un nombre quasiment infini de structures de la chaîne polysaccharidique. R. Laine en 1994 a calculé qu'il pourrait y avoir plus de 10^{12} formes isomères possibles pour des oligosaccharide (Laine, 1994).

Il est par ailleurs commun que les organismes modifient les structures chimiques de leurs polysaccharides selon les conditions physico-chimiques de l'environnement (Barranguet et al., 2003; Jefferson, 2004; Mack et al., 2004; Ozturk and Aslim, 2010).

Chez les bactéries, trois types de polysaccharides principaux sont distingués selon leur localisation cellulaire : (i) les polysaccharides intracellulaires, (ii) les polysaccharides pariétaux, et (iii) les polysaccharides extracellulaires (non-pariétaux).

Les polysaccharides intracellulaires jouent un rôle important dans le métabolisme bactérien. Ils servent souvent de source de carbone et de stockage pour la bactérie (comme par exemple le glycogène bactérien). Ils sont également impliqués dans l'homéostasie cellulaire (pH et pression osmotique), mais aussi dans la motilité des espèces mobiles.

Les polysaccharides pariétaux regroupent l'ensemble des polysaccharides et glycoconjugués entrant dans la composition des parois cellulaires bactériennes. Chez les bactéries Gram-négatives, la paroi de la cellule consiste en une membrane interne et externe séparées par un espace périplasmique (**Figure 7**) (Bos and Tommassen, 2004; Tripathi et al., 2012).

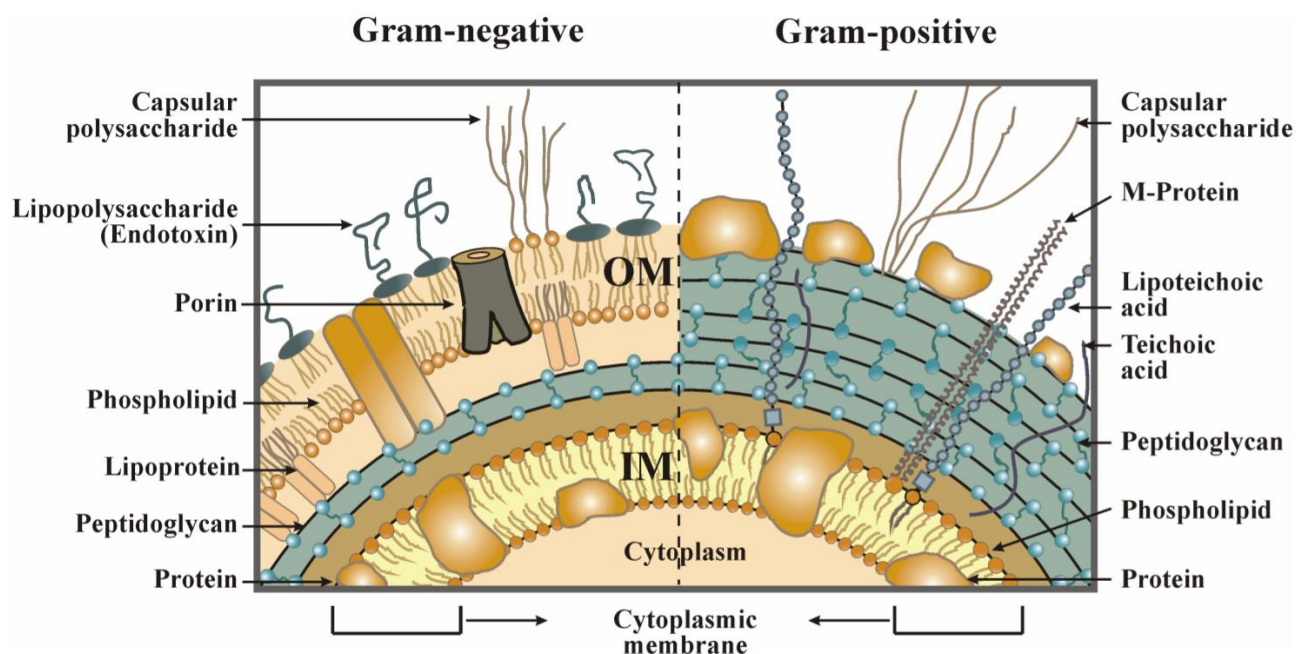


Figure 7 : Représentation d'une paroi bactérienne Gram négative (gauche) et Gram positive (droite) (Shatzmiller et al., 2018)

Le **peptidoglycane** représente le composant structurel majeur du périplasme. Il est constitué de chaînes polysaccharidiques parallèles composées de N-acétylglucosamine et d'acide N-acétylmuramique. Des oligopeptides (L-Alanine chez certaines espèces, acides aminés inhabituels (D-Glutamine et D-Alanine), acide diaminopimélique) lui sont liés de manière covalente. La membrane externe est principalement constituée de **lipopolysaccharides** (LPS) dans le feuillet externe. Les LPS sont composés d'un lipide A, d'un noyau, et de l'antigène-O, ces deux derniers éléments représentant la composante osidique du LPS. Le noyau, constitué de 1 à 4 unités d'acide 3-désoxy-D-manno-oct-2-ulosonique (KDO), permet de faire le lien entre le lipide A et le début de la chaîne polysaccharidique de l'antigène-O. Ce dernier est par la suite constitué d'une répétition de 2 à 8 sucres très diversifiés et cette unité peut être répétée jusqu'à 50 fois (Rietschel et al., 1994). De nombreuses bactéries présentent également **une capsule polysaccharidique** (CPS) qui entoure l'ensemble de la cellule et qui participe à sa protection et aux interactions avec l'environnement extérieur. Les CPS présentent une diversité de structure très importante (> 80 types connus chez *E. coli*). Les bactéries Gram-positives ont une structure de la paroi cellulaire similaire, si ce n'est qu'elles n'ont pas de membrane externe et une couche beaucoup plus épaisse de peptidoglycane avec des polysaccharides spécialisés supplémentaires appelés acides teichoïques.

Finalement, la dernière catégorie de polysaccharides produits par les bactéries regroupe des polysaccharides non pariétaux libérés par la cellule dans le milieu environnant (extracellulaire). Ils sont aussi appelés **exopolysaccharides ou EPS**. La frontière entre EPS et CPS est difficile à définir car les EPS peuvent demeurer à proximité de la cellule mais contrairement aux polysaccharides capsulaires "vrais", ils ne sont pas liés à elle (mis à part de potentielles liaisons électrostatiques) (Roberts, 1996). Certaines définitions incluent parmi les EPS la fraction des CPS dégradée et libérée dans le milieu (Kumar et al., 2007). La majorité des EPS sont cependant des polysaccharides excrétés délibérément par les bactéries dans le milieu environnant (voir paragraphe III.1 et III.2). De manière générale, la différence principale entre EPS et CPS réside par leur composition, leur degré de polymérisation (EPS > 50kDa), ainsi que par leur hydrosolubilité (les EPS sont par nature plus hydrosoluble que les CPS). Les EPS étant utilisés comme substrat dans nos études, ils seront décrits plus en détail dans la partie suivante.

Les EPS bactériens

Biosynthèse - Comme dit précédemment, les EPS sont des polymères hydrosolubles de haut poids moléculaire excrétés en grande quantité dans le milieu environnant. Comparés aux autres types de polysaccharides bactériens (i.e. pariétaux ou intracellulaires), ils représentent la plus grande diversité moléculaire et montrent une large plasticité.

Plusieurs voies de synthèse d'EPS bactériens ont été décrites. Il existe, d'une part, des voies extracellulaires qui impliquent l'assemblage des polysaccharides en dehors de la cellule ou dans l'espace périplasmique. Ces voies concernent principalement la biosynthèse d'homopolysaccharides linéaires, où chaque monosaccharide est ajouté à la suite d'un autre dans la chaîne polysaccharidique. Il existe, d'autre part, des voies de synthèse intracellulaire qui impliquent l'assemblage des EPS dans le cytosol, avant d'être exportés puis libérés dans le milieu extracellulaire. Plus communes, elles permettent l'élaboration d'hétéropolysaccharides plus complexes. Le modèle le plus connu est celui de la formation du xanthane, un EPS ramifié de haut poids moléculaire (500 à 2000 kDa), synthétisé par *Xanthomonas campestris* (**Figure 8**). La biosynthèse de cet EPS comprend 4 étapes : (i) la formation dans le cytosol des précurseurs issus de la source carbonée, (ii) l'assemblage du pentose qui sera lié à un lipide transporteur, (iii) l'assemblage des substituants (pyruvate et acétate), et finalement (iv) la polymérisation de l'EPS et sa libération dans le milieu extérieur (Becker et al., 1998; John A. Leigh, 1992; Kumar et al., 2007). Les phénomènes déterminant l'export des EPS et leur élongation restent cependant méconnus.

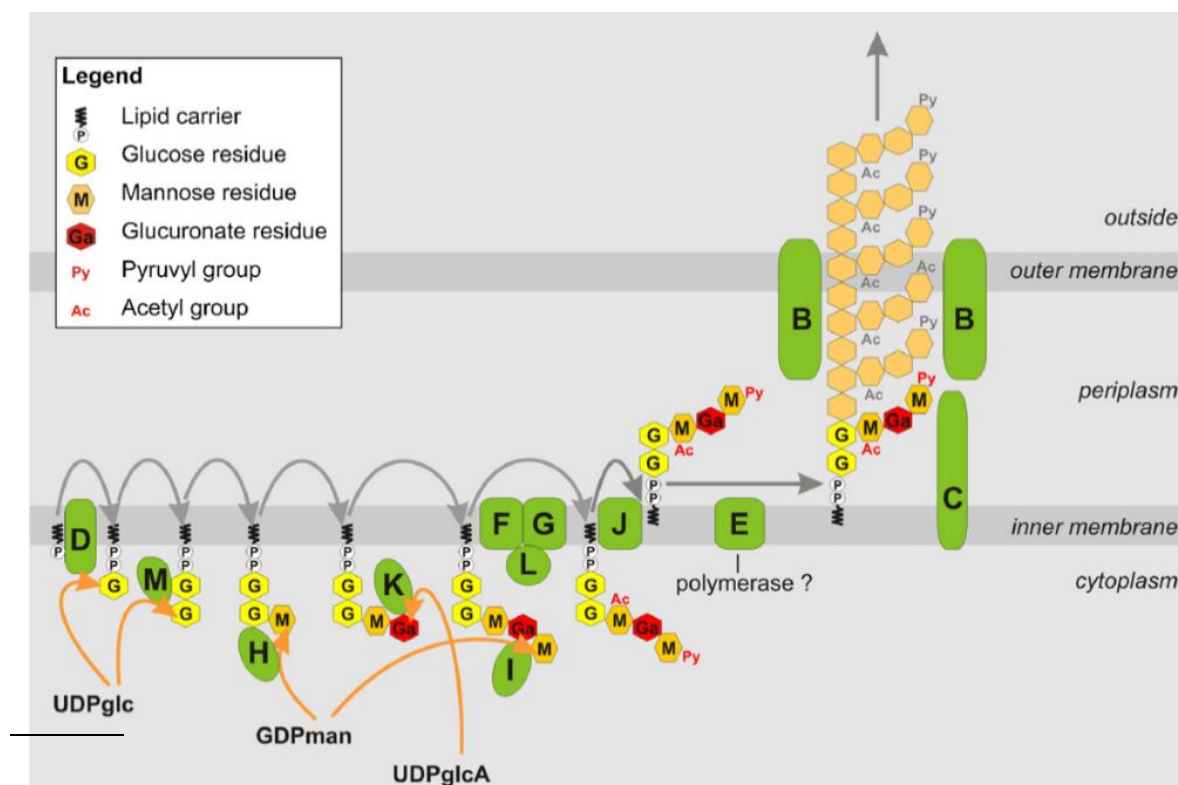


Figure 8 : Schéma de biosynthèse du xanthane, de Vorhölter et al. 2008

Propriétés physico-chimiques - les EPS ont la particularité de présenter les compositions osidiques et les substitutions les plus complexes. On retrouve sur la chaîne principale des oses neutres, oses acides, ou encore des oses rares ou N-acétylés. Certains oses peuvent également être chargés et donc donner aux polysaccharides des propriétés polyélectrolytes (**Table 1**). On compte parmi les substitutions possibles des groupements organiques ou inorganiques, pouvant être ajoutés de manière séquentielle, ou aléatoire (**Table 2**). La variété stéréochimique, l'ajout de ramifications, la présence de groupements de substitution, et les possibilités de liaisons existantes entre monosaccharides rendent le nombre de combinaisons structurales quasiment infini (Sutherland, 2001). Les propriétés physiques des EPS dépendent de cette structure et de la façon dont interagissent les chaînes polysaccharidiques entre elles. Certains types de liaisons inter-monosaccharides

(e.g. les liaisons β -(1- \rightarrow 3) et β -(1- \rightarrow 4)) confèrent une grande rigidité à la chaîne polysaccharidique, tandis que certaines peuvent lui donner plus de flexibilité (i.e. les liaisons α -(1- \rightarrow 6) ou α -(1- \rightarrow 2)). D'autre part, certains embranchements peuvent induire un désordre structurel et donc une grande solubilité en condition aqueuse (Sutherland, 2001). De même pour les groupements substituants et résidus chargés, influençant les propriétés gélifiantes des EPS ou encore des propriétés de chélation vis-à-vis de certains composés ioniques (Iyer et al., 2005; Loaëc et al., 1997).

Table 1 : Monosaccharides présents au sein des EPS (d'après Sutherland I., 2007 et actualisé par Lelchat)

Espèce osidique	Charge conférée à l'EPS	Nombre de carbone
D-arabinose	0	5
D-xylose	0	5
D-lyxose	0	5
D-ribose	0	5
D-glucose	0	6
D-galactose	0	6
D-mannose	0	6
D-fructose	0	6
L-altrose	0	6
L-fucose	0	6
L-rhamnose	0	6
D-talose	0	6
D-rhamnose	0	6
N-acétyl-D-glucosamine	0	8
N-acétyl-D-galactosamine	0	8
N-acétyl-D-mannosamine	0	8
N-acétyl-D-fucosamine	0	8
N-acétyl-D-talosamine	0	8
D-glucosamine	+	6
D-galactosamine	+	6
Ac. D-glucuronique	-	6
Ac. D-galacturonique	-	6
Ac. D-mannuronique	-	6
Ac. D-guluronique	-	6
Ac. D-iduronique	-	6
Ac. D-galactosaminuronique	-	8
Ac. D-mannosaminuronique	-	8
Ac. D-glucosaminuronique	-	8
Ac. 2-déoxy-D-arabino-hexuronique	-	6
Ac. D-riburonique	-	5
Ac. 3-deoxy-D-manno-oct-2-ulosonique	-	8

Table 2 : Types de substitution entrant dans la composition des EPS (d'après Sutherland I., 2007 et actualisé par Lelchat).

Substituants	Nature du substituant	Type de liaison	Charge conférée
Acétate	Organique	ester	0
Glycérate	Organique	ester	-
Pyruvate	Organique	acétalique	-
Succinate	Organique	hémi-ester	-
Lactate	Organique	ester/éther	-
Malonate	Organique	ester	0
Propionate	Organique	ester	0
Hydroxybutanoate	Organique	ester	0
Taurine	Organique	ester	0
Méthyle	Organique	éther	0
Phosphate	Inorganique	ester/diester	-
Sulphate	Inorganique	ester	-

Rôles physiologiques et écologiques - Leur rôle principal est celui de **protection**. Les EPS vont agir telle une barrière de protection physique contre les agressions extérieures. Dans l'environnement, les EPS protègent les bactéries contre les toxines, les molécules biocides (antibiotiques, détergents, antiseptiques), la prédation phagocytaire, ou bien encore les infections virales. La nature hydrophile des EPS permet la formation d'un environnement hydraté favorable à la croissance bactérienne en cas de dessiccation. Les EPS constituent également une protection contre la pénétration des UV, les contraintes thermiques, ou encore les modifications de pH et de pression (Jefferson, 2004; Weiner et al., 1995).

Un autre rôle des EPS est leur participation dans **la réponse au stress**. La production de polymères anioniques permet de favoriser la séquestration de cations et donc de mieux appréhender le stress auquel est confronté la bactérie. La production d'EPS chargés peut aussi permettre la séquestration de métaux ou d'autres ions. La polymérisation d'EPS permet également de réguler les systèmes de production d'énergie, en cas d'apport trop important (Fux et al., 2005). Il a également été démontré un autre rôle des EPS, relatif aux **communications cellulaires** (Von Bodman et al., 2008). Les EPS de *Pseudomonas marina* peuvent par exemple déclencher des métamorphoses chez la larve d'une polychète marine, *Janua brasiliensis* (Kirchman et al., 1981). Les EPS seraient également un réservoir de vésicules membranaires, mais l'étude de ce phénomène est à poursuivre (Nevot et al., 2006; Toyofuku et al., 2015).

Les EPS sont également impliqués dans la **formation des biofilms** bactériens. L'élaboration d'un biofilm d'EPS autour d'une cellule modifie les caractéristiques physico-chimiques de l'environnement immédiat de la bactérie en le rendant plus propice à la survie de la population bactérienne. Un biofilm bactérien peut être constitué de 40 à 90% d'EPS (les bactéries ne représentant parfois que 2% de la biomasse), et le rapport carbone/azote y est en moyenne 5 fois plus élevé que les ratios habituels (Branda et al., 2005; Sutherland, 2001). Les EPS participent à l'adhésion initiale et/ou permanente des bactéries sur les surfaces immergées, mais ils se retrouvent principalement dans la constitution de la matrice du biofilm (Weiner et al., 1995). Les biofilms bactériens sont monospécifiques, et ils deviennent rapidement le lieu d'interaction où s'établissent des synergies entre les différentes souches bactériennes, pouvant alors modifier les propriétés du biofilm (**Figure 9**).

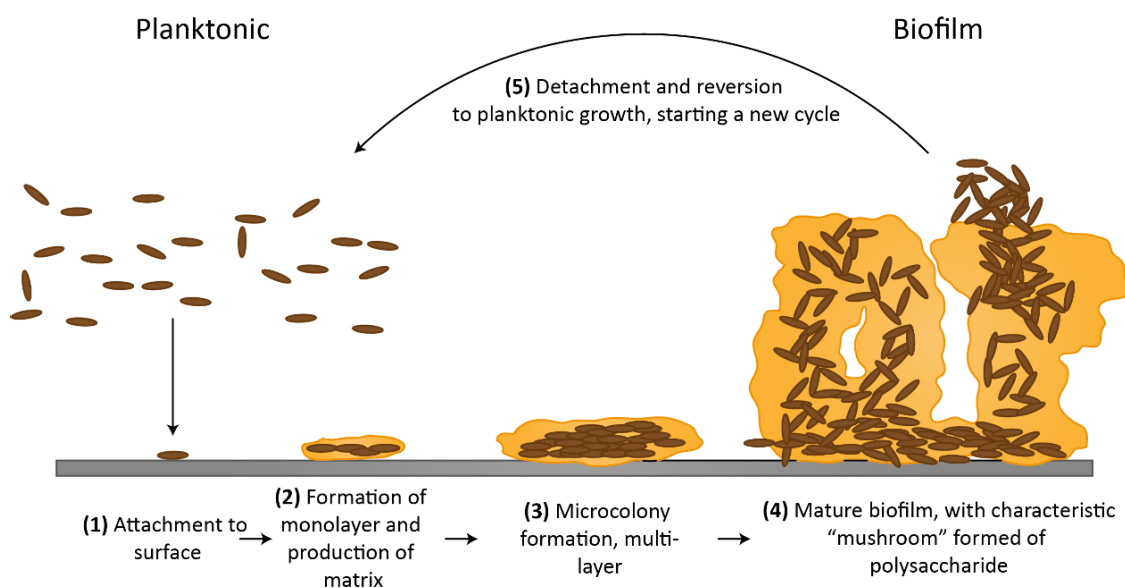


Figure 9 : Etapes de formation d'un biofilm bactérien, de Vasudevan et al. 2014. Premièrement, les bactéries s'attachent à la surface, forment une monocouche et produisent une matrice d'EPS. Par la suite, une microcolonie de plusieurs couches se forme et le biofilm se mature. Cette étape permet le développement des communautés bactériennes avant leur détachement de la matrice et le retour à l'état planctonique.

II.2 - Les polysaccharide dépolymérase

Définition et généralités

Les enzymes qui interagissent avec les polysaccharides sont génériquement définies comme des Carbohydrate Active enZymes (CAZYmes) et regroupent plus de 300 familles de protéines (www.cazy.org, (Levasseur et al., 2013)). Les CAZYmes sont réparties en cinq classes selon leur fonction: (i) les Glycosides Hydrolases qui catalysent l'hydrolyse de la liaison glycosidique, (ii) les Polysaccharide Lyases fonctionnant par un clivage non-hydrolytique de cette liaison, (iii) les Glycosyltransferase permettant la formation de liaisons glycosidiques, (iv) les Carbohydrate Esterases qui catalysent la déacylation des substituent saccharides, et (v) les Activités Auxiliaires, regroupant les enzymes redox fonctionnant avec les autres CAZYmes. Les polysaccharides dépolymérase sont donc (sauf exception) soit des Glycosides Hydrolases (GH), soit des Polysaccharide Lyases (PL).

Les GH fonctionnent suivant deux mécanismes catalytiques principaux. Premièrement, le mécanisme par inversion se déroule en une unique étape, faisant intervenir une molécule

d'eau et opérant un déplacement du groupe hydroxyle anomérique. Les acides aminés opérant directement dans cette catalyse ont chacun deux groupes carboxyliques, l'un va alors agir telle une base, l'autre tel un acide (**Figure 10A**). Secondement, le mécanisme par rétention se réalise en 2 étapes, et cette fois-ci sans déplacement du groupement hydroxyle anomérique. Faisant intervenir également deux groupes carboxyles du site catalytique enzymatique, la première étape consiste en la formation d'un complexe intermédiaire enzyme-substrat par attaque nucléophile du carbone anomérique par un groupement hydroxyle. La seconde étape est une déglycosylation, où le complexe est hydrolysé par l'eau, avec le second résidu hydroxyle jouant le rôle de base et déprotonant la molécule d'eau lors de son attaque (**Figure 10B**).

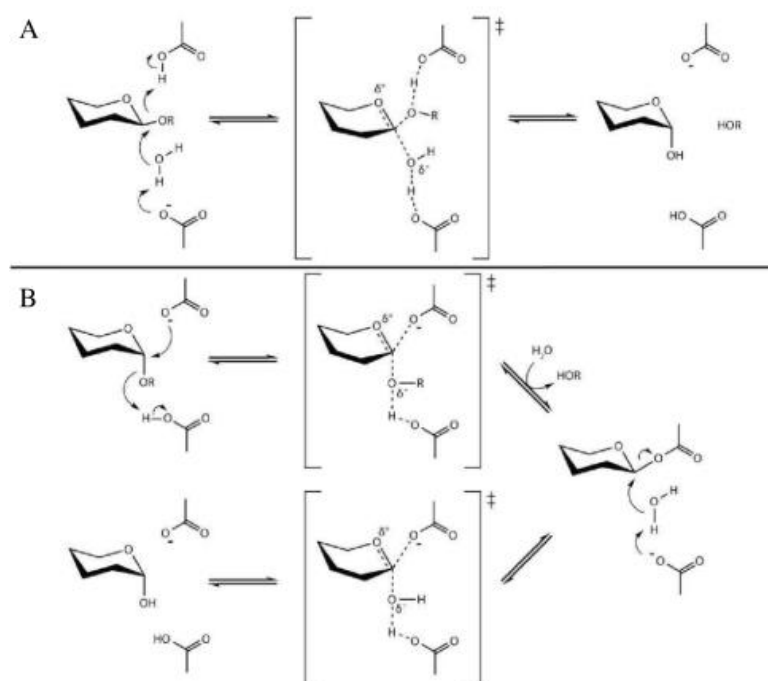


Figure 10 : Mécanismes catalytiques des glycoside hydrolases. (A) Mécanisme par inversion, (B) Mécanisme par rétention.

Les PL ne font pas intervenir de molécule d'eau et le mécanisme fonctionne par une élimination β se déroulant en trois étapes. Une abstraction du proton du carbone C-5 à l'aide d'un cation ou d'un acide aminé basique s'opère d'abord. Il s'en suit une stabilisation par délocalisation de charge sur le groupe carbonyle du carbone C-6, puis un clivage de la liaison O-4 :C-4, facilité par le don d'un proton d'un acide catalytique (**Figure 11**).

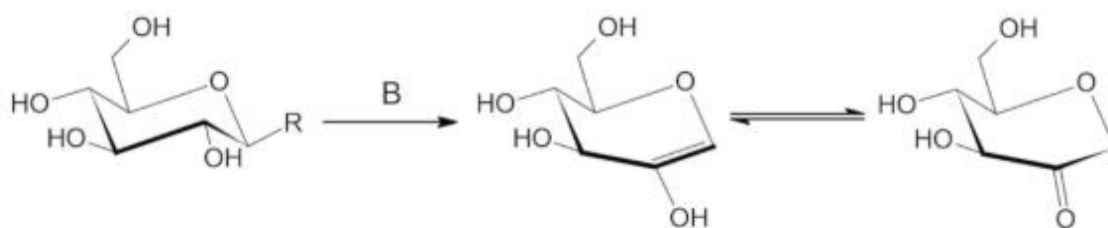


Figure 11 : Mécanisme catalytique d'une polysaccharide depolymerase

Modes d'action

Certaines polysaccharide dépolymérase s'attaquent aux extrémités des chaînes polysaccharidiques, libérant de 1 à 8 résidus par clivage. On parle alors d'un mode d'action exolytique. D'une autre manière, les autres enzymes attaquent les liaisons osidiques en milieu de chaîne polysaccharidique, et ont dans ce cas un mode endolytique. Une enzyme, qu'elle soit endo- ou exolytique, peut également soit changer de chaîne polysaccharidique après clivage de la liaison, soit cliver de manière continue la chaîne jusqu'à son autre extrémité. On parle dans ce dernier cas d'enzyme processive. Ces modes d'action enzymatiques sont étroitement liés à la structure tridimensionnelle des sites actifs des polysaccharide dépolymérase. Dans le cas d'une enzyme endolytique processive, on retrouve le plus souvent des structures enzymatiques avec un site actif en forme de tunnel, par lequel la chaîne polysaccharidique se glisse et peut être dépolymérisée au fur et à mesure. Une forme de site actif en crevasse est en revanche retrouvée chez des endoenzymes à plus faible processivité, car il y aura un maintien moins fort de la chaîne polysaccharidique au niveau du site catalytique. Un site actif en forme de poche sera au contraire plus typique des enzymes exolytiques, sa forme étant optimale pour la reconnaissance de l'extrémité d'un résidu osidique (**Figure 12**).

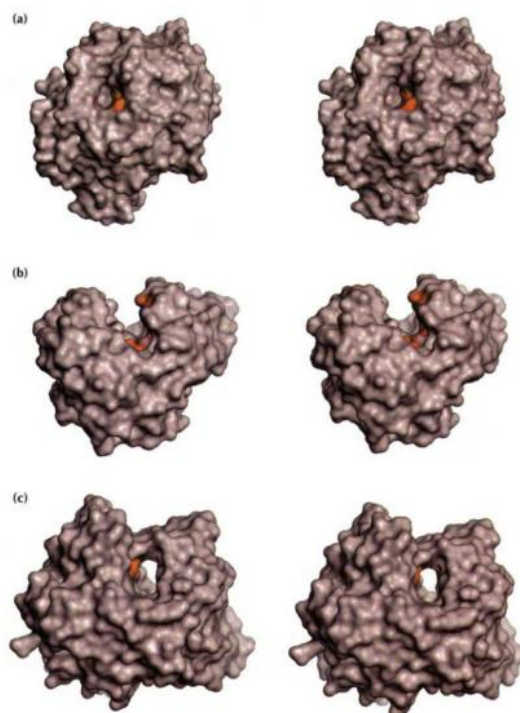


Figure 12 : Topologie du site actif des glycosidases (a) type poche, (b) type crevasse, (c) type tunnel (D'après Davis & Henrissat., 1995)

II.3 - Les polysaccharide dépolymérase virales

Les polysaccharide dépolymérase ont été décrites chez les phages dès les années 50. Ces descriptions faisaient état de « plages de lyses entourées de halos, où le film bactérien était plus fin et plus translucide que la normale » (Humphries, 1948). D'après Humphries, ces halos de dépolymérisation auraient déjà été observés en 1929 par Sertic, qui décrivait alors des variantes de bactériophages adaptées à lyser des formes bactériennes secondaires. Les bactéries prélevées dans ce halo par Humphries étaient cependant viables, et étaient principalement des souches résistantes au phage. Il en conclut à la présence d'une enzyme "dépolymérase" d'origine virale dégradant les polysaccharides de son hôte bactérien (**Figure 13**). Dès lors, de nombreuses études dans le domaine biomédical ont été réalisées et ont révélé le rôle essentiel de ces enzymes durant l'infection virale (Adams and Park, 1956; Bayer et al., 1979; Bessler et al., 1973; Thurow et al., 1974; Yurewicz et al., 1971). « Not a barrier but a key » (Broeker) : la gangue d'EPS ou CPS, jusqu'alors considérée comme une barrière

physique empêchant l'infection, apparaît désormais comme un site reconnaissance du phage (récepteur secondaire) par sa dépolymérase. L'activité de l'enzyme permet au phage de dégrader la matrice d'EPS de son hôte et d'accéder à ses récepteurs membranaires (récepteur primaire) (**Figure 14**). Ce n'est qu'une fois les récepteurs membranaires atteints que le virus s'adsorbe et injecte l'ADN dans son hôte bactérien. La présence de dépolymérases chez le virus pourrait également avoir un effet bénéfique lors de la libération des particules virales après la lyse bactérienne entraînant une libération et une dispersion plus efficace des virions néosynthétisés (Sutherland et al., 2004).

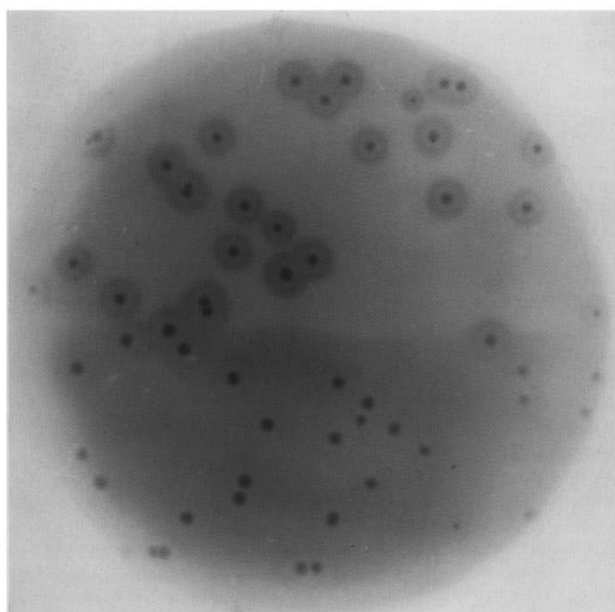


Figure 13 : Photographie montrant des halos de dépolymérisation (moitié haute) formés par le phage *Kp* infectant *Klebsiella pneumoniae* type 2 sur milieu de culture gélosé (par Adams et Park en 1956)

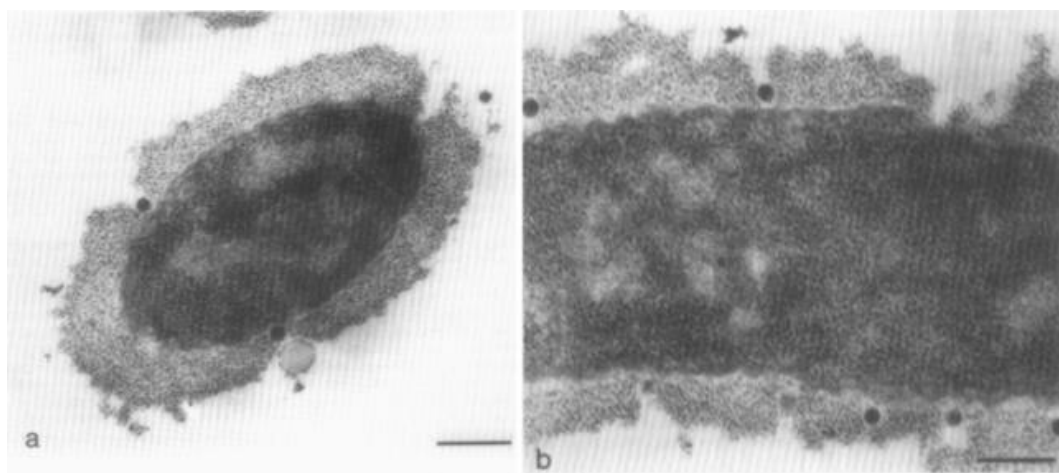


Figure 14 : Cliché de microscopie électronique à transmission montrant la pénétration de la capsule polysaccharidique de *Escherichia coli* (Bi161/42) par le Bactériophage K29 (Bayer 1978)

Mode d'action

Les dépolymérase virales apparaissent sous deux formes : (i) la première est une forme intégrée structurellement au phage, (ii) la seconde est une forme soluble de l'enzyme libérée durant la lyse de l'hôte cellulaire dans le milieu environnant (Drulis-Kawa et al., 2015). Dans une étude récente, 160 gènes codant pour des polysaccharide dépolymérase ont été identifiés dans les génomes de 143 phages (43 *Myoviridae*, 47 *Siphoviridae*, 37 *Podoviridae*, et 16 non-classés) (Pires et al., 2016). Cependant, l'activité de seulement 64 de ces enzymes a été confirmée expérimentalement à ce jour. Les dépolymérase de phage font partie de deux classes principales des CAZymes : les GH et les PL, elles même divisées en sous-groupes suivant leur mode d'action. La majorité des glycoside hydrolases appartiennent au groupe des O-glycosyl hydrolases (EC 3.2.1) comprenant les sialidases, rhamnosidases, levanases, xylanases, et les dextranases (Davies and Henrissat, 1995; Pires et al., 2016). A ce jour, les hydrolases les plus étudiées sont probablement les sialidases (ou neuraminidases EC 3.2.1.18) associées aux phages K1 d'*Escherichia coli* (Gerardy-Schahn et al., 1995; Jakobsson et al., 2007; Kwiatkowski et al., 1983; Leiman et al., 2007; MacHida et al., 2000; Petter and Vimr, 1993; Scholl et al., 2005; Schwarzer et al., 2007; Stummeyer et al., 2006). D'autres sialidases ont été également étudié chez des phages de *Klebsiella* (Hoyles et al., 2015), ou chez d'autres phages de bactéries du genre *Salmonella* ou *Shigella* (Chaby and Girard, 1980; Lindberg et al., 1978). Concernant les polysaccharide lyases de phage, il a été montré l'existence de nombreuses hyaluronate lyases, pectate/pectin lyases, et autres alginate lyases. Les hyaluronate lyases traduisent généralement la présence d'une capsule bactérienne composée d'acide hyaluronique, comme chez *Streptococcus pyogenes* et *S. equi* (Baker et al., 2002; Ferretti et al., 2001; Hynes et al., 1995; Lindsay et al., 2009; Martinez-Fleites et al., 2009; Mishra et al., 2006). Des pectate lyases ont été retrouvées chez des phages de *Pseudomonas* (Cornelissen et al., 2012, 2011), de *Klebsiella* (Lin et al., 2014; Majkowska-Skrobek et al., 2016), de *Vibrio* (Linnerborg et al., 2001), et de *Staphylococcus* (Gutiérrez et al., 2015, 2012) ; tandis que les alginates lyases sont plus spécifiques des phages de *Pseudomonas* et d'*Azobacter* (Wong et al., 2000). Des lyases spécifiques ont également été découvertes chez des phages d'*E. coli* (Clarke et al., 2000; Thompson et al., 2010).

Structure

La reconstruction en 3D des coliphages K1E et K1-5 par Cryo-EM (Leiman et al., 2007) a permis de localiser 1 à 2 sets de dépolymérase (chacune en 6 copies) au niveau de la queue des virions (tailspike), faisant face au milieu environnant (**Figure 15**). Cette position est optimale pour la pénétration du virus au travers des couches d'EPS et pour la libération de l'accès aux récepteurs membranaires. En analysant la position des gènes codant pour des dépolymérase au sein des génomes de phages, Pires et al. (2016) ont montré que plus de 75% des dépolymérase potentielles se retrouvent dans le même ORF que d'autres protéines structurelles de phages. Parmi ces protéines structurelles, on retrouve principalement des fibres caudales, plaques basales, ou encore des protéines de jonction tête/queue (**Figure 16**). Cette découverte corrobore les études en microscopie électronique de Machida et al. en 2000, qui avait alors utilisé des anticorps marqués pour localiser les polysaccharide dépolymérase du coliphage 63D (**Figure 17**) (Machida et al., 2000).

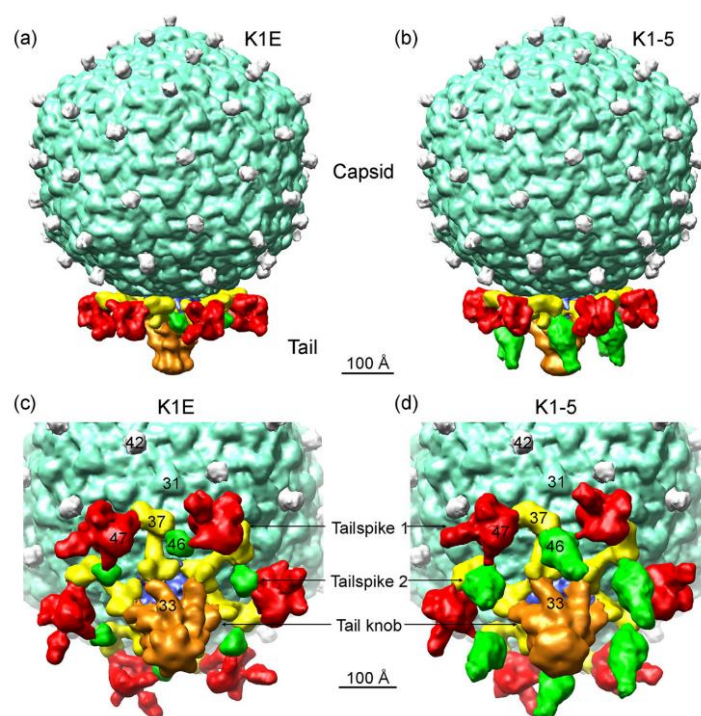


Figure 15 : Identification des depolymerase des phages K1E et K1-5 (en rouge) par cryo-microscopie électronique (Leiman en 2007)

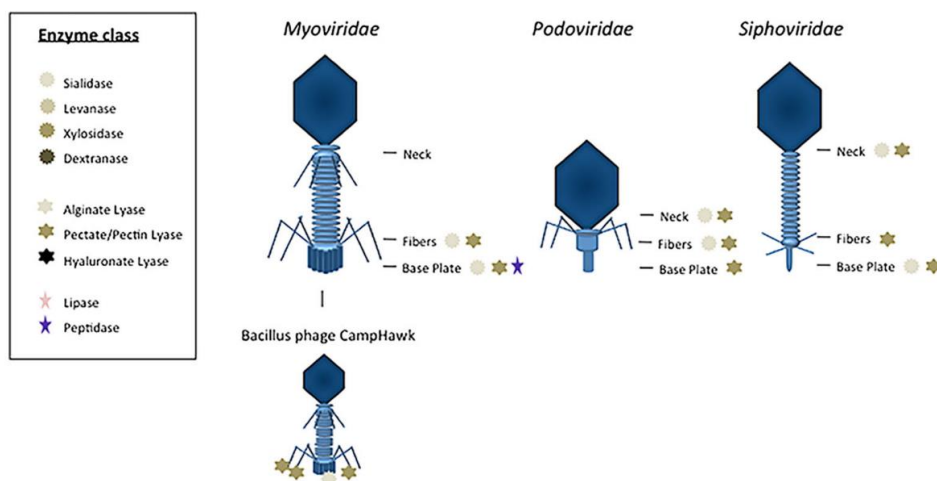


Figure 16 : Localisation des différentes classes de dépolymérase sur les particules virales appartenant à la famille des Caudovirales (Pires et al., 2016)

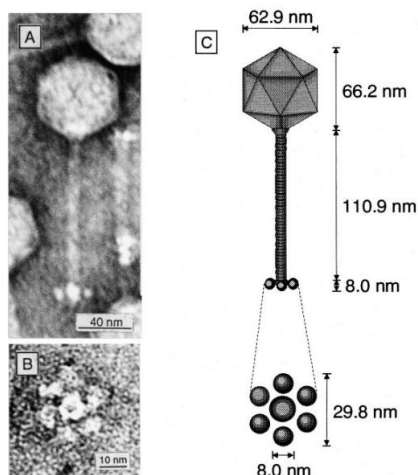


Figure 17 : Identification par microscopie électronique à transmission des dépolymérase (Machida et al., 2000)

Si les polysaccharide dépolymérase présentent une large diversité génétique, certaines caractéristiques structurales semblent conservées. Les premières études structurales furent menées par Steinbacher en 1996, puis Weigle en 2003 (**Figure 18**). Les protéines ayant une activité dépolymérase sont généralement formées d'un long homotrimère ayant la forme d'un « foret », ce qui rejoint de manière imagée leur fonction biologique (Casjens and Molineux, 2012; Steinbacher et al., 1996; Weigle et al., 2003). Ces enzymes semblent être de type fibreuse avec des hélices β composées de brins β parallèles disposés de manière hélicoïdale (**Figure 19**). Cette forme donnerait à la dépolymérase une large surface latérale de contact avec son substrat, et serait déterminante pour la spécificité de l'hôte (Bradley et al., 2001).

Cette structure conférerait aux dépolymérase une grande stabilité, et il a d'ailleurs été démontré une résistance de ces dernières à des températures de plus de 45°C (Majkowska-Skrobek et al., 2016). Ces protéines homotrimériques peuvent être séparées en 3 parties fonctionnellement différentes : (i) une première partie N-terminale permettant une connexion flexible avec la particule virale à la structure caudale ou la plaque basale (ii) une seconde partie centrale assurant la reconnaissance avec l'hôte et l'activité dépolymérase, et (iii) une partie C-terminale responsable de la trimérisation et de la reconnaissance avec le récepteur membranaire de l'hôte (**Figure 20**) (Casjens and Molineux, 2012; Schwarzer et al., 2012; Weigele et al., 2003; Yan et al., 2014).

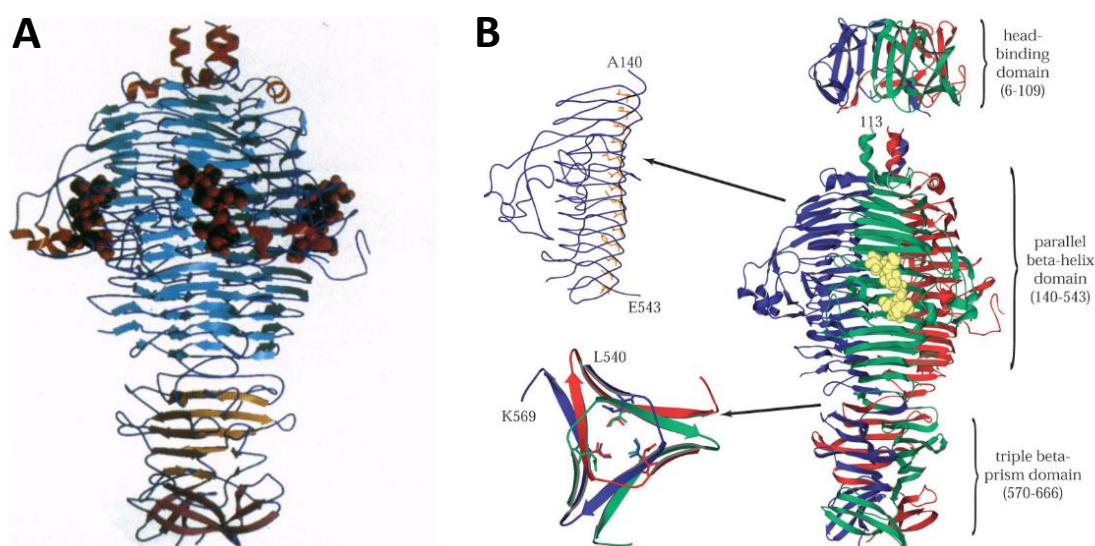


Figure 18 : Premières études structurales de la dépolymérase du phage P22 de *Salmonella enterica*. En A est représenté la protéine trimer tailspike du phage P22, structure résolue par Steinbacher et al. en 1996. Le domaine N-terminal se localise ici en haut de la représentation, et le C-terminal en bas. En B, par Weigele et al. en 2003, est représenté un diagramme plus détaillé de cette protéine, avec (i) à droite la protéine entière avec un nonasaccharide de *S. enterica* fixé (jaune) ainsi que les 3 domaines divergents sur le plan fonctionnel et structural, (ii) en haut à gauche une région constituant une partie de l'hélice β et responsable de la liaison avec des polysaccharides, et (iii) en bas à gauche, une région comportant des résidus hydrophobes et permettant la trimérisation de la protéine et sa stabilisation.

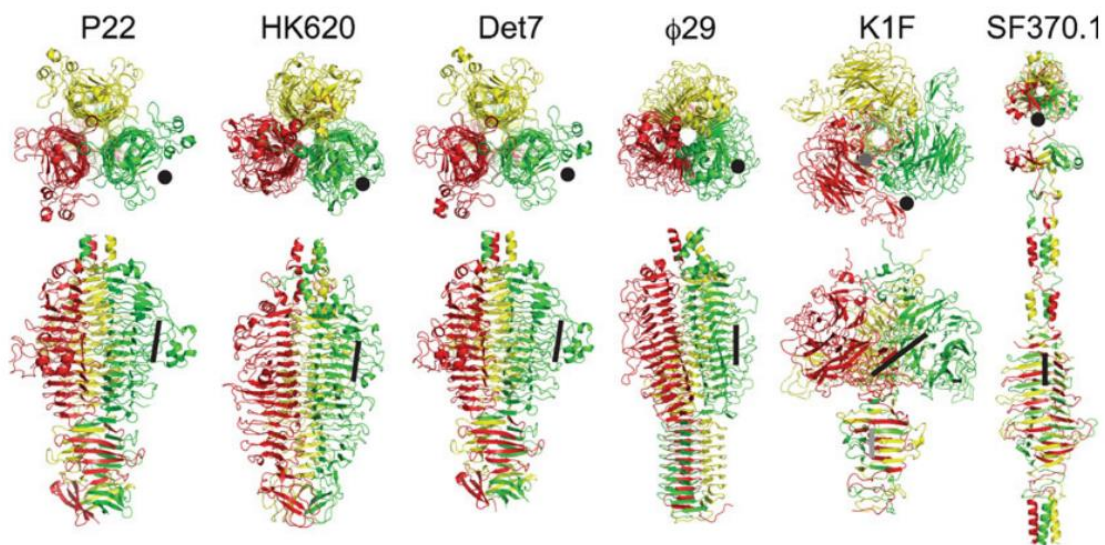


Figure 19 : Représentation structurale des tailspikes viraux résolus depuis 1996 et rassemblées par Casjens et Molineux (2012)

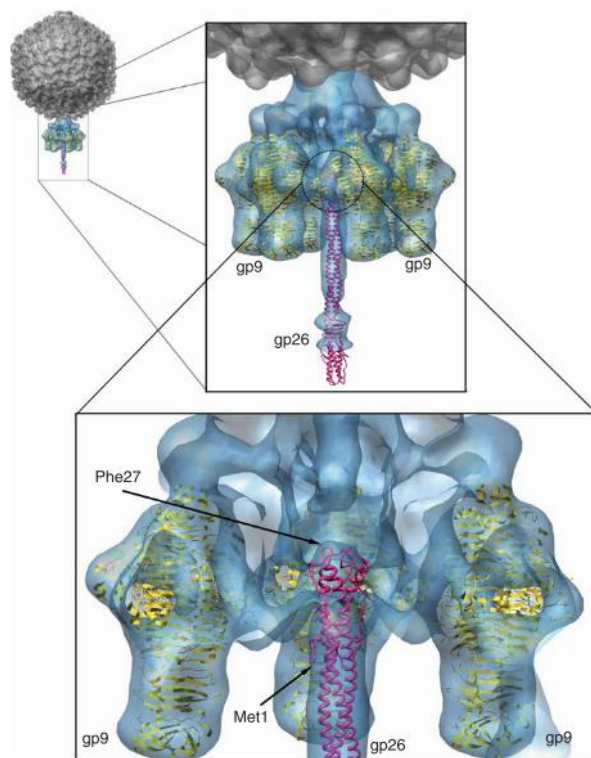


Figure 20 : Description structurale des dépolymérase viraux. Structure de la queue du phage P22 de *Salmonella enterica* avec l'aiguille d'injection (mauve) et les fibres caudales/tail spike (jaune) (Olia et al. 2007).

II.4 - Rôle des dépolymérase virales dans les interactions hôte – virus

Jusqu' alors, la capacité des virus à dégrader les EPS ou CPS de son hôte a été principalement étudiée dans une perspective biotechnologique, notamment pour l'éradication de biofilms ou pour de la phagothérapie. Dans ce contexte, il a pu être démontré que les polysaccharide dépolymérase jouaient un rôle important dans la détermination des spectres d'hôte des bactériophages comme montré chez les coliphages K1E et K1-5. Le phage K1E (*Podoviridae*) présente une endosialidase qui reconnaît et dégrade les CPS de son hôte *E. coli* K1. Le gène codant pour l'endosialidase est situé dans une position génomique occupée par le gène codant pour une protéine caudale (tail spike), à l'instar du phage T7 génétiquement proche de K1E. De la même manière, le phage K1-5, qui infecte également la bactérie encapsulée *E. coli* K1, possède un domaine endosialidase similaire à celui du coliphage K1E au niveau de la tailspike. Fait intéressant, il possède également une deuxième dépolymérase (une polysaccharide lyase) qui dégrade les CPS d'*E. coli* K5, lui permettant au final d'élargir son spectre d'hôte (Leiman et al., 2007).

Par ailleurs, l'étude de la polysaccharide dépolymérase du phage AF qui infecte *Pseudomonas putida* (Cornelissen et al., 2012, 2011) suggère que les CPS peuvent servir de récepteur primaire pour l'infection virale. Ces études montrent que le gène responsable de l'activité dépolymérase chez le phage AF est conservé chez le *P. putida* phage $\phi 15$ (T7-like). Ces 2 phages présentent des spectres d'hôte parfaitement similaires. La tailspike étant le seul composant structural partagé entre les 2 phages, il est probable que cette protéine détermine leur spectre d'hôte. Des expériences complémentaires au laboratoire supportent l'idée que les CPS sont vraisemblablement les récepteurs primaires de ces phages. En outre, cette étude confirme que les phages sont susceptibles d'échanger des dépolymérase par transfert horizontal, leur permettant de modifier la gamme d'hôte qu'ils infectent (Stummeyer et al., 2006).

Les interactions EPS – dépolymérase font l'objet d'une course aux armements entre le phage et son hôte, apportant une pression évolutive constante entre les 2 partis (**Figure 21**) (Labrie et al., 2010). Comme expliqué précédemment, la matrice d'EPS protège la cellule du milieu environnant mais elle constitue également une barrière de défense contre l'infection en masquant les récepteurs membranaires. De son côté, le virus doit s'adapter et acquérir les

dépolymérase nécessaires afin de déjouer cette défense. Cette coévolution entre les phages et leurs hôtes bactériens a permis le développement d'une grande diversité de polysaccharide dépolymérase virales et d'EPS bactériens. Le plus grand niveau de diversité génétique parmi les gènes structuraux de phage est d'ailleurs atteint avec les receptor binding proteins (RBPs) qui incluent les dépolymérase des tailspike (**Figure 19**) (Casjens and Molineux, 2012). Les phages ont développé deux stratégies pour adapter leurs tailspikes à la diversité des EPS produits par leurs hôtes (Leiman and Molineux, 2008). Ils peuvent, d'une part, par échange vertical, changer des résidus du site actif par mutation. L'activité dépolymérase étant en périphérie de la structure interne de la protéine, des modifications à cet endroit précis n'affectent pas la conformation des hélices β . Dans un second temps, les phages peuvent incorporer par transfert horizontal des modules complets au sein de la tailspike afin d'acquérir de nouvelles spécificités. Cette stratégie commune, aussi appelée « cut-and-paste », consiste à conserver les modules N-ter et C-ter, tandis que le module enzymatique peut, lui, être échangé. C'est d'ailleurs ce que l'on retrouve chez les phages d'un même groupe : le domaine central est très variable et peut être changé pour moduler le spectre d'hôte ou s'adapter à un nouvel environnement (Schwarzer et al., 2012; Stummeyer et al., 2006).

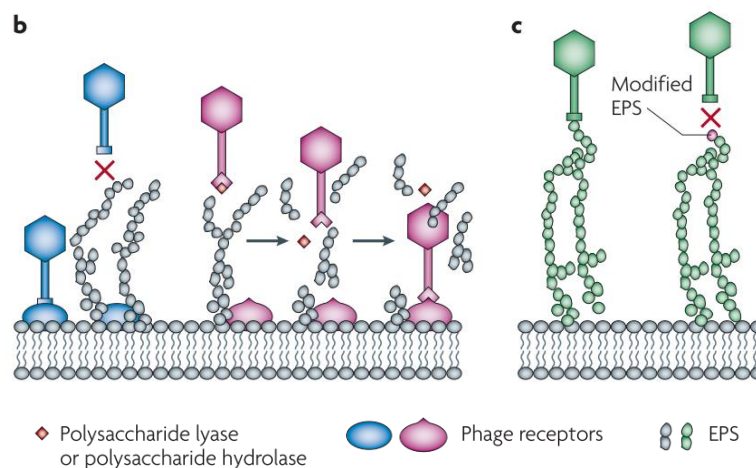


Figure 21 : Différentes stratégies utilisées par les bactéries pour bloquer l'adsorption du phage. Les bactéries utilisent des EPS pour dissimuler leurs récepteurs membranaires, mais les virus ont développé des dépolymérase permettant de dégrader ces EPS pour atteindre les récepteurs bactériens et déclencher l'infection. Il existe une véritable course à l'armement entre phage et bactéries, où chacun va développer de nouveaux outils pour déjouer les moyens de défenses des bactéries (e.g. modifications des EPS ou récepteurs) ou les moyens d'attaques des phages (évolution d'une nouvelle polysaccharide dépolymérase). Labrie et al. 2010.

III - LES POLYSACCHARIDE DEPOLYMERASES DANS L'ENVIRONNEMENT MARIN

Dans l'océan, la dégradation enzymatique des polysaccharides a principalement été étudiée dans un contexte biogéochimique. Les polysaccharides, incluant les EPS bactériens, représentent une fraction importante de la matière organique dissoute (MOD) et particulaire (MOP), dont les dynamiques conditionnent très largement le fonctionnement de la boucle microbienne et les cycles biogéochimiques globaux.

III.1 - Polysaccharides et EPS bactériens dans l'océan

A l'instar des environnements terrestres, les polysaccharides représentent les composés les plus abondants dans l'océan. Ils interviennent par exemple dans le stockage d'énergie et la structure des organismes marins (voir §II.1). Ils représentent également une fraction importante de la MOD (2 à 30%) et jusqu'à 70% de la MOD de haut poids moléculaire (> 1kDa) dont la composition et la dynamique conditionnent largement le fonctionnement des écosystèmes marins (Benner, 2002; Benner et al., 1992; Pakulski and Benner, 1994). La concentration des polysaccharides peut varier entre 200 et 800 nM (Sempéré et al., 2008). A titre de comparaison, la concentration des monosaccharides dans les océans est <50nM et les sucres majoritaires sont le glucose et dans une proportion moindre l'arabinose et du fructose. L'abondance des polysaccharides diminue avec la profondeur, suggérant que ces composés sont particulièrement réactifs en termes de production et de consommation dans les eaux de surface (Pakulski and Benner, 1994). Les polysaccharides peuvent être produits et excrétés par tous les organismes vivants mais dans le milieu marin les macroalgues et les organismes planctoniques (microalgues, bactéries) en sont les producteurs majoritaires (Biersmith and Benner, 1998; Casillo et al., 2018). La diversité globale de ces macromolécules reste toutefois inexplorée car les outils actuels ne permettent pas d'identifier et de caractériser ces espèces chimiques dans des échantillons environnementaux complexes.

Les bactéries alimentent activement le pool de polysaccharides dans le milieu environnant soit par l'excrétion active de CPS ou d'EPS, soit par la libération de matière organique lors d'évènements de prédation des bactéries, ou encore par des processus de lyse cellulaire, notamment causée par les virus (Middelboe and Jørgensen, 2006). En biogéochimie marine, les EPS d'origine bactérienne ont reçu une attention moindre en comparaison des EPS d'origine phytoplanctonique. La libération d'EPS représenterait pourtant 25% du carbone respiré par les bactéries (Stoderegger and Herndl, 1998). Il est donc probable que les EPS bactériens constituent une fraction considérable du carbone organique dissout (COD). La composition osidique des EPS produits par les bactéries marines diffère de celle de leurs congénères terrestres. Les EPS de bactéries marines sont généralement enrichis en acides uroniques (notamment acide D-glucuronique and acide D-galacturonique, (Chi and Fang, 2007)). Cette propriété rend ces macromolécules très polyanioniques (dû à la charge négative des groupes anioniques ; e.g. COO-, CO-, SO₄-) et ainsi particulièrement réactives avec les espèces chimiques de charge opposée (Kennedy and Sutherland, 1987). Les EPS bactériens dans l'océan sont particulièrement reconnus pour leur capacité à complexer des métaux cationiques et éléments traces tel que le fer (C. S. Hassler et al., 2011). Le fer est indispensable à tous les organismes vivants car il intervient dans de nombreux processus métaboliques clés (e.g. photosynthèse, transport d'électrons, fixation du diazote, ...), mais ses faibles concentrations dans les eaux de surface limitent la production primaire dans au moins 40% de l'océan mondial (Fiechter et al., 2009; Kolber, 1994; Martin et al., 1993). Les propriétés physico-chimiques des EPS en font des ligands faibles du fer qui modifient la spéciation chimique et augmentent la biodisponibilité de cet élément pour la communauté phytoplanctonique (C. S. Hassler et al., 2011).

Dans l'océan, les EPS comprennent également une classe spécifique de particules décrites comme mucopolysaccharidiques, encore appelées « Transparent Exopolymeric Particles » ou TEP. Les TEP sont des biogels transparents, que l'on détecte par microscopie optique après coloration au bleu Alcian à pH 2,5, ce qui a pour but de révéler la fraction polysaccharidique de ces molécules (Alldredge et al., 1993). L'abondance des TEP dans la colonne d'eau océanique est de l'ordre de 10⁶ particule par litre, pouvant aller jusqu'à 10⁸ par litre (Bhaskar and Bhosle, 2005; Passow, 2002), en particulier pendant les périodes de prolifération du phytoplancton. Les TEP contribuent de manière significative à ce qui est décrit comme la phase

de gel marin (Verdugo et al., 2004). Ils couvrent un large spectre de taille de par leur capacité à s'agréger (Engel, 2004) et contribuent à la formation de neige marine facilitant l'export de carbone de la surface vers les fonds océaniques (Alldredge et al., 1993; Verdugo et al., 2004). Au cours des dernières années, différentes études ont montré que les EPS bactériens et phytoplanctoniques (et notamment les TEP) peuvent s'accumuler dans la microcouche de surface océanique et par la suite être transportés dans l'atmosphère comme aérosol marin (comme conséquence de forçages tels que le vent, les vagues, la pluie). Un nombre croissant d'études suggère que les EPS marins peuvent agir comme noyaux de condensation pour la formation des nuages (O'Dowd et al., 2004; Russell et al., 2010). Enfin, dans les zones de balancement des marées (ou zones intertidales) les EPS stabiliseraient physiquement le sédiment contre les effets de cisaillement dus aux vagues et les effets de marnage en générant un phénomène d'adhésion entre les particules sédimentaires. Ils auraient également un effet biologique bénéfique en limitant l'impact délétère des chocs osmotiques, du rayonnement UV, de la dessiccation ou de l'amplitude thermique.

III.2 - Dégradation des polysaccharides dans l'océan

Les polysaccharides produits en grande quantité dans les eaux océaniques sont une source de carbone et d'énergie essentielle pour le bactérioplancton. Pour exploiter ces molécules de haut poids moléculaire, les bactéries utilisent des polysaccharide dépolymérase qui catalysent leur dégradation en produits de bas poids moléculaire, utilisables pour leur métabolisme et leur croissance. La biomasse produite sera transférée aux échelons trophiques supérieurs par broutage, et les nutriments régénérés par le métabolisme bactérien bénéficieront aux producteurs primaires. L'ensemble de ces interactions sous-jacentes au transfert de matière et d'énergie constituent la boucle microbienne (voir paragraphe 1.4, **Figure 5**).

Selon leur dégradabilité, et donc leur temps de résidence dans la colonne d'eau, les polysaccharides dissouts, et plus généralement le COD, peuvent être classés en trois catégories : labile, semi-labile, et réfractaire. Les polysaccharides appartenant au pool de

matière **labile** ont un temps de résidence extrêmement court (de l'ordre de la minute ou du jour). Ils sont dégradés et consommés aussi rapidement qu'ils sont produits. Le pool de COD labile ne représente, toutefois, qu'une très faible proportion (<1%) du pool de carbone global. Entre 25 – 50% du COD marin appartient au pool **semi-labile**, sa dégradation et consommation nécessitent plusieurs semaines ou mois. Une hypothèse propose que l'accumulation de COD semi-labile résulte de l'absence d'autres nutriments, notamment le phosphate, qui limitent la croissance bactérienne (Thingstad and Lignell, 1997). Une autre hypothèse, plus plausible, suggère que la nature chimique des substrats (moins favorables énergétiquement) inclus dans ce pool de matière contribue à son accumulation dans les eaux de surface. La plus large fraction du COD marin est, quant à elle, réfractaire aux dégradations microbiennes et persiste dans l'océan pendant plusieurs milliers d'années. Un changement même mineur de la concentration de ce COD réfractaire pourrait affecter de manière significative la séquestration du carbone dans l'océan avec des conséquences sur le climat (Jiao et al., 2010). La capacité qu'ont les bactéries à dégrader les polysaccharides grâce à leurs dépolymérases a donc une influence sur le devenir d'une grande partie de la matière organique. Ces enzymes jouent sans conteste le rôle de «gardiens» du cycle du carbone marin (Arnosti, 2011).

Traditionnellement, il est admis que les polysaccharides de haut poids molécule sont hydrolysés par des dépolymérases en composés de 600 Da avant leur transport dans la cellule (Arnosti, 2011). L'hydrolyse est effectuée par des enzymes extracellulaires qui sont soit associées à la surface des cellules, soit libres dans la colonne d'eau. Les produits de dégradation générés dans le milieu extracellulaire deviennent donc disponibles pour la communauté microbienne présente y compris pour les organismes qui possèdent un éventail enzymatique restreint. Bien qu'il ne fasse aucun doute que les polysaccharide dépolymérases sont omniprésentes et qu'elles digèrent une grande partie de la MOD marine, la quantification de leur activité dans le milieu naturel reste très approximative. L'approche la plus largement utilisée repose sur la dégradation de substrat simple (monosaccharides associés à une sonde fluorescente -MUF) par les communautés microbiennes (Arnosti, 2011; Baltar et al., 2009; Hoppe, 1993). Par ailleurs, les recherches sur la dynamique des polysaccharides sont principalement concentrées sur l'hydrolyse enzymatique de substrats contenant du glucose (tel que MUF- α - et β -glucose; (Kellogg and Deming, 2014; Zaccone et al., 2012)). Ces études ont montré que l'hydrolyse des polysaccharides variait dans le temps et dans l'espace avec

une activité décroissante des côtes vers le large et des eaux de surface vers l'océan profond (Arnosti, 2014, 2003). L'utilisation de ces substrats simples est intéressante pour un certain nombre de raisons (manipulation minimale, facilité d'utilisation...) mais elle présente également des inconvénients importants. D'une part, ces approches ne reflètent pas la complexité naturelle des polysaccharides. D'autre part, ces mesures brutes ne fournissent aucune information sur l'identité des organismes qui produisent les enzymes. Par conséquent, lorsque les taux d'activité varient, il n'est pas possible de savoir si cette variation est liée à des changements dans la composition de la communauté ou à une expression différentielle des enzymes (Arnosti, 2011).

Au cours de la décennie passée, les avancées technologiques en termes d'imagerie et de séquençage ont permis de réviser les paradigmes établis concernant la consommation des polysaccharides par les communautés naturelles. Il est apparu clairement que certains groupes bactériens (e.g. Certains membres du phylum des *Bacteroidetes*) sont spécialisés dans la consommation de polysaccharides complexes tandis que d'autres (e.g. les membres du clade SAR11) consomment des substrats de bas poids moléculaire (glucose) (Elifantz et al., 2007, 2005; Z. Zhang et al., 2015). De la même façon, des préférences en termes de substrats sont observées à l'échelle des communautés bactériennes. Le spectre de substrats qu'elles sont capables d'hydrolyser varie dans le temps et dans l'espace (Arnosti, 2011; Teeling et al., 2016, 2012). L'ensemble de ces observations suggère qu'il existe, d'une part un lien entre la structure de la communauté microbienne et leur fonction enzymatique et, d'autre part, des différences fonctionnelles entre les communautés. Les systèmes enzymatiques utilisés par ces communautés restent toutefois insuffisamment caractérisés à l'échelle moléculaire. En outre, l'existence d'une stratégie alternative d'acquisition des polysaccharides de haut poids moléculaires a récemment été mise en évidence (Reintjes et al., 2019, 2017) (**Figure 22**). En plus de la stratégie dite de « sharing » qui consiste à excréter des enzymes et générer des produits de dégradation extracellulaires directement utilisables par la communauté entière, y compris les organismes opportunistes (« scavengers », e.g. clade SAR11), une stratégie alternative qualifiée de « selfish » (égoïste) peut être mise en œuvre. Les cellules qui développent cette stratégie utilisent des enzymes associées à leur surface pour dégrader partiellement les polysaccharides de haut poids moléculaire. Les substrats sont ensuite directement transportés dans le périplasme pour une dégradation ultérieure. Ce mode de

consommation ne génère pas (ou très peu) de produits d'hydrolyse extracellulaires. Bien que découvert récemment dans le milieu marin, ce mode de dégradation et de consommation des polysaccharides serait courant (utilisé par 26% des bactéries de l'océan Atlantique (Reintjes et al., 2019)). Les organismes utilisant cette stratégie dans le milieu marin ont été identifiés comme appartenant aux *Bacteroidetes*, *Planctomycetes* et *Gammaproteobacteria*. La prévalence de l'une ou l'autre de ces stratégies semble dépendre de la composition initiale de la communauté bactérienne, et du taux de croissance de certaines bactéries. Par ailleurs, cette étude a également démontré qu'un même substrat pouvait être consommé de façon différente selon les membres d'une même communauté, avec des effets différents sur la structure de la communauté bactérienne et le cycle du carbone.

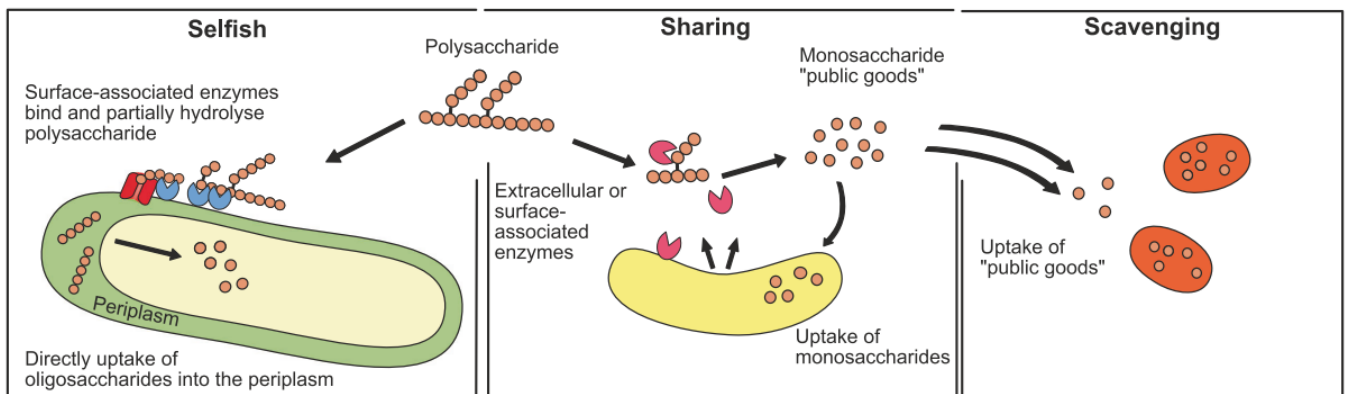


Figure 22 : Diagramme schématique reprenant les trois mécanismes d'assimilation de polysaccharide par les bactéries, réalisé par Reintjes (2019)

Pour résumer, les polysaccharides marins représentent une fraction importante de la MOD et MOP. Leur dynamique (i.e. production versus consommation) conditionne très largement le fonctionnement de la boucle microbienne et les cycles biogéochimiques globaux.

Comprendre la diversité moléculaire et fonctionnelle des dépolymérase qui catalysent la dégradation des polysaccharides est essentiel pour mieux prédire la dynamique de ces composés majeurs dans l'océan actuel et futur. Au cours de la dernière décennie, les avancées en génomique ont permis des progrès majeurs dans la compréhension de l'éventail enzymatique mis en œuvre par un organisme donné pour la dégradation de polysaccharides complexes. Toutefois, la diversité globale de ces enzymes est loin d'être caractérisée. Par exemple, les polysaccharides dépolymérase d'origine virale n'ont jamais été explorées dans

les écosystèmes marins. Compte tenu de l'ubiquité et de la prédominance des virus dans l'océan, il est possible qu'ils agissent de façon importante sur le stock de polysaccharides marins, et qu'ils impactent leur biodisponibilité pour les communautés microbiennes. Par ailleurs, nous anticipons que pour dégrader efficacement les EPS de leurs hôtes, les polysaccharide dépolymérase de virus possèdent des sites actifs adaptés à des séquences uniques de monomères. Cette spécificité pourrait être utilisée pour mieux résoudre la diversité moléculaire (très largement incomprise) et la quantification des EPS microbiens, à l'instar des enzymes de restriction permettant la reconnaissance de séquences génétiques (Roberts, 1983).

IV - CONTEXTE DE LA THESE

IV.1 - Ma position dans l'état de l'art

Les polysaccharides représentent une fraction importante de la MOD dans l'océan. Leur composition et dynamique conditionnent des processus biogéochimiques majeurs. Compte tenu de leur importance, il est aujourd'hui essentiel de comprendre les mécanismes impliqués dans leur production et leur dégradation ainsi que la régulation de ces processus par les facteurs environnementaux. Dans l'océan, la reminéralisation des polysaccharides est principalement réalisée par des communautés microbiennes hétérotrophes (bactéries). Le répertoire enzymatique utilisé par ces micro-organismes pour dépolymériser les polysaccharides et les modes d'assimilation de ces substrats restent encore mal compris. **Identifier le rôle joué par chacun des membres des communautés microbiennes participant à la dégradation de la matière organique (et des polysaccharides plus particulièrement) est crucial pour comprendre les interactions écologiques au sein de ces communautés, ainsi que les facteurs contrôlant le cycle du carbone localement et globalement.**

Les polysaccharide dépolymérases associées aux virus reçoivent une attention croissante depuis quelques années car ils offrent des perspectives biotechnologiques et médicales prometteuses. Dans le milieu marin, ces enzymes sont totalement inexplorées. **Ce manque de connaissance est surprenant car les hôtes principaux des virus marins, i.e. les bactéries et le phytoplancton, sont des producteurs importants d'EPS. Il est donc probable que les virus utilisent des polysaccharides dépolymérases pour infecter leur hôte.** La banque de données CAZY décrit en effet quelques gènes impliqués dans la dégradation des polysaccharides pour des virus marins de bactéries (*Cellulophaga*, *Synechococcus*) et phytoplancton (*Emiliania huxleyi*, *Aureococcus anophagefferens*). Le nombre de dépolymérases virales d'origine marine répertorié dans cette banque de données est toutefois anecdotique en comparaison des enzymes virales d'origine terrestre. Le contenu génétique des virus présente souvent de faibles homologues de séquence avec les banques de données disponibles. Jusqu'à 90% des séquences de métagénomés viraux marins reste sans assignation fonctionnelle (contre 67% pour les microbes) (Hurwitz and Sullivan, 2013; Krishnamurthy and Wang, 2017). Il est donc

possible que les gènes codant pour les polysaccharide dépolymérase soient génétiquement éloignés des molécules connues ayant une fonction similaire, à l'instar des polysaccharides produits par les bactéries marines dont la diversité structurale diffère de celle de leurs congénères terrestres (Chi and Fang, 2007; Kennedy and Sutherland, 1987; Kumar et al., 2007; Sutherland, 2001). Ce manque d'homologie pourrait expliquer que les gènes codant pour ces enzymes dans des génomes de virus ne soient pas détectés en utilisant les outils de BLAST classiques.

Une étude récente à laquelle j'ai participé durant mon stage de Master 2 au laboratoire (Lelchat, Mocaër et al. 2019, Annexe IV) visait à tester l'hypothèse selon laquelle les virus marins possèdent des polysaccharide dépolymérase actives sur les EPS excrétés par leurs hôtes. Pour cela nous avons utilisé 5 phages (appartenant aux familles des *Podoviridae*, *Myoviridae*, et *Siphoviridae*, largement répandues dans l'océan) qui infectent la bactérie productrice d'EPS, *Cobetia marina* DSMZ 4741 (*Gammaproteobacteria*). **Les résultats ont démontré que 4 des 5 phages possédaient des enzymes structurelles qui dépolymérisent des EPS purifiés de *Cobetia marina*.** Les profils de dépolymérisation suggèrent que ces enzymes sont constitutives, à action endolytique, et fonctionnellement diverses. Le suivi des cinétiques d'adsorption des virus sur leur hôte indique que la présence de ces enzymes fournit un avantage significatif (adsorption plus rapide) dans des conditions de production d'EPS intense. Deux phages de *C. marina*, Carin1 et Carin5, ont montré une efficacité supérieure aux autres phages pour la dépolymérisation des EPS de leur hôte bactérien (**Figure 23**).

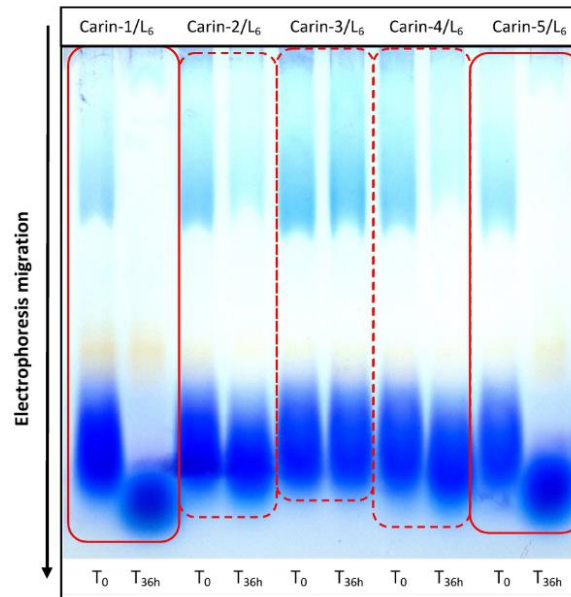


Figure 23 : Profils de dégradation des EPS de *C. marina* (L_6) mis en contact avec les 5 phages Carin1-5. Les EPS ont été incubé durant 36h (T36), puis analysé sur gel d'agarose 1% et coloré au « Stains All ». Ils sont comparés avec les échantillons à T_0 , représentant les EPS natifs. Les polysaccharides apparaissent en bleu, et les protéines en jaune. Les polysaccharides de Haut Poids Moléculaire (HMW) apparaissent en haut du gel, tandis que les polysaccharides de Bas Poids Moléculaire (LMW) se retrouvent en bas du gel. On observe au bout de 36h une dégradation des polysaccharides de haut poids moléculaire et une apparition de produits de dégradation de bas poids moléculaires

La démonstration expérimentale que certains virus marins possèdent des polysaccharidases actives sur les EPS bactériens nous amène à nous questionner sur le rôle de ces enzymes sur la régulation des activités virales dans l'océan. En effet, comme les interactions dépolymérase – EPS constitue la première étape du cycle d'infection, **il est probable que les propriétés biochimiques des dépolymérases d'un virus déterminent son spectre d'hôte et que la régulation de ces activités enzymatiques par les facteurs environnementaux influence la vélocité des taux de lyse virale, voire la perte de virulence des virus.** Par ailleurs, les polysaccharide dépolymérases ont des implications majeures dans le recyclage de la matière organique dissoute (y compris les EPS bactériens). A ce jour, ces activités enzymatiques sont essentiellement attribuées aux communautés bactériennes qui, en retour, conditionnent le fonctionnement de la boucle microbienne et ainsi les flux de matière dans l'océan. **Notre étude suggère que les virus marins pourraient également contribuer à ces processus de dégradation. Compte tenu de la prédominance des virus dans l'océan, en moyenne dix fois plus abondants que les bactéries, de telles études pourraient révéler un processus microbien important qui affecte le cycle du carbone dans l'océan.**

IV.2 - Objectifs et contenu de la thèse

L'objectif global de mon projet de thèse est de caractériser les polysaccharide dépolymérase associées aux virus marins pour mieux comprendre leur rôle écologique et biogéochimique. Pour atteindre cet objectif, nous avons fait le choix d'explorer de manière intégrative, du gène à l'écosystème, des polysaccharide dépolymérase marines issus de phages. Notre démarche se décline en 3 axes de recherche principaux :

Caractériser la diversité génétique des polysaccharide dépolymérase associées aux virus marins (chapitre I) : Comme mentionné ci-dessus, il existe un sévère manque de séquences de référence codant pour des polysaccharide dépolymérase chez les virus marins. Dans le Chapitre 1, nous faisons l'hypothèse que les polysaccharide dépolymérase associées aux virus marins sont génétiquement éloignées de celles de leurs congénères terrestres, ce qui rend leur détection difficile par les outils bio-informatiques classiques. Pour tester cette hypothèse, nous avons séquencé le génome de deux virus (phages Carin1 et Vigo2) qui infectent des bactéries marines productrices d'EPS (*Cobetia marina* et *Vibrio alginolyticus*) et pour lesquels des activités dépolymérase ont été mises en évidence expérimentalement au laboratoire. Cette étude révèle que les gènes codants pour les polysaccharide dépolymérase de ces virus n'ont jamais été décrits préalablement. La surexpression de gènes candidats a permis d'identifier avec certitude la séquence génétique responsable de ces activités pour le phage Carin1 de *Cobetia marina*. Concernant le second phage, Vigo2, la surexpression n'a pu aboutir à l'identification d'un tel gène, mais de nombreux indices portent à croire en la présence d'une séquence de polysaccharide dépolymérase au sein de son génome. Il apparaît que ces deux gènes n'ont aucune similarité avec les bases de données publiques disponibles (GenBank, EMBL, DDBJ, PDB et RefSeq). De la même manière, aucune similarité génétique n'a été trouvée dans les bases de données environnementales. Cette étude suggère que les virus marins constituent un réservoir génétique et enzymatique encore inexploré.

Elucider les propriétés fonctionnelles, structurelles et mécanistiques de ces enzymes (chapitre II) : Dans le prolongement de l'étude précédente, le Chapitre II vise à caractériser les propriétés biochimiques de l'enzyme identifiée et son architecture moléculaire. Ces analyses sont en effet essentielles pour caractériser le mode d'action des enzymes et prédire l'effet des facteurs environnementaux sur leurs activités. Nous faisons ici

l'hypothèse qu'en dépit de ses faibles homologues de séquences génétiques avec les banques de référence, la polysaccharide dépolymérase de Carin1 est structurellement proche d'autres structures viral (e.g. tailspike), ce qui pourrait faciliter leur détection dans l'environnement. La caractérisation biochimique de la polysaccharide dépolymérase de Carin1 purifiée a révélé une activité endolytique, une tolérance thermique large (4 – 60°C) avec une activité optimale à 45°C, un pH optimal à 8.5 et dans un environnement non salé. Cette enzyme présente une forte spécificité de substrat puisqu'elle ne dépolymérise que l'EPS de sa souche hôte. L'enzyme purifiée a permis la formation de cristaux pour résoudre la structure de la protéine par diffraction aux rayons X. En outre, sa structure révèle une architecture moléculaire jamais observée auparavant. Certaines régions sont cependant structurellement similaires aux tailspikes de phages terrestres, dirigeant notre interprétation du rôle de cette protéine en ce sens.

Explorer les implications des polysaccharide dépolymérases virales marines dans le cycle du carbone (Chapitre III) : La capacité de différents virus (phages) de bactéries à dépolymériser les EPS excrétés par leurs hôtes nous interroge sur leurs implications dans le recyclage de ces composés importants pour les communautés bactériennes. Il est communément admis que les bactéries marines hétérotrophes hydrolysent les polysaccharides complexes pour les assimiler. Dans le chapitre III, nous faisons l'hypothèse que les polysaccharide dépolymérases d'origine virale prennent part à la dégradation de polysaccharides dissouts, générant des produits de dégradation plus facilement assimilables par les communautés bactériennes. Pour tester cette hypothèse, nous avons cultivé des communautés bactériennes marines avec deux sources d'EPS bactériens (ceux de *C. marina* ou *V. alginolyticus*) natifs et dégradés par leur phage respectif. Le suivi de différents paramètres physicochimiques, de la croissance et de la diversité bactériennes montre que sur le court-terme (48h) la dégradation virale des EPS bactériens réduit leur biodisponibilité modifie la composition des assemblages bactériens. Cette découverte suggère un rôle insoupçonné des virus marins sur le devenir d'un pool important de matière organique dissoute marine.

IV.3 - Modèles d'études

Deux phages marins ont été utilisés durant cette thèse. Ces deux phages appartiennent à l'ordre des *Caudovirales* et à la famille des *Podoviridae* (i.e. phage à queue courte non-contractile) retrouvée en abondance dans les écosystèmes marins. Des études préliminaires (Thèse de Florian Lelchat 2020 - 2014, mon stage de Master 2) au laboratoire ont mis en évidence que ces phages sont capables de dépolymériser les EPS de leur hôte respectif.

Le phage de *Cobetia marina* Carin1 est un phage lytique isolé de la souche *Cobetia marina* DSMZ 4741. *C. marina* DSMZ 4741 a été isolée dans les eaux côtières au large de Woodshole (USA) (Cobet et al., 1971). Il s'agit d'une bactérie aérobie Gram négative et plutôt halophile (de 0.5 à 20% p/v) appartenant au phylum des *Gammaproteobacteria* (Ivanova et al., 2005, 2002; Kraiwattanapong et al., 1999). Elle est souvent associée aux macro algues et est connue pour sa capacité à former des biofilms. *C. marina* excrète de grande quantité d'EPS, qui sont d'ailleurs visibles sur milieu gélosé par la formation de colonies muqueuses (Arahal et al., 2002; Ivanova et al., 2005; Lelchat et al., 2015). La prévalence des bactéries du genre *Cobetia* apparaît corrélée à l'export de carbone dans les écosystèmes pélagiques, suggérant qu'elles contribuent activement à ce processus (Guidi et al., 2016b). Le phage Carin 1 a été isolé à la station fixe du Service d'Observation du Milieu LITtoral de Brest (SOMLIT-Brest ,4° 33' 07.19 W, 48° 21' 32.13 N). Cette station, localisée à l'interface de la rade de Brest et de la mer d'Iroise, est un système mélangé (système macrotidal) d'une profondeur de 5 à 10 m selon le marnage et est sous influence océanique. Le phage Carin1 est un *Podovirus* qui possède une capsid de 60 nm de diamètre, et une queue courte de 12 à 14 nm (**Figure 24**). La capacité de Carin1 à dégrader les EPS de son hôte a été décrite précédemment (119, voir Annexe IV), révélant la présence d'enzymes à action endolytique, et structurellement lié au virion (**Figure 24**).

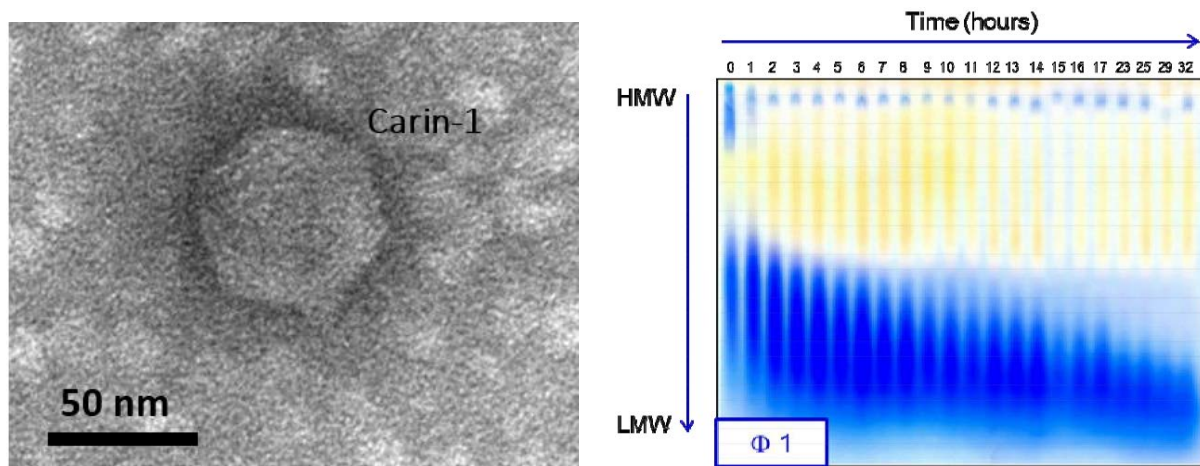


Figure 24 : A gauche, cliché en microscopie électronique à transmission du phage de *Cobetia marina* Carin1 après coloration négative à l'acétate d'uranyle. A droite, cinétique de dégradation des EPS de *C. marina* par Carin1, comme présenté **figure 23**.

Le phage de *Vibrio alginolyticus* Vigo2 a, quant à lui, été isolé sur la souche bactérienne *Vibrio alginolyticus* Va34. Cette dernière est un bacille Gram négatif ubiquiste des communautés bactériennes marines (Sasikala and Srinivasan, 2016). La bactérie *V. alginolyticus* a principalement été étudiée pour sa pathogénicité et sa prépondérance dans l'environnement marin (Pezzlo et al., 1979). Cette bactérie est également reconnue pour sa production d'EPS, formant des colonies mucoïdes sur milieu gélosé. Le phage Vigo2 a été isolé dans les mêmes conditions que Carin1 à la station d'observation SOMLIT-Brest (Thèse Florian Lelchat, 2010-2014). Le diamètre de sa capsid est de 50 nm, et sa queue mesure de 13 à 14 nm. Son activité dépolymérase a également été démontrée précédemment (**Figure 25**) (voir « Blue Biotech Goes Viral: Phages Borne Enzymes for a Better Valorization of Marine Bacterial Exopolysaccharides » et la thèse de Florian Lelchat).

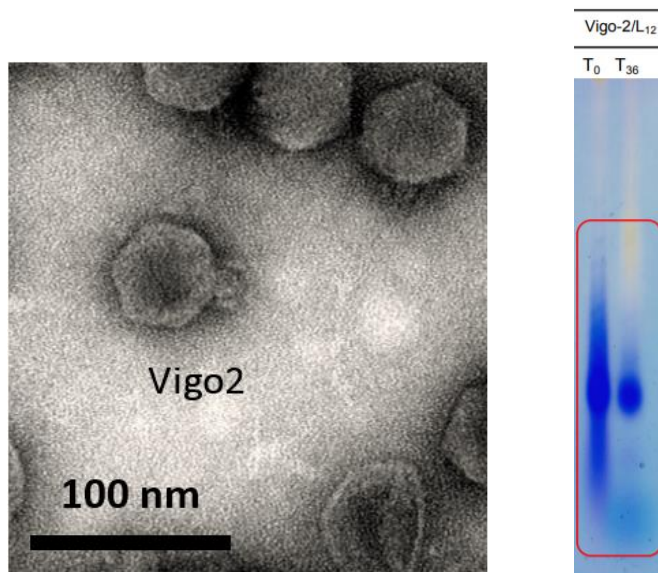


Figure 25 : A gauche, cliché en microscopie électronique à transmission du phage de *Vibrio alginolyticus* Vigo2 après coloration négative à l'acétate d'uranyle. A droite, gel d'agarose après coloration au « StainsAll » révélant la migration de l'EPS natif de *V. alginolyticus* (appelé L12 ici) et leur dégradation par Vigo2 après 36 heures d'incubation. Les polysaccharides apparaissent en bleu, tandis que les protéines apparaissent en jaune. La large population d'EPS dans la condition à T₀ disparaît une fois en présence de Vigo2. Une population reste cependant réfractaire à la dégradation. On peut noter l'apparition de produits de dégradation de bas poids moléculaires en bas du gel après 36h.

CHAPTER I

Genetic identification of polysaccharide depolymerases associated to the marine phages infecting *Cobetia marina* and *Vibrio alginolyticus*

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AUTHOR CONTRIBUTIONS

PYM and ACB devised the project, the main conceptual ideas and proof outline. The EPS production and degradation assays were performed by PYM (methods development, experiment design, optimization, data computation and analysis). PYM carried out the viral production and purification, as well as the flow cytometry viral titrations under supervision of ACB (methods and analysis). PYM conducted the genome extraction and annotation (method, data computation and analysis) under supervision of ACB. Experiments regarding the overexpression of candidate genes were made by PYM (manipulation and method development) under the supervision of RL (methods). Bioinformatics analysis were performed by PYM (genome annotation, gene recruitment), CB (methods, environmental database analysis, supervision) and GM (CAZy database analysis). PYM wrote the manuscript with support of ACB.

INTRODUCTION

Phages (viruses that infect bacteria) represent the most abundant biological entities on Earth. Phage infection contributes to the regulation of microbial population, promotes genetic exchanges among organisms and drives major biogeochemical processes (Clokic et al., 2011; Jiang and Paul, 1998; Suttle, 2007). Phage infection is initiated by the recognition and the adsorption of the virion on the specific bacterial receptors of the host cell. As defence mechanism, the bacterial host can produce structured extracellular polymers (capsular polysaccharides CPS or exopolysaccharides EPS) that provide a physical barrier between the phage and its host receptors. Some phages have evolved polysaccharide depolymerases to specifically recognize, bind, and degrade those polymers (Casjens and Molineux, 2012; Drulis-Kawa et al., 2015; Sutherland et al., 2004). The interaction between the virion's enzymes and the host EPS is considered as one of the first recognition event of the infection cycle. The chemical structure of bacterial EPS varies greatly between bacterial species, but it also depends on the environmental conditions and bacterial growth phase (Corbett et al., 2010; Leiman and Molineux, 2008; Roberts, 1996). EPS display homopolymeric or heteropolymeric composition, linear or branched backbones. Organic or inorganic substituents are also frequently observed on their structures (Freitas et al., 2011). We can thus expect a similarly high or even greater diversity of phage polysaccharide depolymerase. (Delbarre-Ladrat et al., 2014; Guo et al., 2017; Gutiérrez et al., 2015; Hernandez-Morales et al., 2018; Latka et al., 2017; Liu et al., 2019; Majkowska-Skrobek et al., 2016; Mi et al., 2019; Pires et al., 2016; Shang et al., 2015; Yan et al., 2014). The genetic and functional diversity of these enzymes is however not yet fully explored.

In the ocean, there is an estimated 10^{29} infection events that are likely to occur every day leading to the daily demise of up to 40% of the bacterial stock and to the release of one of the largest flux of dissolved organic matter (Breitbart et al., 2007; Suttle, 2007, 2005). Despite the prominent role of virus in the sea, the mechanisms that regulate virus activities are poorly understood. What are the mechanisms involved in virus-host interactions? What are the determinants of virus specificity? How infection processes respond to environmental changes? These fundamental questions are of great relevance for ocean ecology and biogeochemistry; yet, they remain largely unsolved.

Almost all intact or metabolically active marine bacteria exhibit a capsule of polysaccharides that they constantly renew by releasing capsular compounds into the ambient water (Heissenberger et al., 1996; Stoderegger and Herndl, 1998, 1999). They are also known to extensively produce EPS biofilm matrixes for attachment, colonization of immersed surface, or to form aggregates in the water column (Kumar et al., 2007; Nwodo et al., 2012; Orellana and Leck, 2014; Sutherland, 2001). It is thus likely that marine phages use polysaccharide depolymerases to infect their hosts. This hypothesis is consistent with the recurrent observation of opaque halo zone that surrounds phage plaques (Duhaime et al., 2011; Moebus, 1997a, 1997b). Recently, we also provided an experimental demonstration of EPS-degradation activity associated to 4 out of 5 phages that infect the marine bacteria *Cobetia marina* (F. Lelchat et al., 2019). The results of this study suggested that the putative polysaccharide depolymerases were constitutive, endo-acting and, functionally diverse. Viral adsorption kinetics further indicated that the presence of these enzymes provided a significant advantage for phages to adsorb onto their hosts upon intense EPS production conditions. Altogether, these findings suggest that polysaccharide depolymerases are common in marine phages. More detailed genetic and functional studies of these molecules may provide novel fundamental knowledge about the infection process of phages in marine environments. The aim of this study was to identify polysaccharide depolymerases associated to marine phages (*Cobetia marina* phage Carin1 and *Vibrio alginolyticus* phage Vigo2, (F. Lelchat et al., 2019; Florian Lelchat et al., 2019) of ecological interest and for which depolymerase activities was demonstrated empirically in previous studies. Therefore, we have applied a combination of bioinformatics and targeted activity screening. Results from this study suggest that polysaccharide depolymerase associated to these phages probably constitute novel enzyme families and puts forward that marine viruses may represent an unappreciated reservoir of novel depolymerases.

MATERIAL AND METHODS

Bacterial host culture

The bacterial host strains used for this study were *Cobetia marina* DSMZ 4741 and *Vibrio alginolyticus strain Va34*. The bacterial strains were cryo-conserved at -80°C in a 17% glycerol solution. Before experiment, cultures were streaked on a solid autoclaved ZoBell medium (5g peptone, 1g yeast extract, 15g agar diluted in 1L of $0.2\mu\text{m}$ filtered seawater). One day prior to the experiments, a single colony was picked from the solid medium and transferred into autoclaved ZoBell Broth (5g Peptone, 1g Yeast extract in 1L of $0.2\mu\text{m}$ filtered seawater). The culture was incubated overnight at room temperature (approx. 20°C) under vigorous agitation (180 rpm) on a rotary table.

Bacterial EPS production

The production of exopolysaccharide (EPS) was achieved by transferring a single colony in 10 mL of culture medium (ZoBell Broth) supplemented by glucose at 3% (w/v). The 10 mL pre-culture was incubated overnight at 25°C and it was transferred to 500 mL of fresh culture medium supplemented by glucose at 3% (w/v) and maintained at 25°C under vigorous agitation (150 rpm) for 72 hours. Then, cultures were centrifuged at $7,000g$ during 20 minutes to get rid of cells. The supernatants were collected and filtered through $0.45\mu\text{m}$ and $0.2\mu\text{m}$ and concentrated through tangential filtration using a PES vivaflow cartridge with a cut-off of 30 kDa. The concentrated suspension was diluted in 5 volumes of 500 mL MilliQ and further concentrated to remove salts. A 100 mL concentrate was frozen at -80°C overnight and lyophilized (**Picture of lyophilized EPS in Annexes I.1**).

Virus culture

Stock solutions of viral cultures were kept at 4°C in the dark in SM Buffer (NaCl 100 mM, $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ 8 mM, Tris-CL 50 mM, MilliQ water, pH 7.5). Prior to the experiments, the infectivity of the phage suspension was assessed by spot test. Therefore, an aliquot of the stock viral suspension was serially diluted in SM buffer and $5\mu\text{L}$ drop of each dilution was spotted on a host lawn obtained by plating a 1:4 mixture of host culture in molten agar (ZoBell

Broth completed with 0.6 % (w/v) noble agar) onto a Zobell Agar plate. The plate was incubated overnight at room temperature and translucent spots were enumerated to compute the titer of the viral suspension. Large volume of viral cultures was obtained by infecting an appropriate volume of well growing host culture at 1.10^7 cell mL⁻¹ (final concentration) with 1.10^6 infectious virus mL⁻¹ (final concentration) based on the spot assay conducted previously. The mixture was incubated at room temperature under low agitation (60 rpm) on a rotary table for 18 hours. The infection was stopped by centrifugation at 7000g for 30 minutes (Eppendorf 5810R) to remove non-infected host cells. The supernatant was collected and filtered through 0.2 µm polyethylsulfone (PES) filters (Filtration unit Stericup GP Millipore).

Virus purification

The 300mL viral suspension were concentrated by tangential flow filtration using a Vivaflow 200 cartridge (Sartorius Stedim) made of polyethylsulfone (PES) with a 100 kDa cut-off to a volume of 50 mL. Further concentrations were done using a Vivaspin20 centrifugal concentrators (Sartorius Stedim) made of PES with a cut-off of 50 kDa. Repeated runs of centrifugation (8,000g, 15 minutes) were performed until the sample reached a final volume of 1-2 mL. The viral concentrate was then purified on a linear 10-40% sucrose gradient by ultracentrifugation using a SW41Ti rotor at 134074g during 45 minutes; 4°C (Optima XPN80, Beckman). The viral bands were extracted with a 1 mL syringe and washed three times with 15 mL of SM buffer in a 50 kDa PES vivaspin column to remove traces of sucrose. In order to avoid potential contamination with small cellular compounds such as vesicles, an additional chloroform cleaning step was performed. A 500 µL aliquot of each purified virus was vigorously mixed with 250µL chloroform and incubated 30 minutes at room temperature. The mixture was centrifuged for 5 minutes at 4,000g and the aqueous phase was collected and incubated overnight lid open under chemical hood to remove traces of chloroform. The treated sample was purified on a 10-40% sucrose gradient by ultracentrifugation and cleaned with a Vivaspin concentrator as described above.

Bacterial and viral abundance by flow cytometry

The viral and bacterial samples were fixed in 0.5% glutaraldehyde (final concentration) for 15 minutes at 4°C, flash frozen in liquid nitrogen and stored at -80°C until analysis. For flow cytometry analysis, the sample was thawed at 37°C and diluted 100 to 100,000-fold in autoclaved and 0.2µm filtered a TE buffer (Tris-HCl 10 mM, EDTA 1 mM, pH 8.0). Dilutions were stained with the nucleic acid dye SyBRGreen (Life Technologies, 20000 dilution of the commercial stock,). The samples were incubated 15 minutes in the dark at room temperature for the bacterial counts and 10 minutes in the dark at 80°C for the viral counts. After cooling, the samples were analysed using a FACSCanto II equipped with an argon-laser (455 nm). The trigger was set on the green fluorescence and the sample was delivered at a rate of 0.06 mL/min and analysed for 1 min. Virus and bacterial counts were corrected for a blank consisting of TE-buffer with autoclaved 0.2 µm filtered seawater at the appropriate dilution.

Viral genome extraction, sequencing, and annotation

The genome was extracted using a DNeasy Blood & Tissue Kit from Quiagen© and 10 µg of DNA was sent to the GATC-Biotech for sequencing. GATC-biotech used the PacBio RS II technology and conducted genome assembly using HGAP software.

The softwares Glimmer 3 (Delcher et al., 1999) and GenemarkS (Besemer and Borodovsky, 2005) were used to predict the open reading frames (ORF). For each, the Shine-Dalgarno score was evaluated, as well as the presence of Ribosome Binding Site and transcription factor sequences. The predicted ORFs which were smaller than 120 bp were suppressed and each overlapping ORFs was manually checked. The intergenic areas were manually studied. The sequences of these ORFs were compared with BLASTP to the non-redundant protein database of the NCBI (July 2019). The sequences with high scores and expect values lower than 10^{-5} were considered as hypothetical proteins for annotations. PSI-Blast was used to refine the hypothetical functions. The tools tRNAScanSE (Lowe and Eddy, 1997) and Aragorn (Laslett and Canback, 2004) were used to find out the presence of putative tRNA in the viral genomes. The final graphic and annotations were done with the software Geneious version 9.1.3 (<http://www.geneious.com>, (Kearse et al., 2012)). Sequences of polysaccharide depolymerase were compared with blast to *Tara Ocean* metagenomes (OM-RGC_v1). Only sequences with expect values lower than 10^{-35} were considered.

Over-expression of polysaccharide depolymerases candidate genes

Strains, plasmids and culture conditions

During this experiment, we used a pFO4 plasmid (a vector modified from pET15b (Novagen, USA) to be compatible with the BamHI/EcoRI ligation strategy). That plasmid generates a hexahistidine tail at the N-terminal of recombinant protein and contains an ampicillin resistance gene, a LacI gene and as well as a T7 promoter. *Escherichia coli* DH5 α strain was used for standard cloning procedures, and BL21 (DE3) *E. coli* strain was used for gene expression experiments. Both were grown in Lysogeny Broth (LB) liquid or on LB solid medium, as described by Sambrook et al. in 1989 (Maniatis et al., 1989). For expression tests, an auto-inducible ZYP5052 medium (Studier, 2005) was used. All media were supplemented, when necessary, with 100 $\mu\text{g ml}^{-1}$ ampicillin (sodium salt).

Bioinformatics analysis of the target sequences

Genes were selected based on their similarities with fibre, tail or spike protein domains, but also their location on the viral genome sequence (proximity with structural genes and/or lysozyme-like enzymes). For each selected gene, potential signal peptides and transmembrane domains have been predicted using SignalP 5.0 (Almagro Armenteros et al., 2019). The modularity of each targeted protein has been examined using Blast queries against UniProt database and precise delineation of each module has been refined using Hydrophobic Cluster Analysis (HCA) (Gaboriaud et al., 1987). Protein disorder was also examined using MeDor (Lieutaud et al., 2008). For this study, 24 genes or fragment were chosen for Carin1 phage, and 23 genes/modules were selected for Vigo2. Predicted masses ranged between 13 and 127 kDa. The sequences were also compared to the CAZY database (Lombard et al., 2014). Genes were subjected to multiple sequence alignment using MAFFT (Katoh et al., 2002) with the iterative refinement method and the scoring matrix Blosum62.

Primers design and cloning method

Expression vectors (pFO4) were digested either by BamHI/EcoRI (or XhoI/NsiI). For each targeted sequence, restriction sites recognized by BamHI, EcoRI or their isocaudomers (respectively BglII and MfeI) were sought using Geneious software (or XhoI, NsiI / Sall and PstI). The standard scheme for primer design was defined as: for the forward primers, 5'-[hexa-A tail]-[BamHI or BglII]-[Start codon ATG if necessary]-[Hybridization site]-3' and for the

reverse primers, 5'-[hexa-A tail]-[EcoRI or MfeI]-[stop anticodon]-[Hybridization site]-3'. Oligonucleotides for PCR were purchased at Operon Biotechnologies GmbH (Cologne, Germany). PCR amplification was performed on a GeneAmpR PCR System 2700 (Applied Biosystems, USA). The thermocycle utilized was: denaturation at 95°C for 5 min and thirty cycles of denaturing at 95°C for 30 s, annealing at 50°C for 30 s and polymerization at 72°C for 4 min. Template amplification was performed with Q5 polymerase (NEB) and used with the conditions recommended by the supplier. PCR reactions were analysed on 1% agarose gels using standard procedures (Groisillier et al., 2010). The resulting PCR products were purified using the QIAquick™ PCR Purification Kit (QIAGEN, USA), digested with appropriate restriction enzymes and cloned in parallel into the pFO4 expression vector using standard procedures (Groisillier et al., 2010). PCR-screening was performed directly on the DH5α bacterial colonies to verify clones with inserts on expected size, using PCR primers which annealed upstream and downstream of the insertion site of pFO4 and with the same program described above. Plasmid extraction was performed using MiniPrep SV purification Kit (PROMEGA, USA) and recombinant plasmids were used to transform *E. coli* BL21(DE3) expression strain.

Screening for protein expression

Cultures of *E. coli* clones, for which the presence of the expression gene was verified by colony PCR as described previously, were tested for the expression of the desired protein. Screening was done using 2 mL cultures in 24-deep well plates. Cultivation was performed in two phases. First, transformed colonies were grown at 37°C overnight in LB medium containing 100 µg ml⁻¹ ampicillin. Then, cultures were diluted 1:100 with auto-inducible ZYP5052 medium (Studier, 2005) containing 100 µg ml⁻¹ ampicillin and subjected to further incubation at 20°C during 72 hours. Centrifugation at 2,000 rpm for 10 min allowed the recovering of cells that were frozen or used as it is.

Lysis of cells and detection of proteins

For solubility assay, cell pellets from previous cultures were resuspended in 500 µl of lysis buffer (Tris-HCl 50 mM, pH 7.5; NaCl 250 mM; EDTA 1 mM; lysozyme 1 mg ml⁻¹; DNase 0.1 mg ml⁻¹) and incubated at 18°C for 1 hour. The soluble and insoluble fractions were then

separated by centrifugation (12,000 g, 20 min, 4°C). Insoluble pellets were resuspended in 200 µl of lysis buffer and preserved at 4°C. In parallel, soluble fractions were purified using His Microspin columns (GE Healthcare Life Science, USA) according to the protocol recommended by the supplier. Samples from soluble, insoluble and purified fractions were separated by 12% sodium dodecyl-sulphate polyacrylamide gel electrophoresis (SDS-PAGE) using 12% Criterion precast Bis-Tris gels with 26 wells. Targets were scored as positive for expression and solubility if a detectable fusion protein of the correct molecular weight was observed after Coomassie Blue-staining.

Enzyme purification

Among cultures where overexpressed enzymes were soluble, cell pellets were thawed and resuspended in 50mM NaPi with 300mM NaCl (pH8.0) and lysed by three cycles of sonication (2.0min, 30% pulse, 50% power). After centrifugation (10,000g, 4 °C, 20min), the supernatant was filtered (0.45 µm) and loaded onto a 5ml HisTrap FF crude column (GE Healthcare) equilibrated with lysis buffer. After washing, the protein was eluted with 50mM NaPi and 300mM NaCl containing 300mM imidazole (pH8.0). Fractions containing the protein of interest were pooled and desalted using PD-10 columns (GE Healthcare) equilibrated with 50mM NaPi pH7.4.

Depolymerase activity screening

Bacterial exopolysaccharides (0.18 % w/v, final concentration) were incubated with purified virus suspension (1.10^9 infectious virus /mL, final concentration) or with enzymes (undiluted or 1:5 diluted) in a final volume of 500µL in triplicate. The samples were incubated at room temperature for 72 hours, under a gentle agitation. Samples were inactivated at 99°C for 5 minutes and frozen at -80°C until analysis. With enzymes, a LamA control was used in order to exclude the presence of *E. coli* enzyme that could degrade the EPS used.

EPS depolymerization was then detected using agarose gel electrophoresis. The samples were diluted in a loading buffer 4X (1 mL Tris-HCl 0.5M pH 6.8, 1.6 mL of Glycerol 100%, 5 mL of MilliQ water, 80 µL of EDTA 0.5M and 0.4 mL of Bromophenol blue 0.5%) and they were loaded on a 1% agarose gel prepared in TAE 0.5X buffer (TRIS 20mM pH 7.6, Acetic Acid 20mM, EDTA 0.5mM). The electrophoresis was run at 100V during 75 minutes in TAE 0.5X buffer. The gel

was stained using a “Stains All” solution (1-Ethyl-2-[3-(1-ethylnaphtho[1,2-d]thiazolin-2-ylidene)-2-methylpropenyl]naphtho[1,2-d]thiazolium bromide, Sigma) prepared as follow: A 0.1% (w/v) stock solution was prepared in N,N-dimethylformamide. A 10 mL of Stains-All stock solution was mixed to 10mL of N,N-dimethylformamide, 50mL of isopropanol, 10 mL of Tris-HCl 300 mM pH 8.8 and 120 mL of MilliQ water and the gel was transferred into this freshly prepared staining solution and incubated overnight in darkness. The gel was washed for 2 hours in MilliQ water under day light until visualization of the degradation products. The polysaccharide fractions appeared in blue and the protein fractions in yellow/brown. Nucleic acids appeared in purple. Non-treated EPS, EPS without viruses, and viruses without EPS were also used as control.

RESULTS

Viral depolymerase screening

The ability of viruses to depolymerize their host EPS was visualized by agarose gel electrophoresis after Stains All staining (**Figure 26**). The migration of native *C. marina* EPS (host of Carin1), showed two populations with distinct molecular weight (**Figure 26**). The incubation of *C. marina* EPS with purified Carin1 particles resulted a nearly complete depolymerization of the population of high molecular weight (HMW) EPS into products of low molecular weight (LMW). Regarding *V. alginolyticus* EPS (host of Vigo2), the native EPS are characterized by 3 polysaccharides populations (**Figure 26**). After incubation with purified Vigo2, the polysaccharide of higher molecular weight faded, but the intermediate population appeared resistant to viral degradation.

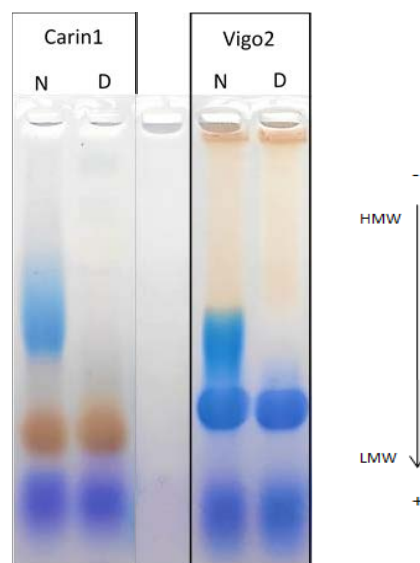


Figure 26: Depolymerase activity screening using agarose gel electrophoresis. Polysaccharides appear in blue, whereas proteins are stained in yellow. N = Native control EPS, D = Degraded with viruses

Genomes, annotations and gene selection

- *Carin1 genome*

Carin1 genome consisted of double stranded linear DNA sequence of 40,505 base pairs (bp) with an average GC content of 60.5%. The GC content did not vary significantly along the genome sequence. The coding density of the genome is 96.5%, with a total of 49 predicted ORFs that were mainly distributed on the reverse strand (43). Of the 49 ORFs, 36 ORFs had significant similarities with genes in public database and a putative function could be assigned to 14 of them. Gene similarities showed that Carin1 genome may be related to the Lessievirus genera. Also, two genes showed similarity to *Pseudomonas putida* phage AF (A hypothetical protein and a putative lysozyme), a phage showing similarities with Uetakevirus and Rauchvirus genera.

Most of Carin1 genes encoding protein with robust functional homologues in GenBank are involved in DNA metabolism. We identified 6 core genes responsible for DNA replication and repair including a replicase (orf2), a recombinase (orf15), a nuclease (orf18), an exonuclease (orf20) and a recombinase (orf21). We also identified a methionine associated tRNA sequence. Other conserved ORFs are those associated with the virion structure, with a phage tail protein (orf46), a major capsid protein (orf43) and a terminase (orf49). Other identifications include proteins cell lysis genes as a pyocine (orf3), a lysin (orf28), a lysozyme (orf33) and a peptidase (orf44) (**Figure 27 and Table 3**).

None of the predicted ORFs had homolog encoding polysaccharide depolymerase in GenBank. We search for putative polysaccharide depolymerase in Carin1 genome using expert database CAZY and we found that orf31 had distant homologs (e-value = $1e^{-5}$) that belonged to the glycoside hydrolase family 28 (GH28). The GH28 family comprised polygalacturonase (EC 3.2.1.15); α -L-rhamnosidase (EC 3.2.1.40); exo-polygalacturonase (EC 3.2.1.67); exo-polygalacturonosidase (EC 3.2.1.82); rhamnogalacturonase (EC 3.2.1.171); rhamnogalacturonan α -1,2-galacturonohydrolase (EC 3.2.1.173); xylogalacturonan hydrolase (EC 3.2.1.-). Additionally, we found a synteny between Carin1 and with *Pseudomonas* phage AF from orf25 to orf38 (**Figure 28**). Despite weak similarity between their genetic sequence (except the lysozyme and orf32 of Carin1), the gene order is conserved in this sequence fragment, which interestingly include a polysaccharide depolymerase (phage AF gp19

protein). Location of the polysaccharide depolymerase on the phage AF genome corresponds to the location of orf31 in Carin1.

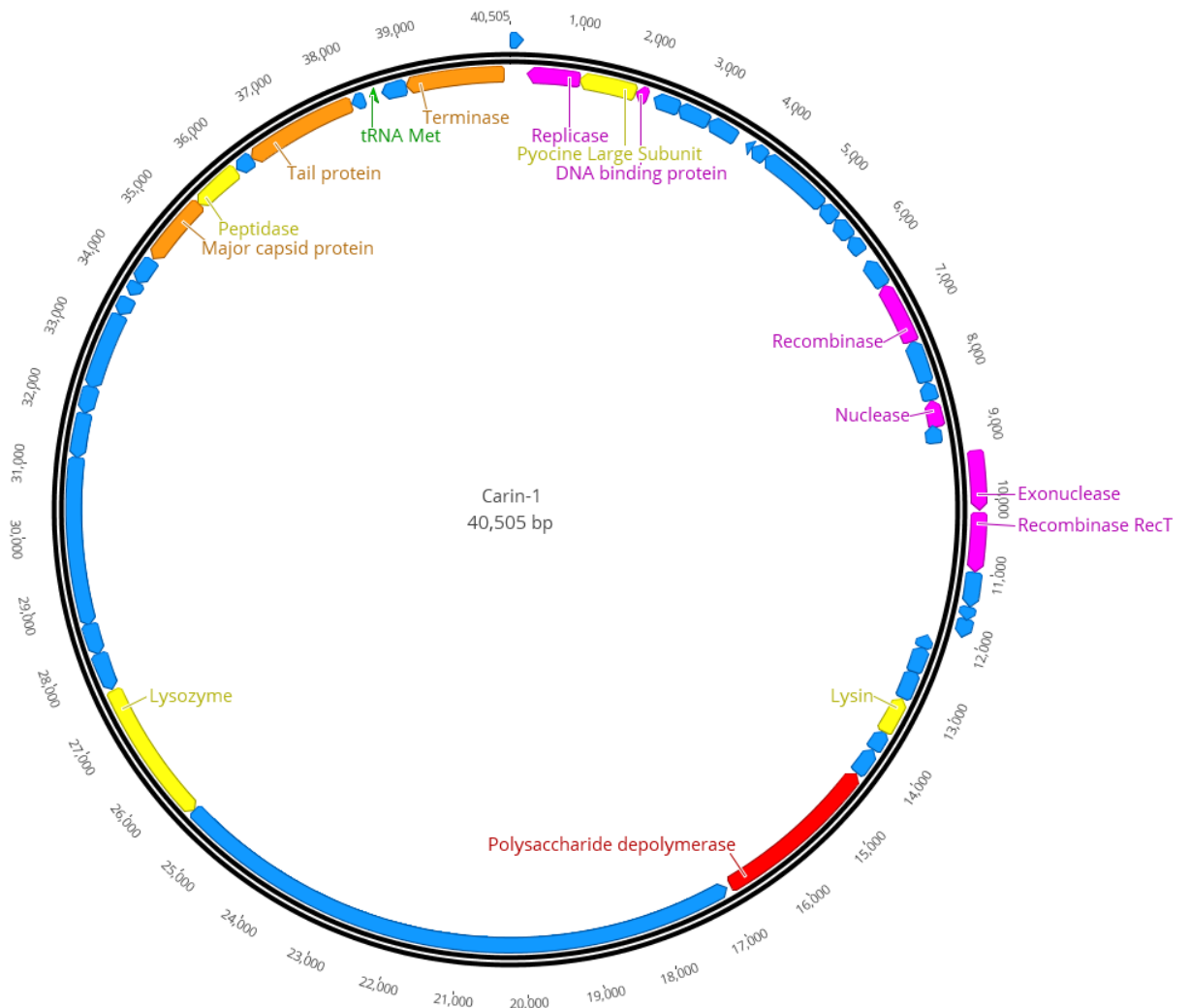


Figure 27: Carin1 genome annotated. Hypothetical proteins are in blue, DNA replication and recombination are in magenta, cell lysis genes are in yellow, and structural genes are in orange. The tRNA appear in green, and the identified Polysaccharide depolymerase appears in red

Table 3: Carin1 genome annotation

Orf	Annotation	E-value	Start	End	Length	Viral homologs
Carin-1_orf01	Hypothetical protein	none	1	187	187	
Carin-1_orf02	DNA replication protein DnaC [Vibrio phages]	8.00E-40	261	1043	783	
Carin-1_orf03	Pyocine Large Subunit [Pseudomonas bauzanensis]	2.00E-53	1040	1891	852	
Carin-1_orf04	DNA binding protein	1.00E-09	1891	2085	195	
Carin-1_orf05	Hypothetical protein [Halomonas sp.]	9.00E-15	2177	2572	396	
Carin-1_orf06	Hypothetical protein [Halomonas sp.]	1.00E-04	2556	3056	501	
Carin-1_orf07	Hypothetical protein	none	3053	3523	471	
Carin-1_orf08	Hypothetical protein	none	3693	3809	117	
Carin-1_orf09	Hypothetical protein	none	3809	4048	240	
Carin-1_orf10	Hypothetical phage protein	4.00E-45	4058	5143	1086	
Carin-1_orf11	Hypothetical protein	none	5140	5427	288	
Carin-1_orf12	Hypothetical protein	none	5450	5767	318	
Carin-1_orf13	Hypothetical protein [Halomonas sp.]	1.00E-06	5769	6047	279	
Carin-1_orf14	Hypothetical protein [Halomonas sp.]	6.00E-16	6217	6639	423	
Carin-1_orf15	Recombination-associated protein Rdc [Cobetia marina]	7.00E-45	6642	7577	936	
Carin-1_orf16	Hypothetical protein [Pseudomonas phage KPP25]	1.00E-13	7577	8215	639	
Carin-1_orf17	Hypothetical protein [Vibrio phage 1.127.O._10N.286.52.E12]	7.00E-09	8212	8487	276	
Carin-1_orf18	hypothetical protein [Burkholderia virus DC1], Nuclease	5.00E-32	8496	8897	402	BceP22like
Carin-1_orf19	Hypothetical protein	none	8869	9132	264	
Carin-1_orf20	Exodeoxyribonuclease VIII [Proteus phage vB_PvuS_Pm34]	6.00E-89	9310	10134	825	
Carin-1_orf21	Recombinase RecT	1.00E-56	10161	10985	825	
Carin-1_orf22	hypothetical protein [Pseudomonas phage]	5.00E-47	10982	11470	489	
Carin-1_orf23	Hypothetical protein	none	11467	11655	189	
Carin-1_orf24	Hypothetical protein	none	11645	11908	264	
Carin-1_orf25	Hypothetical protein [Halomonas sp.]	1.00E-12	12007	12192	186	
Carin-1_orf26	Hypothetical protein [Synechococcus phage S-CBP2]	1.00E-14	12189	12581	393	
Carin-1_orf27	Hypothetical protein [Prokaryotic dsDNA virus sp.]	2.00E-17	12565	12999	435	
Carin-1_orf28	Putative N-acetylmuramoyl-L-alanine amidase [Halomonas phage QHHSV-1]	9.00E-55	13030	13584	555	T4-like
Carin-1_orf29	Hypothetical protein	none	13565	13897	333	
Carin-1_orf30	Hypothetical protein	none	13894	14316	423	
Carin-1_orf31	Polysaccharide depolymerase	identified	14332	16818	2487	
Carin-1_orf32	Hypothetical protein [Pseudomonas putida phage AF]	0	16901	25474	8574	
Carin-1_orf33	Putative structural lysozyme [Pseudomonas phage AF]	4.00E-54	25474	27633	2160	
Carin-1_orf34	Hypothetical protein [Halomonas xianhensis]	9.00E-15	27646	28203	558	
Carin-1_orf35	Hypothetical protein [Halomonas xianhensis]	6.00E-62	28207	28668	462	
Carin-1_orf36	Hypothetical protein [Halomonas xianhensis]	0.00E+00	28655	31147	2493	
Carin-1_orf37	Hypothetical protein [Halomonas xianhensis]	1.00E-60	31147	31815	669	
Carin-1_orf38	Hypothetical protein [Idiomarinaceae bacterium]	3.00E-38	31819	32217	399	
Carin-1_orf39	Hypothetical protein [Vibrio phage vB_VmeM-32]	2.00E-24	32214	33368	1155	T4-like
Carin-1_orf40	Hypothetical protein	none	33368	33646	279	
Carin-1_orf41	Hypothetical protein [Halomonas xianhensis]	3.00E-13	33692	33907	216	
Carin-1_orf42	Hypothetical protein [Halomonas xianhensis]	1.00E-39	33919	34311	393	
Carin-1_orf43	Major capsid protein [Vibrio phage 1.021.A._10N.222.51.F9]	2.00E-121	34350	35384	1035	
Carin-1_orf44	Peptidase [Halomonas xianhensis]	1.00E-59	35396	36136	741	
Carin-1_orf45	Hypothetical protein [Halomonas xianhensis]	5.00E-08	36142	36435	294	
Carin-1_orf46	Phage tail protein	1.00E-177	36432	38102	1671	
Carin-1_orf47	Hypothetical protein	none	38118	38327	210	
Carin-1_orf48	Hypothetical protein [Pseudomonas putida]	4.00E-52	38584	38952	369	
Carin-1_orf49	Terminase	0	38956	40425	1470	



Figure 28: Similarities in gene order between *Carin1* and *Pseudomonas phage AF*. Only the lysozyme (*orf33*) and the long hypothetical protein (*orf32*) of *Carin1* showed sequence similarities with genes from *phage AF*.

- *Vigo2* genome

Vigo2 has a double stranded genome sequence of 35,145 base pairs, which displays an average GC content of 44.4% with no significant variations along the sequence. The coding density of *Vigo2* genome sequence is 89% with a total of 45 predicted ORFs. All except 2 ORFs are distributed in the same direction. 27 ORFs do not have significant similarities with genetic sequence in public databases (71%). 12 ORFs showed homology with known phage proteins, which some are related to Lessievirus and Enquatrovirus genera.

Most of *Vigo2* genes encoding protein with robust functional homologs in GenBank are involved in the structure of the phage. We identified a tail fibre proteins (*orf2*), two tail proteins (*orf31* and *orf34*), two unknown structural proteins (*orf21* and *orf32*), a major capsid protein (*orf36*), a head-tail connector (*orf39*) and a terminase (*orf42*). We also identified two genes encoding proteins involved in DNA/nucleic acid metabolism (*orf12*, an endonuclease and *orf44*, a putative RNA binding protein), and a lysozyme (*orf16*). (**Figure 29 and Table 4**).

There were no significant hits of *Vigo-2* genes to known polysaccharide depolymerase in the expert database CAZY. However, *orf20*, which is located near a structural tail proteins (*orf21*), showed distant similarities with putative pectate lyase of *Serratia rubidaea*. Moreover, prediction of structure showed that *orf20* may display a three β -helixes the C-terminal extremity, which is a feature of phage polysaccharide depolymerases.

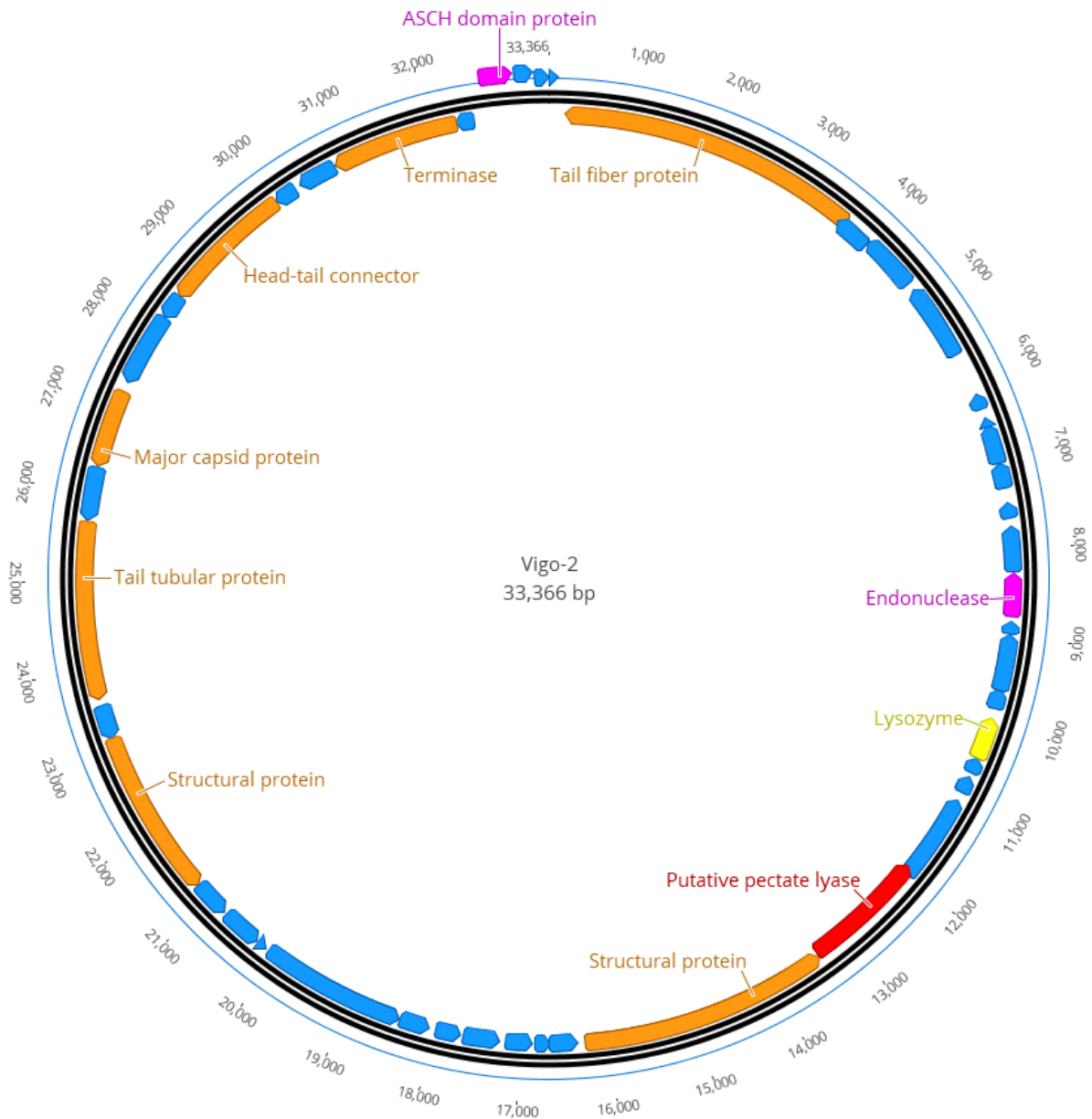


Figure 29: Annotated genome of Vigo2. Hypothetical proteins are in blue, DNA replication and recombination are in magenta, cell lysis genes are in yellow, and structural genes are in orange. The putative Polysaccharide depolymerase appears in red

Table 4 : Gene annotation of *Vigo2* genome

Orf	Annotation	E-value	Start	End	Lenght	Viral homologs
Vigo-2_01	Hypothetical protein	None	33223	108	252	
Vigo-2_02	Putative Tail fibre protein [Vibrio phages]	3.00E-76	195	3698	3504	
Vigo-2_03	Hypothetical protein	None	3610	4038	429	
Vigo-2_04	Hypothetical protein	None	4025	4696	672	
Vigo-2_05	Hypothetical protein	None	4782	5678	897	
Vigo-2_06	Hypothetical protein	None	6175	6351	177	
Vigo-2_07	Hypothetical protein	None	6453	6566	114	
Vigo-2_08	Hypothetical protein	None	6557	7000	444	
Vigo-2_09	Hypothetical protein	None	17002	7295	294	
Vigo-2_10	Hypothetical protein	None	7464	7649	186	
Vigo-2_11	Hypothetical protein	None	7739	8257	519	
Vigo-2_12	Endonuclease [Stenotrophomonas phage vB_SmaS-DLP_1]	2.00E-12	8268	8777	510	Bcep22like
Vigo-2_13	Hypothetical protein	None	8842	8976	135	
Vigo-2_14	Hypothetical protein	None	8973	9623	651	
Vigo-2_15	Hypothetical protein	None	9620	9847	228	
Vigo-2_16	Lysozyme [Vibrio phages]	3.00E-29	9966	10448	483	
Vigo-2_17	Hypothetical protein	None	10448	10627	180	
Vigo-2_18	Hypothetical protein	None	10672	10875	204	
Vigo-2_19	Hypothetical protein [Prokaryotic dsDNA virus sp.]	3.00E-07	10971	12005	1035	
Vigo-2_20	Hyp. Prot. [Vibrio phage VBP47] / Putative lyase [S. rubidaea]	2.00E-05	11921	13381	1461	N4-like
Vigo-2_21	Structural protein [uncultured Mediterranean phage uvMED]	4.00E-80	13393	16257	2865	
Vigo-2_22	Hypothetical protein [Vibrio cholerae]	4.00E-29	16352	16669	318	
Vigo-2_23	Hypothetical protein	None	16666	16833	168	
Vigo-2_24	Hypothetical protein	None	16862	17179	318	
Vigo-2_25	Hypothetical protein [Vibrio cholerae]	4.00E-12	17246	17665	420	
Vigo-2_26	Hypothetical protein	None	17694	17990	297	
Vigo-2_27	hypothetical protein [Vibrio phage phi 2]	8.00E-14	18064	18414	351	
Vigo-2_28	Hypothetical protein	None	18419	20113	1695	
Vigo-2_29	Hypothetical protein	None	20146	20265	120	
Vigo-2_30	Hypothetical protein [Vibrio phage CKB-S2]	2.00E-31	20275	20748	474	unclassified
Vigo-2_31	Hypothetical protein [Vibrio phages]	1.00E-24	20794	21207	414	
Vigo-2_32	Structural protein [Vibrio phage phiVC8]	6.00E-24	21217	23163	1947	Lambdalike
Vigo-2_33	Hypothetical protein	None	23166	23567	402	
Vigo-2_34	Tail tubular protein B [uncultured Mediterranean phage uvMED]	1.00E-59	23632	25692	2061	
Vigo-2_35	Hypothetical protein [Candidatus Pacearchaeota archaeon]	7.00E-27	25702	26313	612	
Vigo-2_36	Major capsid protein [uncultured Mediterranean phage uvMED]	4.00E-44	26322	27227	906	
Vigo-2_37	Hypothetical protein	None	27354	28202	849	
Vigo-2_38	Hypothetical protein	None	28207	28500	294	
Vigo-2_39	Head-tail connector protein [uncultured Mediterranean phage uvMED]	1.00E-54	28501	30042	1542	
Vigo-2_40	Hypothetical protein	None	30042	30320	279	
Vigo-2_41	Hypothetical protein	None	30375	30830	456	
Vigo-2_42	Terminase [Vibrio phage CKB-S2]	3.00E-108	30833	32308	1476	unclassified
Vigo-2_43	Hypothetical protein	None	32301	32519	219	
Vigo-2_44	ASCH domain protein / putative RNA binding domain [Vibrio phage]	4.00E-29	32623	32985	363	
Vigo-2_45	Hypothetical protein	None	32995	33198	204	

To identify the genes coding for the polysaccharide depolymerases of both phages, several candidate genes including Carin1_orf31 and Vigo2_orf20 were selected for targeted activity screening. Genes were selected based on their sequence similarities with tail proteins (fibres, tube, spike domains) and their location on the genome sequence (proximity with structural or lysozyme-like encoding proteins).

Overexpression and Enzyme screening

A total of 24 candidate genes or Carin1 and 23 candidates for Vigo2 (including full gene sequences and gene fragments) were cloned in expression strains. (selected genes are listed in **Annexe I.2**). All genes and fragments were successfully amplified by PCR and inserted in the cloning vector (**Annexe I.3**). Cloning of candidate genes in NEB 5 α *E. coli* strains was successful for 36 genes (78%) (**Annexes I.4 and I.5**). Control of gene insertion in the vector by *HincII* digestion validated the correct insertion of all the other candidates, and cloning *E. coli* expression strains was successful. A total of 15 genes were then overexpressed, among which 11 proteins were successfully expressed. At the end, seven expressed proteins were soluble and purified (**Figure 30**).

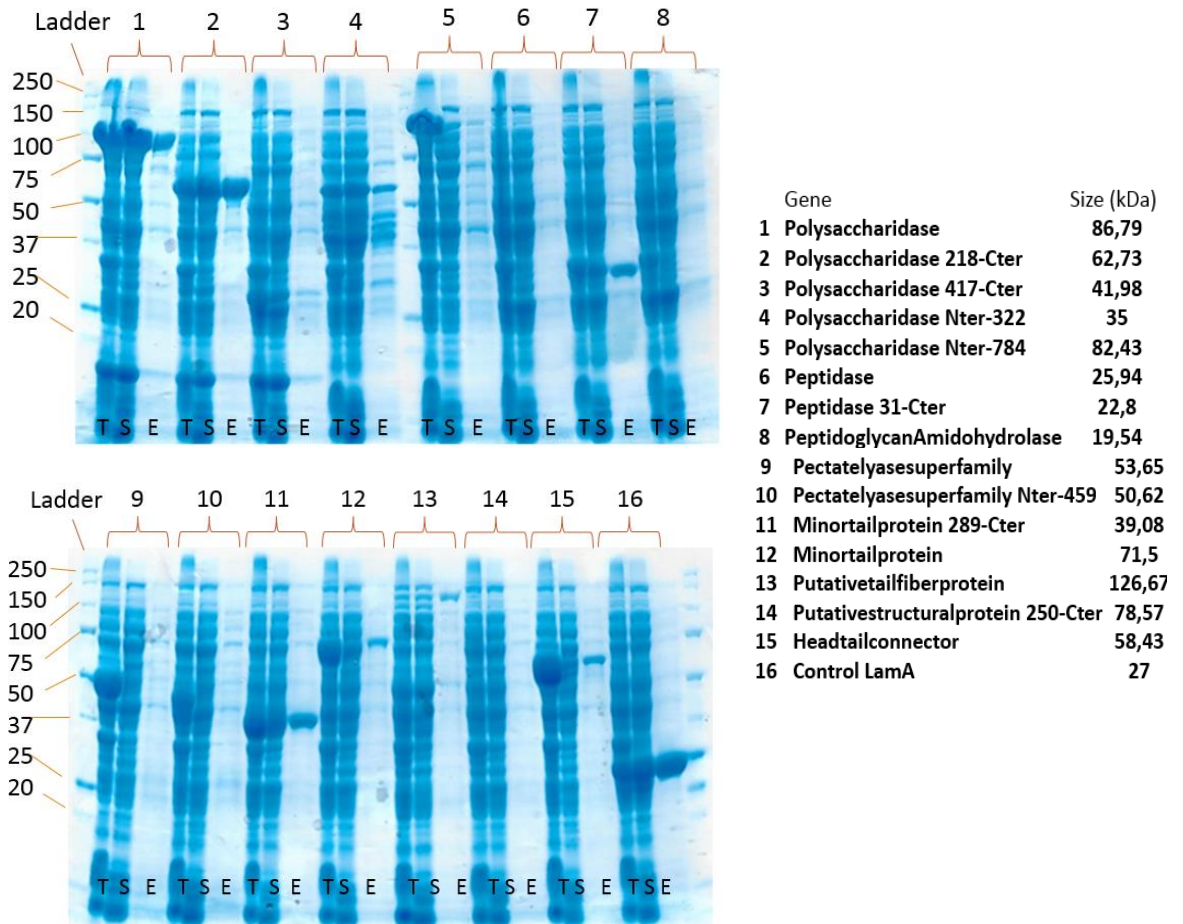


Figure 30: SDS-PAGE assay to evaluate the solubility of the recombinant proteins. T = Total fraction, S = Soluble Fraction, E = Eluate/Purified protein

For the candidate genes of Carin1, overexpressed protein 1 and 2 (corresponding to the ORF 31) showed a depolymerase activity on the *C. marina* host EPS. The depolymerization pattern was similar to that observed with the purified virions (positive control) (**Figure 31**). None of the Vigo2 proteins overexpressed and purified showed an EPS depolymerase activity. Experiments were also conducted with the insoluble fractions of the pectate lyase family related protein, but the assays failed (**Figure 31**).

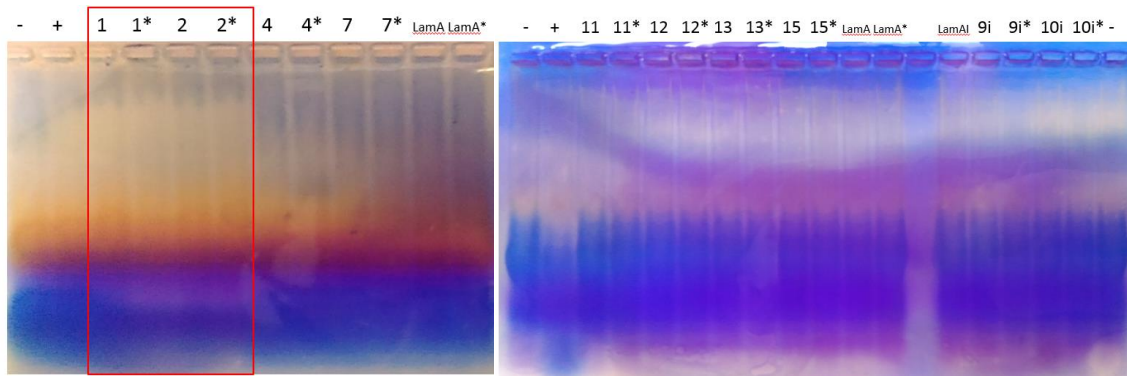


Figure 31: Polysaccharide depolymerase screening with recombinant proteins. Left are shown results with *Carin1* recombinant proteins; Right with *Vigo2* recombinant proteins.

Comparison of identified genes with environmental databases

The global distribution of *Carin1* and *Vigo2* depolymerases were investigated using marine viromes collected from various waters including Atlantic Ocean, Mediterranean Sea, Pacific Ocean and Indian Ocean.

Both of the genes were BLAST-ed to marine environmental metagenomics databases (Tara Ocean, Global Ocean Survey, Ocean Sampling Day). No consistent hits were found.

DISCUSSION

EPS depolymerases associated to viral particles can play an important role during the infection process. Many phages were shown to use these enzymes to degrade the layer of exopolysaccharides (EPS) of their host in order to gain access to their receptor. Over the past decade, phages with EPS-degrading properties have received a growing interest, mostly for the search of novel tools to eradicate biofilms and pathogenic bacteria. By comparison, the presence of EPS depolymerase in marine phages and their implication for viral activities in the sea has been barely explored. Here, we report, for the first time, the study of two marine phages, *Cobetia marina* phage Carin1 and *Vibrio alginolyticus* phage Vigo2, with EPS-degrading properties.

Identification of two novel genetic sequences encoding phage polysaccharide depolymerases

A combination of bioinformatics and targeted activity screening revealed that EPS depolymerase associated to *Cobetia marina* phage Carin1 belong to an undescribed CAZyme family. The genome analysis of *C. marina* phage suggests that Carin1 may be related to the Lessievirus. This *Podoviridae* genus includes phages that infect members of the genus *Burkholderia* (previously part of *Pseudomonas*). Carin1 also display similarities with *Pseudomonas putida* phage AF, which display EPS depolymerase activities (Chang et al., 2010; Cornelissen et al., 2012; Guichard et al., 2013). Despite weak sequence identity between Carin1 and phage AF, the genetic environment of their respective depolymerases and lysozymes is similar. We detected a functional synteny over a genome region of 8 genes including the EPS depolymerase. Whether gene synteny can be used to retrieve additional sequences of EPS depolymerases remains to be investigated. There is, to our knowledge, no other marine phages that belong to this group reported to date. The *Vibrio alginolyticus* phage Vigo2 showed gene similarities to Lessievirus, but also Enquatrovirus viruses, that infect *E. coli* and *Pseudomonas*. Some phages from this genera display polysaccharide depolymerase activities including phage G7C and phage Ab09 (Latino et al., 2019; Prokhorov et al., 2017; Yang et al., 2015). Although we demonstrated experimentally that Vigo2 possesses EPS degrading properties, the gene encoding these activities could not be confirmed in this study.

Our best candidate gene encoding depolymerase (orf20) could be amplified and overexpressed but the targeted protein was insoluble after purification. Our confidence in the correct identification of this gene is however strengthened by the reasonable sequence identity with tail fibre proteins (2.10^{-7}). Additionally, we found BLASTP hits (2.10^{-5}) to a pectate lyase related proteins on the N-terminal domain of orf20. This observation was also made by Liu et al. in 2019, who used this information to identify experimentally a phage polysaccharide depolymerase (Liu et al., 2019). Finally, we could predict a putative β -helix structure in C-terminal domain, a typical fold of viral polysaccharide depolymerases (Casjens and Molineux, 2012; Nobrega et al., 2018). Additional biochemical and structural analyses are surely needed to classify these depolymerases but it is likely that they belong to novel CAZYme families. Although hundreds of marine phage genomes were sequenced, gene encoding polysaccharide depolymerases (other than lysozymes) are seldom detected, as exemplified by the study of Carin1 and Vigo2. To the best of our knowledge, the expert database CAZy reports depolymerases (polysaccharide lyases) for only 12 marine *Cellulophaga* phages and one uncultured Mediterranean phage, mostly related to lysozymes. By comparison, 160 sequences of polysaccharide depolymerases were described from 143 phages genomes (including 37 *Podoviridae*) that infect genera of terrestrial bacteria (mostly human and plant pathogens). The majority of phage depolymerases (126 proteins) are encoded in the same open reading frame of phage structural proteins (mostly on tailspike, tail fibres, base plates, but sometimes also in the neck) or in close proximity to genes that are structural proteins (Pires et al., 2016). We may suspect marine bacterial EPS to differ from their terrestrial counterparts, as they are dealing with different environmental conditions (Arnosti et al., 2014; Limoli et al., 2015). In that way we may suggest that the genes encoding polysaccharide depolymerase in marine viruses are distantly related to known molecules with similar function. Our findings suggest that marine viruses may represent an untapped reservoir of novel polysaccharide depolymerases.

No recruitment in environmental databases

The polysaccharide depolymerases that we identified during this study appeared to be rare in the ocean as suggested by metagenomics recruitment analysis. Similar analysis conducted on the whole genome sequence of the Carin1 and Vigo2 phages provided the same results (data not shown). The polysaccharide lyases detected in *Cellulophaga* phages were comparatively more widely distributed (Annexes I.6). Several reasons could explain these results. The hosts of Carin1 and Vigo2 (*Cobetia marina* and *Vibrio alginolyticus*, respectively) are widespread in the ocean. They are however mostly found associated to marine aggregates, macroalgae and organisms (Arahal et al., 2002; Guidi et al., 2016a). Likewise, marine phages can occur at high concentrations on aggregates and in/on organisms (Comeau and Suttle, 2007; Mari et al., 2007). Yet, marine viromes essentially describe the genetic diversity of free-living viral communities. The observed absence of Carin1 and Vigo2-like phage and their associated EPS depolymerases in these metadata may thus result from an inadequate sampling. In addition, the chemical structure of bacterial EPS is extremely variable between and within bacterial species. It is thus likely that the specific depolymerase targeting this EPS display a similar or even greater level of molecular diversity. Our previous study on the EPS degrading properties of five phages that infect *C. marina* strongly suggest that EPS depolymerase are functionally, and mostly likely genetically, diverse. Alternative method of gene/protein recruitment (e.g. using protein structure rather than their sequences) may improve the detection of these unusual enzymes. The finding that the EPS depolymerases identified in this study are rare led us to speculate that they are not prone to extensive genetic exchange. Horizontal gene transfers of EPS-depolymerase, tail spike, or fibre proteins have been described previously and occurs across phage genera, sub-families, and even unrelated phages that infect the same host (Cornelissen et al., 2012; Schwarzer et al., 2012; Stummeyer et al., 2006). The genomic analysis of the other phages of *Cobetia marina* with EPS degrading activity would certainly to help to test this hypothesis.

CONCLUDING REMARKS

Altogether, the results from this study suggest that the polysaccharide depolymerases associated to *C. marina* phage Carin1 and *V. alginolyticus* phage Vigo2 probably belong to undescribed enzyme families and put forward that marine viruses may represent an unappreciated reservoir of novel depolymerases. The identification and characterization of these molecules should not only provide novel reference sequences to enrich public databases and current repertoire of carbohydrate active enzymes but also provide novel fundamental knowledge about the mechanisms of marine phage infection. Besides these fundamental aspects, the discovery of these bioactive molecules could provide novel research tool to elucidate the structure of polysaccharides, to produce oligosaccharides, or to eradicate marine bacterial biofilms.

CHAPTER II

Biochemical and structural characterization of dpo31, a novel marine depolymerase from the *Cobetia marina* phage Carin1.

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AUTHOR CONTRIBUTIONS

PYM and ACB devised the project, the main conceptual ideas and proof outline, with the great help of TR and MC. PYM and TR carried out the experiments regarding the enzyme production and purification (Supervised by TR). The EPS production and degradation assays were performed by PYM (methods development, experiment design, optimization, data computation and analysis). The EPS analysis by SEC-RI was carried out by PYM under the supervision of DJ (methods development, experiment design, optimization, data computation and analysis). Protein crystallization were performed by PYM and TR (supervised and optimized by TR). The structure resolution of Dpo31 was performed by TR, PL and SS. SAXS analyses were performed by MC. PYM wrote the manuscript with support of ACB and MC. TR and SS participated mainly in the writing of the structure resolution method. MC contributed mainly in the writing of the SAXS method and results.

INTRODUCTION

During the infection process, numerous host – virus recognition events occur. Some of them involve the extracellular polysaccharides (i.e., lipopolysaccharides, capsular polysaccharides (CPS) and exopolysaccharides (EPS)) of the bacterial host (Kumar et al., 2007; Roberts, 1996). EPS and CPS are structured polymers produced by the bacteria to protect the cell against environmental threats (desiccation, starvation, etc) but they can also serve as a physical barrier between the phage and their membrane receptor (Jefferson, 2004; Weiner et al., 1995). Some phages have evolved polysaccharide depolymerases to degrade these polymers to gain access to the bacterial membrane (Hughes et al., 1998; Sutherland et al., 2004). These enzymes, frequently located at the phage tail (tailspike protein, fiber protein, base plate), act as “adjuvant” for viral infection (Pires et al., 2016). (Casjens and Molineux, 2012; Leiman et al., 2007; Smith et al., 2005; Stummeyer et al., 2006; Weigele et al., 2003). They confer a great benefit for successful infection in biofilm (Hughes et al., 1998; Yan et al., 2014) and during intense EPS production of the host (F. Lechat et al., 2019). Viral polysaccharide depolymerase are also involved in host specificities. Phages can exchange and/or acquire new polysaccharide depolymerases to modify/ enlarge their host range, (Cornelissen et al., 2011; Leiman et al., 2007; Schwarzer et al., 2012).

The biochemical and structural characterization of polysaccharide depolymerase are essential for a better understanding of their functional mechanisms and evolution. Viral polysaccharide depolymerases belong to two main enzyme classes: the Glycoside hydrolases (involving a water molecule) and the Polysaccharide lyases (involving a β -elimination) (Latka et al., 2017). Only few studies report on their molecular architecture but they pointed conserved structural features. These enzymes are usually constituted of three monomers forming a long homotrimer characterized by a triple-strand β -helix (Casjens and Molineux, 2012; Weigele et al., 2003). Such structure increases the enzyme stability as suggested by their generally broad thermal tolerance (up to 45°C (Liu et al., 2019)). Structural studies defined three functional regions : (i) a N-ter domain that encode a flexible module involved into the attachment to the phage capsid, (ii) a central part involved in the host recognition and the polysaccharide depolymerization activity, and (iii) a C-ter domain allowing the trimerization of the protein and involved in the recognition of the host membrane receptor (see Figure 18 and 19 in

Introduction) (Casjens and Molineux, 2012; Weigele et al., 2003). Changes in host specificity can occur by the horizontal uptake of catalytic and recognition modules while the N-terminal domain seem to be conserved (“cut-and-paste” strategy) (Leiman and Molineux, 2008). So far, most studies of viral polysaccharide depolymerases were conducted on phages that infect pathogenic bacteria (Casjens and Molineux, 2012; Latka et al., 2017). Recently, we identified polysaccharide depolymerase activity associated to several phages isolated from marine environments, among which the *Podoviridae* *Cobetia marina* phage Carin1 (Lelchat et al. 2018, 2019, Chapter I). We suggest this marine phage to be related to Lessievirus. The gene encoding the polysaccharide depolymerase of Carin1 (orf31 hereafter referred to as **Dpo31**) appears to be divergent from known protein with similar function (Chapter I), suggesting that it may constitute a novel protein family. In this study, we conducted a biochemical and structural characterization of Dpo31 to get more insight into the functional mechanisms of this unusual enzyme. Therefore, we overexpressed and purified Dpo31 in order to determine its mode of action, tolerance to changes in pH, temperature, and salinity as well as its substrate specificity. The purified enzyme allowed the formation of crystals that were diffracted by X-ray. Data collected were used to resolve the structure of Dpo31. This study revealed that Dpo31 exhibit unique structural features but also several conserved domains of *Podoviridae* tailspike proteins.

MATERIAL AND METHODS

Enzyme production and purification

Escherichia coli clone of interest (containing orf31, see Chapter I) was grown at 37°C overnight in LB medium containing 100 µg ml⁻¹ ampicillin. Then, culture was diluted 1:100 with auto-inducible ZYP5052 medium (Groisillier et al., 2010; Studier, 2005) containing 100 µg ml⁻¹ ampicillin in a final volume of 500 mL and subjected to further incubation at 20°C during 72 hours (baffled flasks, 300 rpm). Cells were recovered by centrifugation (35 min, 4°C, 3,000 g) and they were resuspended in 20 mL of buffer A (TRIS 50 mM pH 8, NaCl 500 mM, imidazole 20 mM) and chemically lysed as described previously (Chapter I, (Groisillier et al., 2010)). Afterwards, the lysate was clarified at 12,000 g for 30 min at 4°C and the supernatant filtered on 0.22 µm. The supernatant was loaded onto an IMAC column charged with NiCl₂ (0.1 M) and pre-equilibrated with buffer A. The column was washed with buffer A and Dpo31 was eluted with a linear imidazole gradient produced by the mixing of buffer A and buffer B (TRIS 50 mM pH 8, NaCl 500 mM, imidazole 500 mM) at a flow rate of 1 mL min⁻¹. The different fractions were analyzed by SDS-PAGE and the fractions of interest were concentrated on Amicon Ultra 15 (Cellulose, 10 kDa) (Merck Millipore) to reach a volume of 2 mL. Then, the protein was injected onto Sephacryl S-200 size exclusion column (GE Healthcare) pre-equilibrated with buffer C (TRIS 50 mM pH 8, NaCl 300mM). Absorbance at 280 nm was followed and fractions containing Dpo31 were collected and pooled. A concentration step was added using an Amicon Ultra 15 until reaching a 1-2 mL volume.

SelenoMethionine (SeMet) incorporation and SAD phasing were used to resolve Dpo31 structure. Cultures using SeMet were done as previously, besides that the auto-inducible ZYP5052 medium was replaced by PASM-5052 (Studier, 2005).

EPS production

The EPS production was done as described previously (see Chapter I). Briefly, the production was achieved by incubating *C. marina* bacterial strain in Zobell culture medium supplemented by glucose at 3% (w/v) at 25°C under vigorous agitation (150 rpm) for 72 hours. The supernatants were then collected, filtered through 0.45µm and 0.2µm, and concentrated

through tangential filtration using a cut-off of 30 kDa. The concentrated suspension was diluted in 5 volumes of 500 mL MilliQ and further concentrated to remove salts. A 100 mL concentrate was frozen at -80°C overnight and lyophilized

EPS degradation assays

Bacterial exopolysaccharides at 0.2% (w/v) in SM Buffer (NaCl 100 mM, $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ 8 mM, Tris-CL 50 mM, MilliQ water, pH 7.5) were incubated in triplicate with Dpo31 at 100 ng mL^{-1} in a final volume of 200 μL . The samples were incubated during 1 hour at 200 rpm. Samples were then inactivated at 90°C for 5 minutes and frozen at -80°C until analysis.

Temperature assays were conducted in SM buffer. pH assays were done in 100 mM buffer at 25°C including Gly-NaOH buffer (Glycine, Sigma), Tris-HCl buffer (TRIZMA base, sigma) and Tris-Maleate buffer (Tris(hydroxymethyl), sigma and maleic acid, sigma, equimolarly). 0.2 M solutions were beforehand prepared, 1M NaOH (Sigma) or 1M HCl (Sigma) were added until the targeted pH, and then diluted with MilliQ water until 0.1M (with pH control). Salt assays were done in Tris-HCl buffer at 100 mM and at 25°C . Relative activity of Dpo31 was calculated using the highest value of the series as a 100% efficiency.

EPS analysis by Size-Exclusion Chromatography

Prior to analysis, samples were thawed, diluted 1:2 (to reach 1 mg mL^{-1} of EPS concentration), and filtered through a $0.45\text{ }\mu\text{m}$ PES syringe filter (Sartorius). Analyses were performed using an UltiMate 3000 HPLC system. Volumes of 50 μL were injected on a Superdex 10/300 column (GE Healthcare) at 0.5 mL min^{-1} . SM Buffer (NaCl 100 mM, $\text{MgSO}_4\cdot 7\text{H}_2\text{O}$ 8 mM, Tris-CL 50 mM, MilliQ water, pH 7.5) was used as solvent. Signal was acquired by refractometry during 120 min after injection.

Protein crystallization and structure resolution

Crystallization experiments of the His-tagged Dpo31 (orf31, 838 residues, see Chapter I) from phage Carin1 was performed by hanging-drop vapor-diffusion methods at room temperature by mixing 2 μL of protein (12 mg/ml) with 1 μL of reservoir solution containing 1.2 M Ammonium sulfate and 100 mM Citric acid pH 5.0. The crystals were flash-frozen in liquid nitrogen in the reservoir solution containing 20% (v/v) glycerol. X-ray diffraction data were

collected at beam line PROXIMA 1 at the SOLEIL synchrotron (St Aubin, France). Data processing was performed using the XDS package (Kabsch, 2010). Experimental phases were obtained from a selenium derivative crystal (SeMet), where single-wavelength anomalous diffraction (SAD) datasets were collected at the peak of Se K absorption edge ($\lambda = 0.979$). The Selenium sites were identified using HKL2MAP (Pape and Schneider, 2004) and experimental phases calculated with PHASER (McCoy et al., 2007). Initial maps were of poor quality and could not be interpreted. The Phased Rotation Function (PRF) implemented in MOLREP (Vagin and Teplyakov, 2010) was used to model a 130-residue fragment of a homology model (3SUC.pdb) in the electron density, combined with experimental amplitudes and phases. The search model used in the PRF (pdb entry 3SUC) was identified using HHpred (Zimmermann et al., 2018). Subsequently PHASER+PARROT were used for solvent flattening and improvement of the phases. The resulting model underwent iterative cycles of manual reconstruction in COOT (Emsley et al., 2010) and refinement in BUSTER (Blanc et al., 2004). Phase extension was subsequently carried on using the final model for molecular replacement against a higher resolution native dataset. Model was evaluated using MolProbity (Chen et al., 2010) (N.B. Model validation has not been done yet).

SAXS analyses

SAXS measurements were conducted at the French Synchrotron SOLEIL (St. Aubin, France) on the SWING beamline and were performed at 15 °C. The SAXS data were measured using the same buffer as for crystallization trials. The scattering vector was defined as $q = 4 \pi/\lambda \sin\theta$, where 2θ is the scattering angle. Data were collected covering a q range of 0.005– 0.5 Å⁻¹. Data were recorded using an AVIEX170170 CCD detector at a distance of 1.807 m ($\lambda = 1.033$ Å). Stock solution of Dpo31 was prepared at a final concentration of 12 mg ml⁻¹. To isolate the various species in solution, SAXS data were collected on samples eluted from an online size exclusion high-performance liquid chromatography (SEHPLC Bio-SEC3, Agilent) column and directly connected to the SAXS measuring cell. A volume between 60 to 120 µl of protein sample was injected and eluted directly into the SAXS capillary at a flow rate of 0.2 ml/min. The elution buffer consisted of 50 mM Tris HCl pH 8.0 and 100 mM NaCl. Two hundred fifty SAXS frames were collected continuously during the elution at a frame duration of 1.5 s and a dead time between frames of 0.5 s. One hundred frames accounting for buffer scattering were

collected before the void volume. SAXS data were normalized to the intensity of the incident beam and background (i.e. the elution buffer) subtracted using the program FoxTrot (David and Pérez, 2009) the Swing in-house software. The experimental curves were analyzed using Primus to calculate the R_g value in the Guinier region and GNOM to calculate the $P(r)$ and D_{max} value. The experimental curve was compared with the theoretical diffusion curve of the crystal structure (M44B as of the model in October 2019) calculated with CRY SOL.

RESULTS

Overexpression and purification of Dpo31

In a previous genomic/proteomic study, the *C. marina* phage Carin1 was shown to encode a polysaccharide depolymerase (Dpo31) active on *C. marina* EPS (see chapter I). To characterize the enzymatic activities and the structure of Dpo31, this gene was overexpressed and the recombinant protein was purified using a nickel affinity chromatography and size exclusion chromatography. Affinity chromatography was effective with a narrow elution peak triggered by increasing concentration of imidazole. Elution started with an imidazole concentration of 175 mM (35% of buffer B) (**Figure 32**).

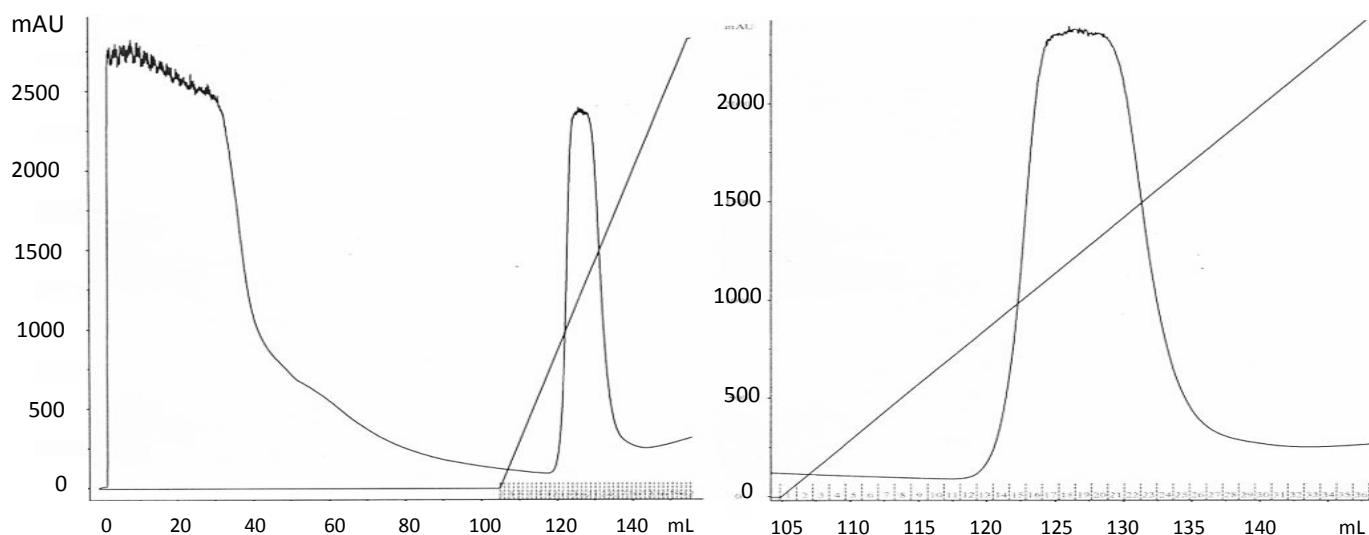


Figure 32: Monitoring of the affinity chromatography step by following absorbance at 280 nm. The left panel shows the total record; the right panel shows a close up at the peak of elution. The diagonal represents the increasing imidazole gradient (from 20 to 500 mM).

The size-exclusion chromatography step allowed a greater purification and was followed by SDS-PAGE. Fractions containing Dpo31 were collected and merged (18 to 26) (**Figure 33**).

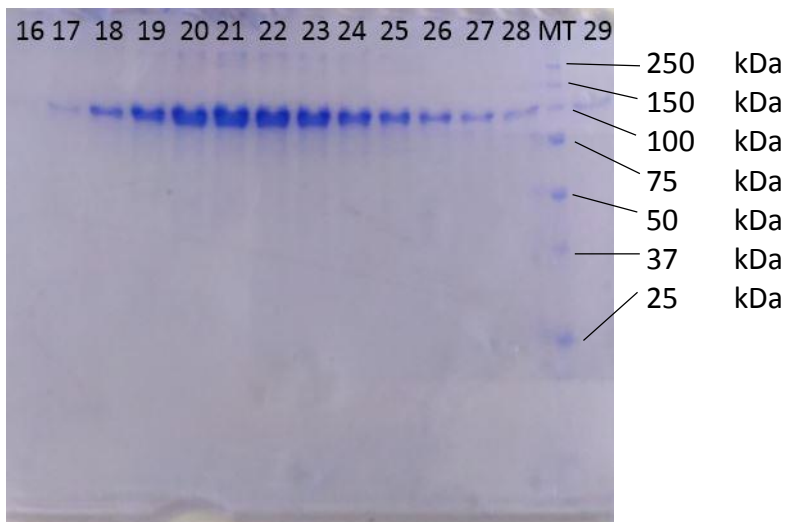


Figure 33: SDS PAGE of proteins from the size-exclusion chromatography purification step.

This two-step purification yielded high amount of recombinant protein with a final yield of 47.32 mg produced by 500mL of culture. The apparent molecular mass of the recombinant protein ranged between 85 and 90 kDa based on the size markers loaded on SDS-PAGE, which is compliant with the theoretical size of Dpo31 calculated from the gene sequence (86.79 kDa).

Regarding the production and purification of the protein labeled with selenomethionine, 500 mL of culture yielded 16.4 mg of protein. The protein reacted differently on the IMAC column and was eluted at an imidazole concentration of 80 mM (16% of buffer B) (data not shown)

Dynamic Light Scattering (DLS) data discriminated two populations with different hydrodynamic diameters. The first population (monomer) had an estimated diameter of 10.1 nm (± 2.2) and the second population (polymer) had a diameter of 141.8 nm (± 43.2).

A DLS analysis was conducted over a large temperature range (4-68°C) in order to determine the melting temperature of the protein (T_m). The obtained T_m was $43.72 \pm 0.15^\circ\text{C}$ (**Figure 34**).

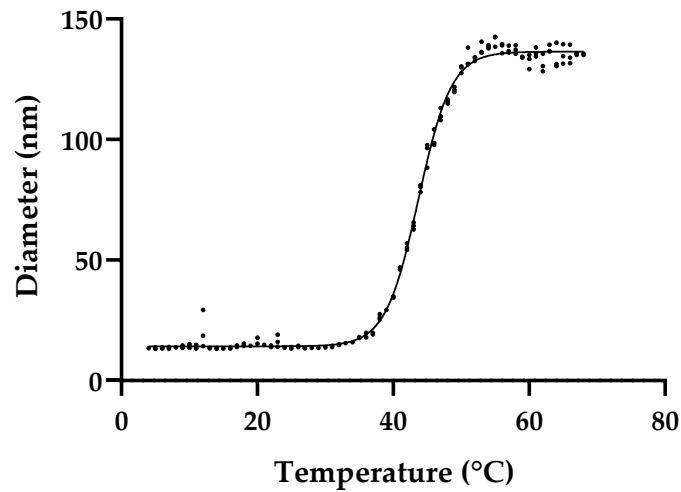


Figure 34: *T_m curve obtained using DLS. Measures were done each degree between 4 and 68°C in triplicate. T_m obtained was 43.72 ± 0.15°C*

Enzymatic mode of action

A common test to determine if an enzyme is a lyase or a hydrolase is based on an absorbance monitoring at 235 nm during the enzymatic reaction. If the reaction produces unsaturated bonds characteristic of a lyase activity, the signal increases in this spectral region. In our case, the action of Dpo31 on *C. marina* EPS did not induce any increase in absorbance, suggesting that the enzyme is most probably a glycoside hydrolase (**Figure 35**).

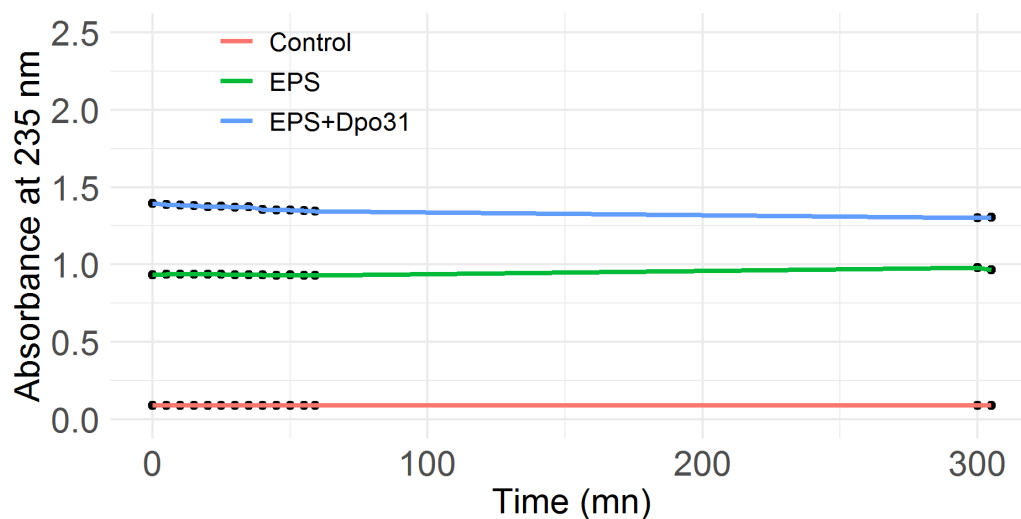


Figure 35: *Monitoring of the degradation activity by absorbance at 235 nm during 300 minutes (5 hours).*

We tested different methods to detect and quantify the depolymerase activity of Dpo31. The reducing sugars assay is a colorimetric method that quantifies reduced sugar extremities produced by the enzymatic reaction. No signal was detected during the reaction of Dpo31 on its substrate in spite of multiple optimizations (reaction volume, reagent and EPS concentration, and incubation times) (data not shown). As an alternative, we used size-exclusion chromatography coupled with a refractometer (SEC-RI) to monitor the Dpo31 activity.

SEC-RI analyses of native and degraded *C. marina* EPS are shown in Figure 36. The SEC-RI profile of native EPS showed two distinct populations. A population of HMW compounds (1) that was eluted at 30 minutes with a substantial front shoulder that was eluted between 26-28 minutes; and a large population of smaller size species (2) that peaked between 44 and 63 minutes of elution (**Figure 36A**).

After 1h of incubation of *C. marina* EPS with Dpo31 at 15°C (which corresponded to a partial degradation of *C. marina* EPS), the peak amplitude of the HMW species decreased considerably and we detected additional populations at 37 minutes (3), (2a), 59 (2b) and 62-63 (2c) minutes of elution (**Figure 36B**).

After 1h of incubation at 45°C (which corresponded to a complete degradation of *C. marina* EPS), the populations of intermediate size (elution time) disappeared and a unique peak of small-sized product was eluted at 62-63 minutes (2c) (**Figure 36C**). This last peak was considered as the final degradation products and its area and was monitored to assess Dpo31 activity under different pH, temperature, and salinity conditions.

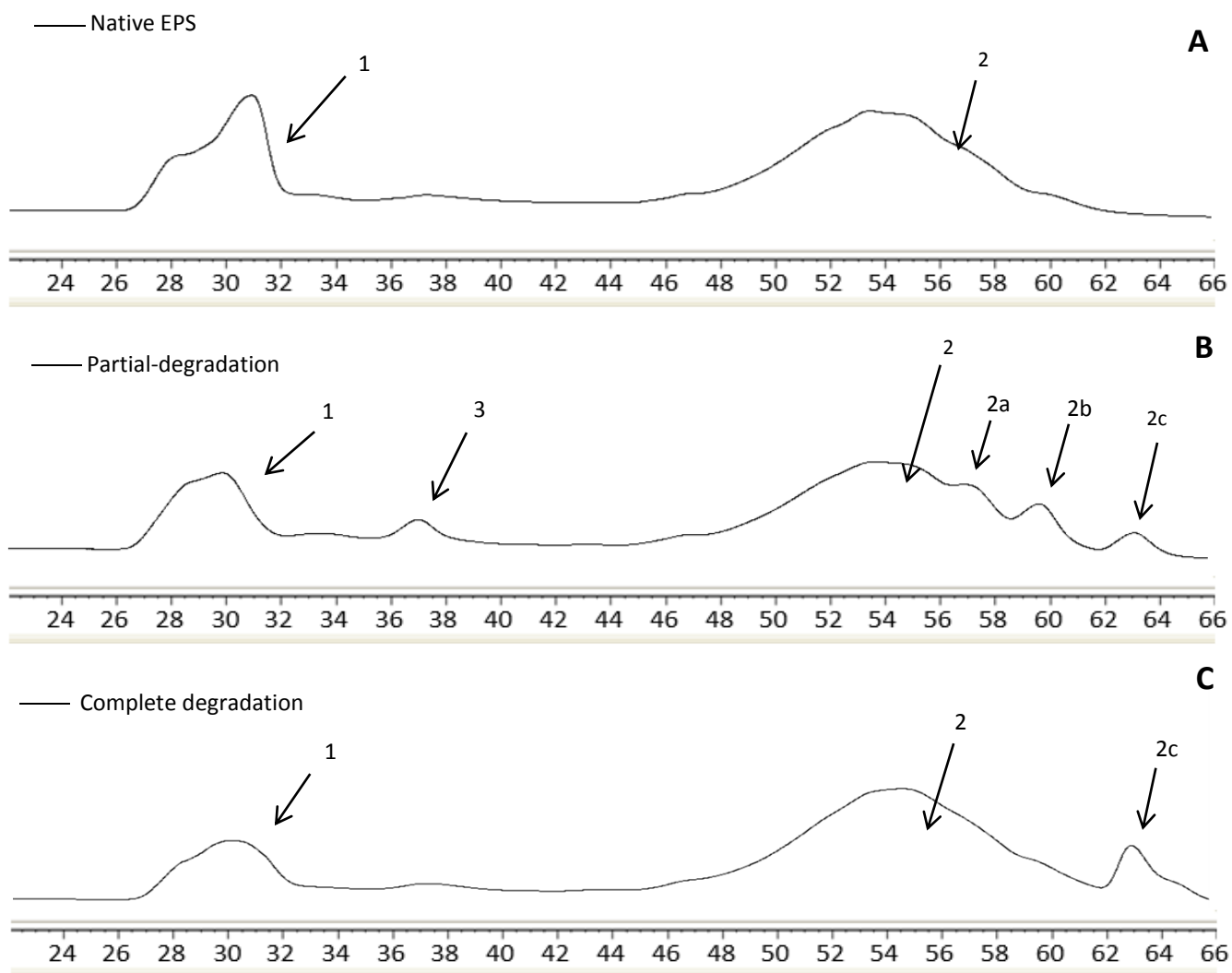


Figure 36: SEC-RI analysis of Native EPS (A), partially degraded EPS (B), and completely degraded EPS (C). Refractory Index is shown against time (in minute). Numbers are showing different populations.

Biochemical characterization of Dpo31

The activity of Dpo31 increased with increasing temperature from 4°C to 45°C, which corresponded to the optimal temperature. At this temperature, the activity is four-fold higher compared to the one at 13°C (average seawater temperature at the isolation site of Carin1). Above 45°C, the enzyme activity decreased rapidly. Minute activity was recorded at 60°C (Figure 37).

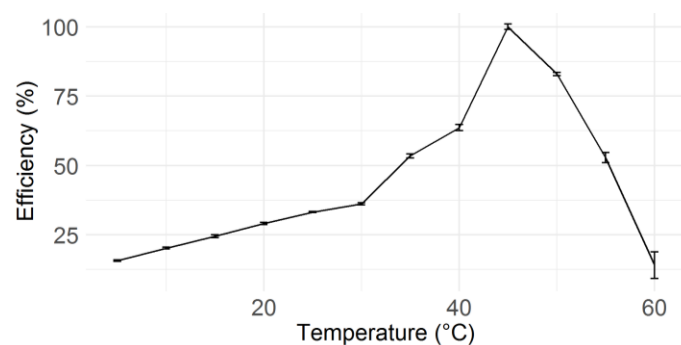


Figure 37: Graph showing depolymerization efficiency (relative activity) of Dpo31 depending on the temperature. Maximum efficiency was defined with the highest value recorded in this condition

The addition of salt reduced Dpo31 activity. The maximal activity was recorded with no NaCl, and decreased until a concentration of 350 mM. No more activity is observed at NaCl concentration higher than 600 mM. The addition of cations (Mg^{2+}) increased the depolymerization activity but the same pattern is observed. The activity decreased until NaCl concentration of 300 mM and remains low at higher concentrations (Figure 38).

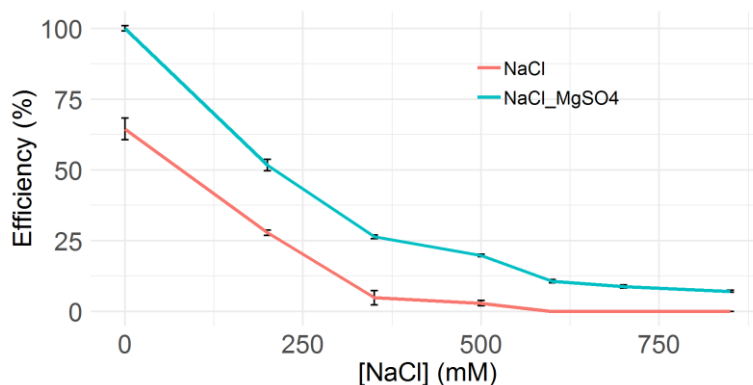


Figure 38: Graph showing depolymerization efficiency (relative activity) of Dpo31 depending on the Salt concentration and in presence of cations (Mg^{2+}) or not.

Dpo31 activity was detected between pH 5.5 and 10 with an optimal activity recorded at pH 8.5 in Glycine-NaOH buffer. The buffering molecule influenced the enzymatic activities, with the highest values obtained with Gly-NaOH. Dpo31 activity at pH 8.5 in Gly-NaOH were 2 fold higher than in Tris-HCl. Likewise, the enzyme activity at pH 8.0 in Tris-HCl were three-fold higher than in Tris-Maleate (**Figure 39**).

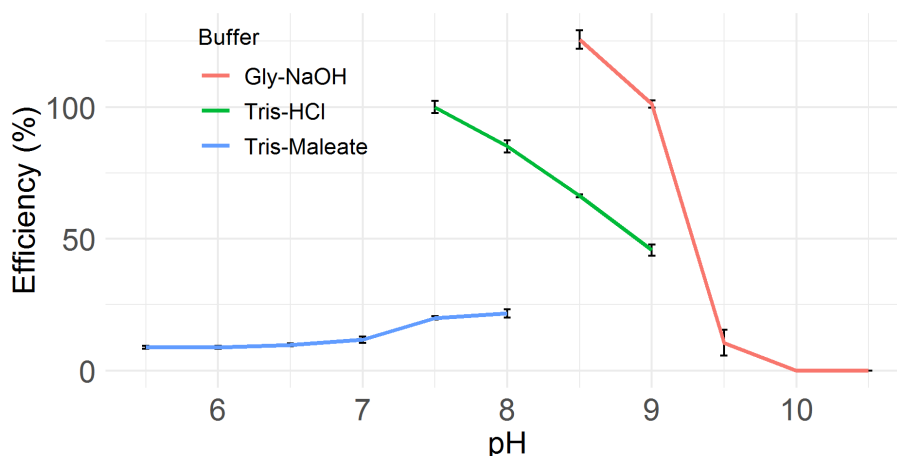


Figure 39: Graph showing depolymerization efficiency (relative activity) of Dpo31 depending on pH and with different buffers.

Substrate specificity of Dpo31

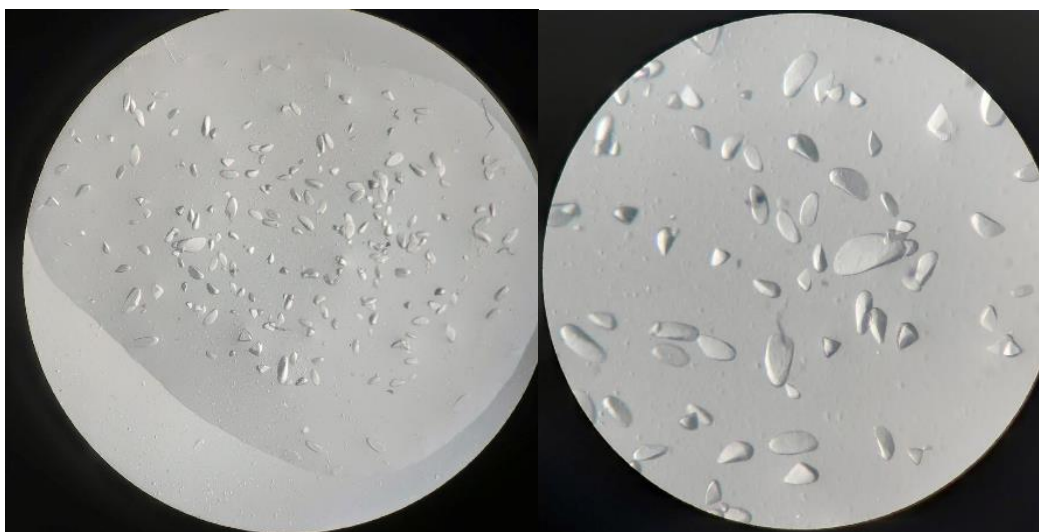
In order to determine the substrate specificity of Dpo31, we produced and purified EPS of bacteria strains that belonged to (i) different strains (*C. marina* LMG2218t1, LMG6798, LMG6842), (ii) different species (*Halomonas* family), and (iii) different genera (*Vibrio* and *Pseudoalteromonas* classes) as Carin1 host. The degradation of these bacterial EPS was monitored by agarose gel electrophoresis and “Stains All” coloration (**Table 5 and Annexes II.1**).

Table 5: Table gathering degradation results using bacterial EPS different from the host EPS

Genus	Species	Strains	Degradation
<i>Cobetia</i>	<i>marina</i>	DSMZ 4741	+
<i>Cobetia</i>	<i>marina</i>	LMG2218t1	-
<i>Cobetia</i>	<i>marina</i>	LMG6798	-
<i>Cobetia</i>	<i>marina</i>	LMG6842	-
<i>Vibrio</i>	<i>alginolyticus</i>		-
<i>Vibrio</i>	<i>coralliilyticus</i>		-
<i>Vibrio</i>	SWAT3		-
<i>Pseudoalteromonas</i>	<i>carraghenovora</i>		-
<i>Pseudoalteromonas</i>	<i>atlantica</i>		-
<i>Pseudoalteromonas</i>	<i>tunicata</i>		-
<i>Pseudoalteromonas</i>	<i>undina</i>		-
<i>Halomonas</i>			-

Crystallography and structure of Dpo31

First crystals appeared with the pHClear Suite screening kit (Quiagen) condition with 1.6 M Ammonium sulfate, pH 5.0 buffered with Citric Acid 0.1 M. Optimization steps were required, starting from a screening step assisted by a robot, and subsequent modification of salt concentrations, pH and buffer concentration, addition of additives and modification of drop/tank volume ratio. The optimized conditions were of 1.2 M Ammonium sulfate and 0.1 M Citric acid pH 5.0 (**Figure 40**).

**Figure 40:** Pictures of the crystals that were selected for X-ray diffraction

X-ray diffraction data were collected on protein crystals to determine the 3D crystal structure of the enzyme. In a first step, the dataset collected on the Se-met labeled protein crystals (3.2 Å resolution) was used to solve the phase problem using SAD (single anomalous diffraction) combined with molecular replacement using as a model a tail-spike protein (pdb id 3SUC). Then data from a native protein crystal that diffracted to 2.2 Å were used to iteratively construct the 3D structural model into electron density. The data collection parameters and refinement statistics are listed in **Table 6**. Crystals contain 54.05 % of solvent and one molecule in the asymmetric unit, and belong to the rhombohedral space group $H\ 3\ 2$ (hexagonal settings). The unit cell dimensions are as follows: $a = b = 88.2\ \text{Å}$, $c = 643.34\ \text{Å}$; $\alpha = \beta = 90^\circ$, $\gamma = 120^\circ$.

The structure of the polysaccharide depolymerase is currently being resolved. The current model contains 650 out of 828 residues. N-terminal and C-terminal domains are currently being constructed but are poorly defined within the electron density.

The asymmetric unit containing only a single molecule, the biological trimer is formed around the threefold crystallographic axis. The homotrimer is 236 Å long and 68.8 Å large (**Figure 41A**; measured within the program COOT). The N-terminal area is characterized by 3 coiled-coil domains separated by unstructured domains. The C-terminal domain is represented by a β -helix in which modules are anchored. Those modules are part of the monomer β -strands sequence and are 84 amino acids long (**Figure 41B et 41C**). A central domain with interconnected α -helix and β -strand appears between the N-ter and C-ter domains. A carbohydrate molecule is found in this domain within the trimer conformation (**Figure 41D**).

Table 6: The data collection parameters and refinement statistics for Carin1 native protein and SeMet protein. * Values in parentheses refer to the highest resolution shell.

	CARIN1-PS † SeMet	CARIN1-PS § Native
Data collection*		
Space group	<i>H32</i>	<i>H32</i>
Cell dimensions		
$a = b, c$ (Å)	88.0, 644.4	88.2, 643.3
α, β, γ (°)	90.0, 90.0, 120.0	90.0, 90.0, 120.0
Resolution range (Å)	50 – 2.9 (2.98 – 2.9)	50 – 2.2 (2.26 – 2.2)
$I/\sigma(I)$	16.8 (1.0)	17.7 (2.4)
R_{merge} (%)	21.0 (449.8)	11.7 (97.0)
R_{pim} (%)	3.3 (78.1)	2.6 (31.3)
Completeness (spherical, %) ¶	99.9 (100.0)	99.2 (97.6)
Completeness (spherical, %)	75.3 (22.2)	43.3 (6.2)
Completeness (ellipsoidal, %)	92.7 (92.7)	89.3 (69.5)
Redundancy	41.9 (33.5)	21.2 (9.9)
CC(1/2)	1.000 (0.570)	1.000 (0.777)
Refinement		
Resolution range (Å)		46.4 – 2.2
No. reflection work set/test set		22237/1087
R/R_{free} (Buster, %)		22.6/27.2
No. atoms		
Protein		4667
Ligand/ion		36
water		11
R.m.s. deviation bonds (Å)		0.009
R.m.s. deviation angles (°)		1.17
Average B-factor (Å ²)		
From atoms		55.8
From Wilson plot		48.4
Ramachandram plot		
Most favored (%)		89.4
outliers (%)		1.56
Molprobit score		2.44

¶ Values before applying the elliptical cut with the STARANISO program.

† Diffraction data collected from two crystals, which diffracted anisotropically to 3.33 Å along 0.894 a^* - 0.447 b^* , 3.33 Å along b^* and 2.59 along c^* .

§ Diffraction data collected from two crystals, which diffracted anisotropically to 3.25 Å along 0.894 a^* - 0.447 b^* , 3.25 Å along b^* and 2.09 Å along c^* .

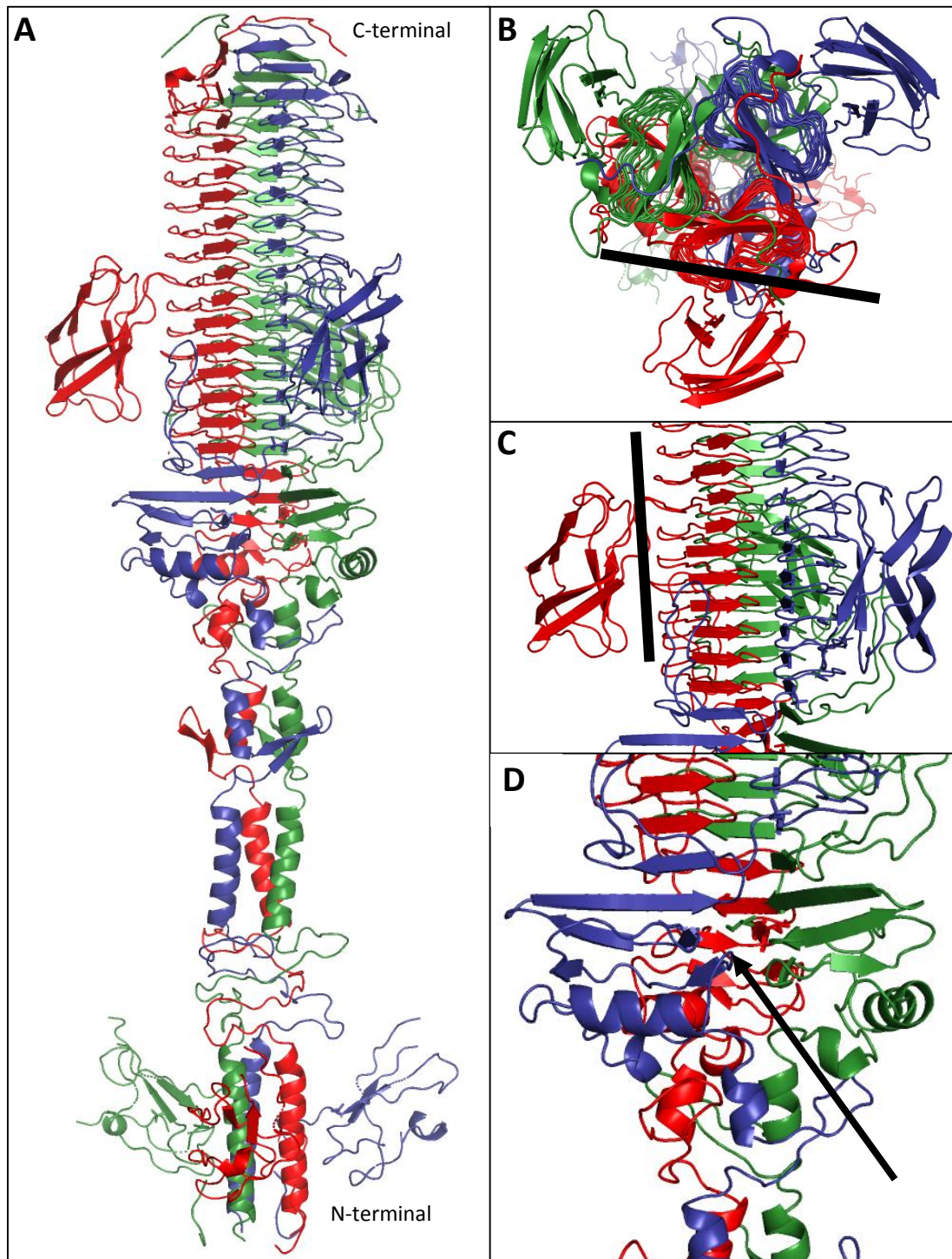


Figure 41: Molecular architecture of Carin1 polysaccharide depolymerase. In **A** is represented the protein in its length, with the C-terminal region at the top of the diagram and the N-terminal region at the bottom. In **B** is represented the C-terminal domain form top view. **C** represents this module from the side, separated by a black line. In **D** is represented the central domain were a sugar unit is attached to the protein (pointed by the arrow). The black line defines the module anchored in the β -strands among the β -helix.

SAXS analyses

The R_g value from the experimental curve was calculated to be $R_g=70.4 (\pm 2) \text{ \AA}$ for the data points in the low Q values ranging from point 12 to 27 ($sR_g=1.03$) (**Figure 42**)

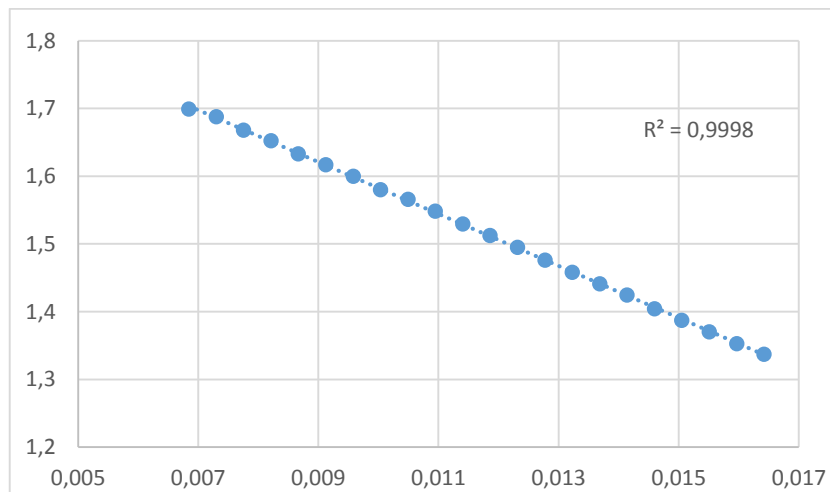


Figure 42: Guinier-plot showing no aggregation within sample

The R_g value from the $P(r)$ calculation is $69.5 (\pm 2) \text{ \AA}$ and the estimated D_{max} value is 228 \AA . The R_g “envelope” value calculated with CRY SOL from the trimeric crystal structure is $73 (\pm 1) \text{ \AA}$ and the diameter indicated is 239 \AA . When calculating the longest distance between atoms in the coordinate file with COOT, the D_{max} is 230 \AA . The CRY SOL using the molecular trimer of Carin1 gives a much lower χ^2 (17.5 versus 25.9) fit to the experimental curve than the monomer (**Figure 43**).

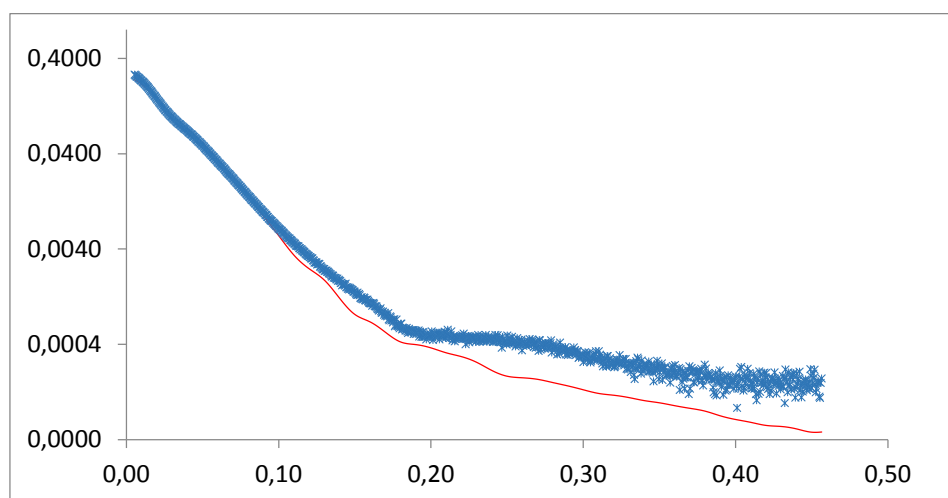


Figure 43 : CRY SOL fit (red line) and Carin1 experimental SAXS curve (blue stars)

DISCUSSION

Polysaccharide depolymerases facilitate viral infection and seem to be determinant in host specificities of many phages. Despite the prominence and the important biogeochemical role of phages in the environments, their functional mechanisms have been barely explored. Previously, we identified polysaccharide degrading activity in several marine phages and we found that the gene encoding this function is distantly related to known proteins. This study reports, for the first time, the biochemical characterization of a polysaccharide depolymerase associated to a marine phage, and provides a preliminary reconstruction of this unusual enzyme.

Enzymatic mechanism of Dpo31

The biochemical characterization indicated that Dpo31 is a glycoside hydrolase with a probable endo-lytic mode of action. The SEC-RI methods indicated that the size of the degradation products decreased with time. The appearance of products of intermediate size at early stage of the reaction is indicative of an endo-acting mechanism as reported for most phage depolymerase (Latka et al., 2017; Sutherland, 1999). The cleavage of glycosidic bonds all along the polysaccharide chain enable an efficient phage penetration in the EPS layer, even for polymers of high viscosity (Sutherland, 1999). The end-products were eluted shortly prior to the salt peak suggesting that they comprise two to three monomers. Detailed analyses are required to confirm and characterize end products. A collection of the different fractions followed-up by a composition analysis may give information about the species released, the original polysaccharide chain, and the mechanism of the enzyme.

Dpo31 can withstand range of temperature and pH that largely encompass the natural variation of these parameters in the environment. Optimal activity of this enzyme was recorded at 45°C and pH 8.5. These values are in the range of reported T°C and pH optima for depolymerases of *Klebsiella* phages, such as *K. pneumoniae* phage B5055 (40°C, pH 7.5) (Kassa and Chhibber, 2012; Majkowska-Skrobek et al., 2016). Surprisingly, NaCl significantly reduced Dpo31 activity (no activity at NaCl concentration higher than 350 mM) yet the addition of a cation decreased this effect. These results suggest that electrostatic interactions are

important for the enzymatic mechanism, which may be disturbed by the presence of salts. EPS are known for their ability to complex cations (C. S. Hassler et al., 2011; C. S. C. Hassler et al., 2011; Nichols et al., 2005). If released during depolymerization, those cations may take part in the reaction by stabilizing the catalytic domain and thus supporting the depolymerization process.

Further experiments may include the use of whole phage particles to investigate Dpo31 depolymerase activity. Polysaccharide depolymerases may surround the short phage tail by six copies, as observed with tailspikes of P22 (Lander et al., 2006). This molecular conformation may induce modification of the depolymerization activity, that would be interesting to investigate.

A novel protein structure suggesting a new glycoside hydrolase family

The structure analysis of Dpo31 revealed an original architecture, but also similarities of some sub-domains to tail-spike proteins. SAXS and crystallography data are congruent regarding the trimeric conformation of the protein and its elongated shape. The 236-Å-long homotrimer displays 2 major, structurally different domains, with β -helices at the C-terminal domain and intertwined α -helices at the N-terminal region. The orientation of the protein and attachment site to the phage capsid could not be solved.

The β -helix structures resolved at the C-ter domain are typical of phage tailspike and tail fiber proteins. Interestingly, some of these tailspike proteins display binding sites with polysaccharides (O-antigen, LPS) (Steinbacher et al., 1996; Weigele et al., 2003) or show capsule-depolymerase activity (Smith et al., 2005). The substrate binding sites of tailspike proteins are generally located at protruding modules anchored to the β -strands (Casjens and Molineux, 2012). Our enzyme displays such modules and we could expect similar interactions with polysaccharides at those sites. The surface of these module show pockets with reactive amino-acids which may take part in the hydrolytic reaction (for example Asp-522 and Asp-527), which could be expanded by analogy to potential sugar/polysaccharide binding domains.

Regarding the N-terminal region, the coiled-coil structure with the intertwined α -helices is reminiscent of the architecture of phage needles (Bhardwaj et al., 2011). These proteins are used by bacteriophages to penetrate the bacterial membrane and inject their DNA material. However, the reconstruction of this domain was challenging due to areas of poor electron

density, indicating that this N-terminal region is somewhat flexible. It is thus unlikely that this protein displays the needle function, although this hypothesis cannot be totally discarded thus far. But if in that way, we can expect a similar role of anchoring of the protein to its host, with these potential needle-like domains acting like harpoons on the bacterial membrane.

A central domain constituted of an entanglement of β -strands and α -helices was resolved between these two regions. Interestingly, a ligand, potentially a sugar unit, was found inside this domain. This ligand might have been incorporated during the overexpression method and stayed bound to the protein after the purification steps. This finding suggests that this domain potentially displays affinity to carbohydrates.

CONCLUDING REMARKS

We are currently refining the reconstruction of this molecule, yet the actual structure shows weak similarities to known molecules. Data collected thus far cannot provide a straightforward identification of the catalytic active site. The two potential sites of interactions with polysaccharides (at the C-ter and the central domains) need to be further investigated in order elucidate their functional roles. The lack of information about the orientation of the protein and its attachment to the virion is also a concern. Two potential scenarios can be envisioned. In the first scenario, the N-terminal domain is anchored to the phage capsid. The C-terminal domain would act as flexible arms terminated by three catalytic modules associated to the β -helix structure. This model would ensure an efficient depolymerization activity, with several flexible arms directed towards the bacterial EPS. In the second scenario, the C-terminal domain comprises the attachment module to the capsid. Catalytic modules would be less flexible, but the phage would display N-terminal anchors (or harpoons) targeting the host membrane. Such orientation would ensure both EPS depolymerization activity and firm anchoring of the phage to the host membrane.

CHAPTER III

Marine viruses influence the fate of bacterial exopolysaccharides

Pierre-Yves Mocaër, Marion Urvoy, François Thomas, Florian Lelchat, Sophie Le Gall, Marc Vernet, Christel Hassler, Mirjam Czjzek, Claire Boisset, and Anne-Claire Baudoux.

AUTHOR CONTRIBUTIONS

PYM and ACB devised the project, the main conceptual ideas and proof outline, with the help of FT and CB. Viral strains were isolated by FL and ACB, under supervision of ACB. PYM and MU carried out the virus production and purification, as well as the EPS production and degradation (methods development, optimization). The EPS analyses were performed by PYM (manipulation, methods adaptation and optimization). DOC analysis were performed by CH. The osidic composition analyses were performed by SLG (method development, manipulation, data analysis) and to a lesser extent, PYM (manipulation). PYM designed the microcosm experiment, supervised by ACB. Prokaryotic abundance measure was performed by PYM and MU (manipulation, adaptation). Bacterial production was performed by PYM and MV (manipulation), under the supervision of MV (methods). Extracellular hydrolytic activity assays were done by PYM (manipulation, method development, optimization) and MU (manipulation). Sugar content measurement were measured by PYM (methods development, optimization, manipulation). Bacterial diversity was assayed by MU (data analysis) and PYM (data analysis), under the supervision of FT. Statistical analysis were performed by PYM. PYM and ACB wrote the manuscript, supervised by ACB and with support of ACB, MC, FT and CH.

INTRODUCTION

The dissolved organic matter (DOM) represents the largest reservoir of marine carbon and it plays a pivotal role in the ocean functioning. Bacteria have been identified as an important source of DOM in the ocean. Components of bacterial cells are released as DOM in the ambient waters through variable biological processes, including the direct excretion of extracellular polymeric substances (EPS) but also the predation by viruses or metazoans (Culley et al., 2007; Lønborg et al., 2013; McCarthy et al., 1996; Middelboe and Jørgensen, 2006). Most metabolically active bacteria are surrounded by a capsular layer, which serve to the formation of aggregates, biofilms, nutrients storage, and provide cell protection (Elsakhawy, 2017; Nwodo et al., 2012). Natural bacteria assemblages were shown to constantly renew their capsular envelope by releasing dissolved parts of the capsule in the surrounding water (Heissenberger et al., 1996; Stoderegger and Herndl, 1999). This process comprises, on average, 25% of the bacterial respiration rate (Stoderegger and Herndl, 1998). Because bacteria account for the largest biomass compartment in the ocean, the release of capsular compounds likely form a major component to the total oceanic DOM (Benner et al., 1992; Heissenberger et al., 1996). Yet, the fate of these EPS in marine ecosystems is not well understood.

EPS produced by marine bacteria consist mostly of polysaccharides (hereafter referred to exopolysaccharides) but also proteins, lipids and DNA. The polysaccharidic moiety displays a wide chemical diversity with homopolymeric or heteropolymeric composition, including numbers of rare and unusual monosaccharides, a linear or branched backbones, and inorganic or organic substituents (Bramhachari and Dubey, 2006; Decho and Gutierrez, 2017; Drouillard et al., 2018; C. S. Hassler et al., 2011; Kokoulin et al., 2014; Nichols et al., 2005; Vincent et al., 1994). Compared to terrestrial counterparts, the exopolysaccharides released by marine bacteria typically contains higher level of uronic acids (D-glucuronic and D-galacturonic acid) (Chi and Fang, 2007; Delbarre-Ladrat et al., 2014), that provide a negative charge to these macromolecules. Laboratory experiments using natural bacterial assemblages amended with bacterial EPS suggested that these macromolecules are barely accessible to microbial utilization (Stoderegger and Herndl, 1998, 1999; Z. Zhang et al., 2015), with the *Flavobacteriia* (*Bacteroidetes*) identified as the main contributor to these utilization. It was thus proposed

that bacterial EPS represent a considerable fraction of semi-labile to refractory DOM and contribute to carbon storage in the oceans.

The huge molecular diversity of bacterial EPS implies that specific enzymes are required for their remineralisation, which would make them naturally resistant to bacterial ectoenzymes. Over the past two decades, EPS depolymerases have however been described in great number of viruses of bacteria (bacteriophages or phages). These enzymes are typically located on the virion's tail and viruses use them to degrade their host capsules in order to access their primary membrane receptors. Viral EPS depolymerases mostly include polysaccharidases (hydrolase and lyase) and they display a high level of functional and molecular diversity (Cornelissen et al., 2012, 2011; Latka et al., 2017; Leiman et al., 2007; Scholl et al., 2005; Sutherland, 1999; Yele et al., 2012). Interestingly, Lelchat et al. (2019) demonstrated that marine viruses can also display EPS depolymerases active on the dissolved exopolysaccharides released by their host. These enzymes have been shown to be active regardless of the substrate concentration, endo-acting (i.e. cleaving the polymer mid-chain), and they appear to be functionally diverse (F. Lelchat et al., 2019). There are an estimated 10^{30} viruses in the ocean, most of which are viruses that infect bacteria. It is commonly assumed that at least one, but usually multiple, viruses infect any living bacteria in ocean. This leads us to question whether virally-mediated EPS degradation could enhance their organic carbon lability and hence bacterial utilization with cascading impacts on the structure microbial communities.

The aim of this study was to examine how viral degradation influences the microbial utilization of bacteria EPS. To address this question, we selected two virus-bacteria host model systems for which viral ability to degrade their host EPS was demonstrated empirically (F. Lelchat et al., 2019). Native and virally degraded EPS were amended as the unique carbon source to seawater cultures of natural bacterial assemblages and short-term changes (over 48 hours) in carbohydrate concentration, bacterial activity and diversity were monitored during the course of the experiment.

MATERIAL AND METHODS

Bacterial and viral strains

Cobetia marina (DSMZ 4741) and *Vibrio alginolyticus* (VA34) are respectively infected by Carin1 and Vigo-2, two short tailed viruses from the *Podoviridae* family isolated from the surface water of the bay of Brest at the long-term monitoring station SOMLIT (4° 33' 07.19 W, 48° 21' 32.13 N) (F. Lelchat et al., 2019; Lelchat, 2017). Briefly, the seawater was concentrated using 100 kDa Vivaflow® 200 ultrafiltration units (Sartorius) and filtered through a 0.2 µm polyether sulfone (PES) syringe filter (Sartorius) as described in Lelchat et al. 2019. The filtrate was used in a plaque assay with either *C. marina* or *V. alginolyticus* grown in ZoBell medium (5 g/L peptone, 1 g/L yeast extract, 15 g/L agar in 80 % GF/F filtrated aged seawater and 20 % MilliQ water) and incubated at 20 °C. After 24 h, well defined plaque-forming units (PFU) were picked and eluted in sterilized ultrafiltered (0.2 µm) seawater and the procedure was repeated twice to ensure clonal phage population. Clonal virus lysates were then stored at 4 °C in autoclaved SM buffer (5.8 g/L of NaCl, 2 g/L of MgSO₄, 50 mL of 1 M Tris-Cl at pH 7.5 in MilliQ water).

Bacterial EPS production

Cryogenized stocks of *Cobetia marina* and *Vibrio alginolyticus* were streaked onto ZoBell petri dishes and incubated overnight at 20 °C. A single colony was then pre-cultivated overnight at 25 °C and 200 rpm in liquid ZoBell medium enriched with 30 g⁻¹L of glucose in order to induce EPS production. Once the exponential phase was reached, the pre-culture was inoculated (1% v/v) in 500 mL of glucose-amended ZoBell medium (3% w/v) and incubated for 72h (25°C, 150 rpm).

To purify the produced EPS, cultures were centrifuged at 7,000 g for 20 min (4 °C). Supernatants were collected, filtrated on 0.2 µm Stericup® filtration units (PES membrane, GP Millipore) and concentrated to approximately 50 mL via tangential flow filtration using 30 kDa cut-off Vivaflow® 200 cassette. Concentrate was dialyzed against MilliQ water (5 x 500 mL) in order to desalt the medium. Finally, the solution was frozen to -80 °C and lyophilised.

Virus production

Stock solution of viruses was reactivated by performing a plaque assay and eluting plaques in 7 ml of SM buffer. The titer of the viral lysates was determined by spot test. Briefly, the viral lysates were serially diluted in SM buffer (ten-fold increment) and spotted onto a host bacterial lawn obtained by mixing 1:4 of host grown in ZoBell supplemented with 0.6 % w/v of agar. After an overnight incubation at 20 °C, PFU were enumerated and the viral titer was then determined. The virus production was realised by incubating 10^7 host cells/mL with 10^6 PFU/mL in ZoBell medium for 18 h at 20 °C under an agitation of 60 rpm. Host precultures were performed as previously described and cellular abundances were determined by flow cytometry as described below. Infection was stopped by pelleting bacterial debris by centrifugation at 7,000 g and 4 °C for 30 min. Supernatant was then filtrated onto 0.2 µm PES Stericup® filtration unit to ensure a cell-free filtrate. The viral lysate was concentrated down to 1 mL by ultrafiltration using a 100 kDa Vivaflow® 200 unit followed by repeated centrifugation onto 50 kDa PES cut-off membrane Vivaspin® 20 columns (6,000 g, 4 °C). The concentrated viral cultures were then loaded on top of a sucrose linear gradient (10 to 40 % w/v) and ultracentrifuged at 134 074g for 45 min at 4 °C (rotor SW41 in a Beckman Optima XPN-80 centrifuge). The viral bands were collected using a sterile syringe needle, washed with SM buffer and concentrated using 50 kDa Vivaspin® columns three-times to remove trace of sucrose. The viral titer of the purified virus suspension was finally determined using the spot test method.

Viral degradation of EPS and purification of hydrolysis products

Viral degradations of EPS were performed by inoculating the purified viruses Carin1 and Vigo-2 (5.10^9 PFU mL⁻¹ final concentration) in, respectively, 0.2% (w/v) of the EPS of *C. marina* and *V. alginolyticus* diluted in SM buffer. The mixture was homogenised and incubated in the dark at 20° for 48h under gentle agitation. Low molecular weight hydrolysis products were then recovered after filtration onto a 100 kDa Vivaspin column. Controls containing the EPS alone were processed and incubated under similar conditions to check for potential auto-hydrolysis phenomena. The degradation patterns were visualized by running the native EPS and virus+EPS suspension on an agarose gel electrophoresis

Agarose gel electrophoresis

Samples at 4 mg mL⁻¹ were loaded for each bacterial EPS using a 4X loading buffer (Tris-HCl 62.5 mM pH 6.8; Glycerol 20%; DT 5 mM; Bromophenol Blue 0.025%) as follow: (i) the native EPS (N), (ii) the degraded EPS before removing of viruses and non-degraded parts (DnP), and (iii) the degraded EPS purified of viruses and High Molecular Weight (HMW) polysaccharides (D). The electrophoresis was performed for 90 minutes at 120V. The gel was then transferred in a 25% isopropanol bath (v/v) for 1 hour before overnight staining in a Stains All solution bath (10 mL of 3,3'-diethyl-9-methyl-4,5,4',5'-dibenzothiacarbocyanine 0.1% (w/v) in N,N-dimethylformamide, 10 mL of N,N-dimethylformamide; 50 mL of isopropanol; 10 mL of 300 mM Tris HCl pH 8.8, completed to 200 mL with MilliQ water, Sigma Aldrich). Destaining was finally performed by transferring the gel in distilled water for 2-hours under natural light.

EPS analyses

Carbohydrate, protein, DNA and DOC content of both intact bacterial EPS and hydrolysis products were quantified. Total dissolved sugars were dosed using Dubois method (Dubois et al., 1956). Briefly, 100 µL of 5 % phenol followed by 500 µL of 95 % sulphuric acid were added to 200 µL samples, allowed to stand at 80 °C for 30 min. Absorbance was read at 490 nm in a Spark plate reader (Tecan). Results were expressed in xanthan equivalent. Proteins were quantified according to the Bradford protocol modified by Bio-Rad for microplate reading. 160 µL of sample were mixed with 40 µL of Bio-Rad "Protein Assay Dye Reagent Concentrate". Absorbance was read at 595 nm using a Spark Tecan after a 5-min incubation at ambient temperature. Bovine Serum Albumin (BSA) was used as a standard. Double stranded DNA content was measured using the Qubit dsDNA High-Sensibility Assay Kit (Thermofischer) and the Qubit 2.0 Fluorimeter (Thermofischer). The DOC concentration was measured as follow: Samples were collected in glass tubes (washed with 10 % hydrochloric acid (HCl), washed MilliQ water and combusted 4-h at 500 °C prior to sampling). Samples were gravimetrically diluted with MilliQ water, acidified using HCl and quantified using a Shimadzu TOC-LCPH analysis system. Blanks consisting of MilliQ water were made.

Osidic composition analysis

The osidic composition of EPS was analysed by the BIBS platform (Research Unit BIA, INRA, Nantes). Quickly, identification and quantification of neutral sugars were performed by gas-

liquid chromatography (GC) after sulphuric acid degradation (Balti et al., 2018). 20 mg of EPS were hydrolysed using 1M H₂SO₄ at 110°C during 2h. Sugars were converted to alditol acetates as described in Blakeney et al. (Blakeney et al., 1983). They were then separated by chromatography on a TG-225 GC Column (30 x 0.32 mm ID) using TRACE Ultra Gas Chromatograph (Thermo Scientific, 205°C, carrier gas H₂). Standard sugar solution and inositol as internal standard were used for calibration. Standards refer to Sorbitol, Mannitol, Xylitol, myo-Inositol, N-acetylglucosamine, N-acetylgalactosamine, D-(-)-Fructose, D-(+)-Mannose, D-(+)-Galactose, D-(+)-Glucose, Glucuronic acid and Galacturonic acid (Sigma Aldrich). Uronic acid concentrations were measured using the metahydroxydiphenyl colorimetric acid method (Blumenkrantz and Asboe-Hansen, 1973).

Microcosm experiments

Microcosms design

Natural bacterial communities were sampled from surface waters of the SOMLIT station Astan off Roscoff (18/04/2018, 48°46'40" N, 3°56'15" W). This station is representative of the permanently mixed water column of the Western English Channel. 20L of water was filtered under gentle vacuum through 1 µm filters (Isopore PC membrane, Millipore) and concentrated using a 0.2 µm cut-off Vivaflow[®] 200 ultrafiltration unit. Bacterial concentrate was then diluted to initial volume in autoclaved aged seawater filtrated through GF/F filters and 30 kDa Vivaflow[®] unit.

A volume of 600 mL of bacterial inoculum were distributed into each microcosm and amended with 100 mg_{DOC}/L of native or virally degraded EPS from of either *C. marina* or *V. alginolyticus*. A control microcosm was inoculated with an equivalent volume of SM buffer. Microcosms were incubated for 48-h at 20 °C in triplicate and shaking was manually performed at each sampling time.

Sampling for prokaryotic abundance, bacterial production, extracellular hydrolytic activities and sugar content was sterilely done at 0, 12, 18, 24, 39 and 48-h. Additional samples were taken at 0, 24 and 48-h for bacterial diversity (**Schema summarizing the protocol, Annexes III.1**).

Prokaryotic abundance

Prokaryotic abundance was quantified using flow cytometry according to Marie et al. (1999). 1 mL samples were fixed with 0.5 % glutaraldehyde, incubated 10 min at ambient temperature, flash-frozen in liquid nitrogen and stored at – 80 °C. Prior to analysis, samples were thawed at 37 °C, diluted in 0.2 µm filtered TE buffer (50 mM of TRIS at pH 8.7, 1 mM of EDTA in milliQ water) and stained with 2 % (v/v) of 200X SYBR Green (Thermofischer) for 15 min in the dark. Cell enumeration was carried out using a BD FACSCanto II flow cytometer (Beckton Dickinson) equipped with a blue laser (488 nm) at medium flow rate.

Bacterial production

Bacterial secondary production was measured using the rate of incorporation of ³H-leucine as described by Kirchman et al. 1985 and modified by Smith and Azam 1992. 1.5 mL samples were amended with radioactive leucine at final concentration of 20–25 nM and incubated at 20 °C for 1 h in the dark. Leucine uptake was stopped by addition 0.2 mL of cold 45 % Trichloroacetic acid (TCA) and 50 µL of BSA (3.4 g/L). Samples were concentrated by centrifugation and rinsed with 5% TCA two times prior to radio assay by liquid scintillation counting in Ultima Gold cocktail (Perkin-Elmer, Waltham, MA). Measurements were performed in triplicates and blanks were done, skipping the incubation step.

Extracellular hydrolytic activity assay

Extracellular hydrolytic activity was assayed as described by Hoppe 1993, using six fluorogenic substrates consisting of monosaccharides (α -D-glucoside, β -D-glucoside, α -galactoside, β -galactoside, β -glucuronide and N-acetyl- β -D-glucosamine) labelled with 4-methyl-umbelliferone (MUF) (Sigma Aldrich). Stock solution of substrates were first dissolved in a volume of 2-methoxyethanol, and then volume was completed with MilliQ water. 195 µL of samples were mixed with 5 µL of substrate (100 µM final concentration) in a 96-wells microplate (CytoOne) and the increase in fluorescence was monitored every 5-min for 1.5-h using a Spark plate reader (Tecan) with respective excitation and emission wavelength of 364 and 460 nm. Enzymatic activities were determined as the slope of the linear fluorescence curve obtained. Standard solutions ranging from 120 to 1400 nM of MUF were simultaneously measured.

Sugar content

2 mL samples were centrifuged 15 min at 8,000 g and 4 °C. 1 mL supernatant was then frozen at – 80 °C. Total sugars were determined with Dubois assay as described above. Detection limit was observed at 4µg mL⁻¹.

Bacterial diversity

Bacterial diversity was determined using metabarcoding analysis of the V3/V4 region coding for 16S ribosomal unit. Initial diversity (0-h) was determined by collecting three-times 2 L of 1 µm filtrated natural seawater onto 0.2 µm Sterivex filters (Merck). 24 and 48-h sampling were done by collecting respectively 200 and 400 mL from each microcosm.

Sampling. Sterivex were frozen at – 20 °C until analysis. DNA extraction was performed by incubating each Sterivex with 1.5 mL of lysis buffer (128 g of sucrose, 25 mL of 0.2 µm filtrated Tris-base 1 M pH 8, 40 mL of 0.2 µm filtrated EDTA 0.5 M, completed to 500 mL with nuclease-free water) and 100 µL of 20 mg/mL lysozyme at 37 °C for 45 min under shaking. 20 µL of proteinase K (20 mg/mL, Macherey-Nagel) and 100 µL of 10 % SDS (Bio-Rad) were then added and incubated for 1 h at 55 °C under shaking. Sterivex content was transferred into clean Falcon tube and Sterivex were washed with 1 mL lysis buffer. A volume of isoamyllic phenol-chloroform (Eurobio) was added for each volume of lysed sample, tubes were centrifuged 15 min at 4,500 rpm and upper phase was transferred into a clean tube kept on ice. Nucleic acids were finally purified using a NucleoSpin PlantII Macherey Nagel kit.

Amplification and sequencing. Amplification and sequencing was performed by Fasteris company. DNA was amplified using standard primers (Forward: 5'–CCTACGGGNGGCWGCAG–3'; Reverse: 5'–GACTACHVGGGTATCTAATCC – 3', 550 bp amplicon, Herlemann et al. 2011). Sequencing was performed using MiSeq Flow Cell (Illumina) with a 15 million read coverage.

Data processing. Reads were demultiplexed using Fasteris internal script, adaptators and bad quality reads (< 15) were trimmed using Trimmomatic package and reads were joined into a single contig by pair-end joining using ea-utils “Command-line tools for processing biological sequencing data” (version 1.1.2 revision 537). Contig data were further processed using Frogs pipeline. Reads with correct size and primer sequences were selected (1 mismatch allowed), clustered into OTU (operational taxonomic unit) using Swarm method (aggregation distance = 1) and chimera were removed. Reads were further filtered by selecting sequences present

in at least two samples. Finally, data were mapped against SILVA 16S database (RDP assignation) and exported to R.

R analysis. OTUs corresponding to mitochondria and chloroplasts were removed prior to analysis. Phyloseq package was used for data rarefaction (*rarefy_even_depth* function, *rngseed* = 33 333), alpha (Shannon Index and specific richness) and beta diversity and Bray Curtis based NMDS. ANOVA analysis was done using *aov* function completed by *post-hoc* Tukey test (*TukeyHSD* function) from R-core package. PERMANOVA test was done using *adonis* function and variance homogeneity was checked for using *betadisper* test (Vegan package). DESeq2 package was used to checked for OTU abundance significant variations. Adjusted p-value inferior to 0.05 were considered significant.

Statistical Analysis

A one-way analysis of variance (ANOVA) was carried out to establish any significant differences in the experiment regarding the source and condition of the EPS. The differences were expressed as the mean \pm standard error and the level of significance was established at $P < 0.05$ (R package 'stat' (version 3.5.3) (Team, 2019)).

RESULTS

Degradation of EPS by viral enzymes

The ability of the selected viruses to degrade their host EPS was controlled by incubating host EPS with purified virus suspension and the depolymerization pattern was visualised by agarose gel electrophoresis (**Annexes III.2**). As observed in Lelchat et al. 2019, the migration of native *Cobetia marina* EPS (L₆) showed two EPS populations with distinct molecular weight. The incubation of L₆ with purified Carin1 induced a nearly complete depolymerization of the native EPS into products of low molecular weight. Regarding *Vibrio alginolyticus*, the incubation of native EPS with Vigo-2 resulted in the depolymerization of the products of high molecular weight, yet a population of intermediate molecular weight appeared to be resistant to viral degradation. The purification of the degradation products by ultrafiltration isolate the degradation products (<100 kDa) from viruses. Part of non-degraded *V. alginolyticus* polysaccharides however remained in the <100kDa fraction. As mentioned in methods and to eliminate this bias, all microcosms were inoculated with the same amount of measured DOC (100 mg L⁻¹ final).

Composition of bacterial EPS before and after viral degradation

C. marina and *V. alginolyticus* EPS comprised considerable amount of dissolved organic carbon (DOC). The initial DOC concentrations were of 2.49 g L⁻¹ for native *C. marina* EPS and 2.50 g L⁻¹ for native *V. alginolyticus* EPS. The DOC concentrations did not change significantly after viral degradation with values of 2.61 g L⁻¹ and 2.99 g L⁻¹ for virally degraded *C. marina* EPS and *V. alginolyticus* EPS, respectively ($P > 0.05$, ANOVA) (**Annexes III.3**). The proportions of proteins (< 0.35 %) and DNA (< 0.01 %) expressed in EPS dry weight were low, showing that the purification of the virally degraded product did remove all viral contaminants from the samples. The initial carbohydrates concentrations accounted for 25% and 18% of the EPS dry weight from *C. marina* and *V. alginolyticus*, respectively. After viral degradation, the proportion of carbohydrates decreased to 16.4% and 11.3% for *C. marina* and *V. alginolyticus* EPS, respectively (**Table 7**). Concerning the osidic composition of the EPS, the polysaccharide moiety of *Cobetia marina* EPS consisted of large amounts of neutral sugars (70%) and uronic acids (18%) while *Vibrio alginolyticus* EPS is composed of relatively higher proportion of uronic acids (34%), and a lower amount of neutral sugar (50%) (**Table 8**).

	<i>Cobetia marina</i>		<i>Vibrio alginolyticus</i>	
	Native	Degraded	Native	Degraded
DNA	0.96 ± 0.03	0.27 ± 0.01	0.18 ± 0.01	0.02 ± 0.01
Protein	3.5 ± 0.2	2.4 ± 0.2	3.2 ± 0.2	0.8 ± 0.1
Carbohydrate	251.6 ± 2.4	163.5 ± 1.2	180.4 ± 4.5	112.9 ± 4.9

Table 7: Composition of the bacterial EPS used in the experiment. In this table are shown the differences in DNA, protein, and carbohydrate composition for *Cobetia marina* EPS native or degraded by viruses, and for *Vibrio alginolyticus* EPS native or degraded. All data are represented in mg per gram of EPS.

		Rib	Man	Gal	Glc	GlcA	GalA	GalNAc	GlcNAc	%Total (Dw mass)
<i>Cobetia marina</i>	Native	0.56 ± 0.05	3.77 ± 0.06	1.03 ± 0.15	0.97 ± 0.15	1.28 ± 0.03	0.34 ± 0.02	0.15 ± 0.07	0.72 ± 0.04	8.83 ± 0.24
	Degraded	0.00	2.3 ± 0.55	0.8 ± 0.02	0.6 ± 0.02	1.2 ± 0.04	0.2 ± 0.04	0.1 ± 0.1	0.6 ± 0.19	5.8 ± 0.54
<i>Vibrio alginolyticus</i>	Native	0.00	5.63 ± 0.18	0.5 ± 0.06	0.7 ± 0.15	1.90 ± 0.08	3.22 ± 0.06	1.00 ± 0.11	1.21 ± 0.05	14.12 ± 0.56
	Degraded	0.00	6.15 ± 0.44	0.31 ± 0.11	0.34 ± 0.03	0.86 ± 0.39	2.80 ± 0.32	0.57 ± 0.02	1.10 ± 0.02	12.17 ± 0.60

Table 8: Osidic composition of the bacterial EPS used in the experiment. Rib: Ribose, Man: Mannose, Gal: Galactose, Glc: Glucose, GlcA: Glucuronic Acid, GalA: Galacturonic Acid, GalNAc: N-acetyl Galactose, GlcNAc: N-acetyl Glucose.

Utilisation of native and degraded EPS by marine bacterioplankton

To examine the impact of viral degradation of EPS on their utilisation by natural bacterial communities, we used 600 mL of seawater cultures with natural bacterial assemblages and added native or virally degraded EPS of *C. marina* or *V. alginolyticus* as the unique carbon source. Control microcosms that did not receive native or degraded EPS were also included. The influence of these amendments on total carbohydrate concentration, bacterial abundance, bacterial production, extracellular enzymatic activities and the diversity of bacterial communities were monitored during 48-h. These series of microcosm experiments with native, degraded, or no EPS as substrates indicated variable responses of the bacterial community.

Carbohydrate concentration

Carbohydrates were the most representative chemical compounds that we identified in bacterial EPS. The total carbohydrate concentration was highly dynamic throughout the experiment suggesting a succession of carbohydrate uptake and release in microcosms (**Figure 44**). Initial carbohydrate concentrations were of 27.8 and 18.1 $\mu\text{g mL}^{-1}$ for *C. marina* EPS, and of 17.7 and 11.4 $\mu\text{g mL}^{-1}$ for *V. alginolyticus* (native and degraded, respectively). The carbohydrate dynamics however differed depending on the EPS source and whether it was degraded by viruses. Regarding the *C. marina* EPS, carbohydrate concentration varied widely in the microcosms supplemented with native EPS with two cycles of consumption-release of carbohydrates ($P < 0.05$, ANOVA). By comparison, we did not record any significant changes in carbohydrate concentration in the microcosms amended with virally degraded EPS. Regarding *V. alginolyticus* EPS, two successive production peaks were observed with native EPS ($P < 0.05$, ANOVA), whereas microcosms amended with degraded EPS showed no significant decrease or increase in carbohydrate concentration ($P > 0.05$, ANOVA).

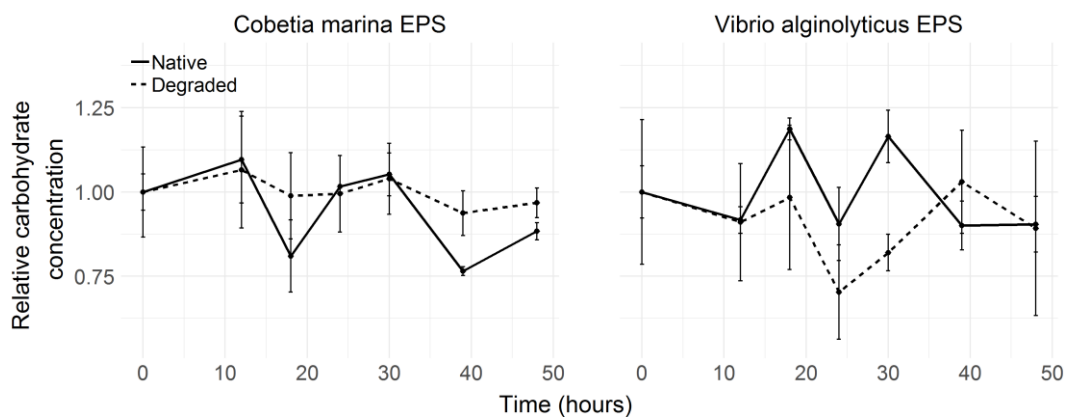


Figure 44 : Carbohydrate dynamics in microcosms analyzed over time. Values were normalized to the carbohydrate concentration at T0 for each treatment. Error bars represent standard deviation (n= 3).

Protein and DNA concentrations were not monitored as their concentrations were under the detection limit. Carbohydrate concentrations in control microcosms without EPS addition were under the detection limits (data not shown).

Abundance

The initial bacterial abundance in all microcosms were of 4.22×10^4 cell mL⁻¹. Control microcosms (**Figure 2**, no EPS addition, dotted line) showed a slight increase in bacterial abundance reaching 2.85×10^6 cell mL⁻¹ at the end of the experiment. The addition of EPS, regardless of its origin, induced a considerable bacterial growth compared to the control ($P < 0.001$, ANOVA). In the microcosms supplemented with native EPS, the bacterial grew exponentially between 20 and 30 hours of incubation and reached a concentration of 3.00×10^7 cell mL⁻¹ with *C. marina* EPS (**Figure 45A**) and 4.04×10^7 cells mL⁻¹ with *V. alginolyticus* EPS (**Figure 45B**) after 48 hours. Compared to native EPS, the addition of degraded EPS was less efficient in promoting bacterial growth and final bacterial concentrations (Both *C. marina* and *V. alginolyticus* EPS, $P < 0.001$, ANOVA). Overall, bacterial abundances were three- and two-fold lower at the end of the experiment for *C. marina* and *V. alginolyticus*, respectively.

Bacterial production

Initial bacterial productions in microcosms were 1 to 10 ngC L⁻¹ h⁻¹. The production in control microcosms increased after 30 hours of incubation and reached $4 \mu\text{g}_\text{C} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ at 48 hours. Addition of EPS induced a significant modification of the bacterial production regardless of its origin or its treatment. Patterns observed are in accordance with the bacterial abundance results with an increasing of values between 18h and 24h of incubation. In microcosms amended with native EPS, the bacterial productions reached a maximum of $4.9 \mu\text{g}_\text{C} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ at 30 hours of incubation with *C. marina* EPS and a maximum of $11.3 \mu\text{g}_\text{C} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ at 24 hours for *V. alginolyticus* before decreasing to $2.4 \mu\text{g}_\text{C} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ and $5.1 \mu\text{g}_\text{C} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$, respectively. Compared to native EPS, bacterial production was lower once EPS were degraded by viruses, with a maximum difference at 30 hours for *C. marina* ($P < 0.001$, ANOVA) and at 39 hours for *V. alginolyticus* ($P < 0.001$, ANOVA). Maxima of production reached $1.5 \mu\text{g}_\text{C} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ and $7.8 \mu\text{g}_\text{C} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$ at 24 hours for *C. marina* and *V. alginolyticus* EPS, respectively, before decreasing to 0.9 and $3.2 \mu\text{g}_\text{C} \cdot \text{L}^{-1} \cdot \text{h}^{-1}$, respectively (**Figures 45C and 45D**).

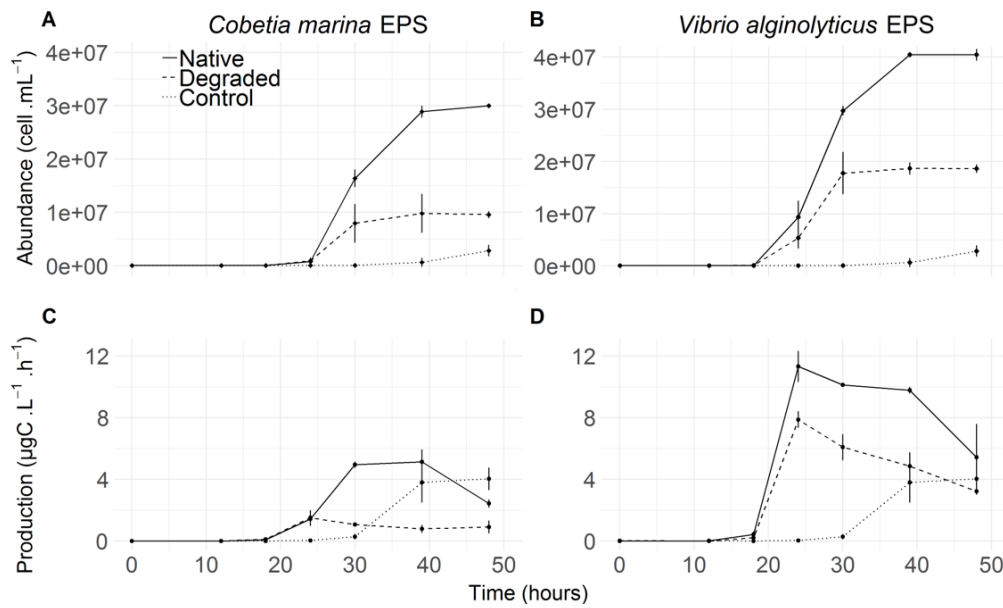


Figure 45: Bacterial abundance (A, B) and production (C, D) over the course of experiment. The control microcosms with no EPS added are shown dotted lines. Error bars represent standard deviation ($n=3$).

Extracellular Enzymatic Activity (EEA)

EEA were measured after 1-h of incubation with MUF substrates and were detectable only during the first 24 hours of the experiment. No detectable EEA activities could be recorded in the control microcosms (no EPS addition). In microcosms amended with EPS, EEA was recorded with the six selected MUF substrates and relatively high EEA was recorded in presence N-acetyl β D-glucosamine substrate (**Figure 46**). For *C. marina* EPS, no differences in EEA were observed between microcosm amended with native or degraded EPS ($P > 0.05$, ANOVA). Regarding the *V. alginolyticus* EPS, significant reduction in EEA was observed with degraded EPS with the B-glucosidase, B-glucuronidase ($P < 0.05$), B-galactosidase and the N-acetyl B D-glucosaminidase activities ($P < 0.001$, ANOVA). However, the α -glucosidase activity was greater ($P < 0.05$, ANOVA).

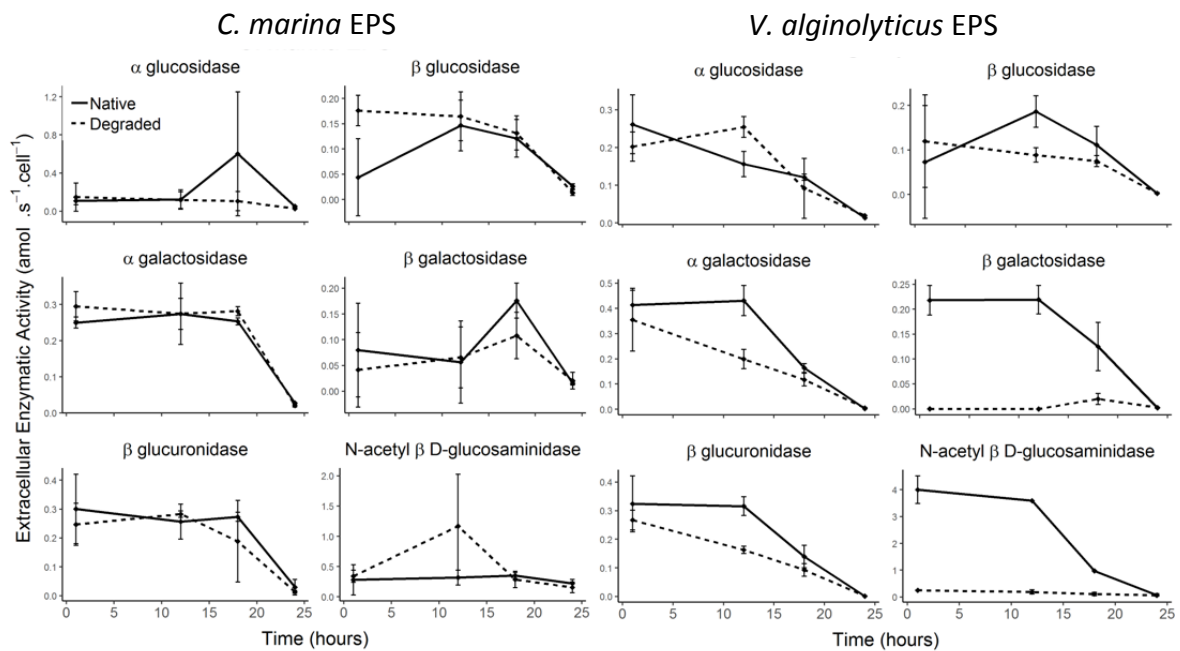


Figure 46: Extracellular Enzymatic Activities (EEA) in microcosms over 24-h. Error bars represent standard deviation ($n=3$).

Diversity and structure of the bacterial communities

To understand the impact of the EPS degradation by viral enzymes on the bacterial community, bacterial diversity was analysed in the different microcosms at 0, 24 and 48 hours. Rarefaction curves (**Annexes III.4**) showed that the sub-sampling of sequences does not affect the quality of the data. After quality controls and filtering, a total of 3,434,320 sequence reads were obtained, which were classified into 3092 different OTUs. Of these OTUs, 3029 and 2748 OTUs were classified at class and family levels, respectively.

The initial bacterial community was mainly composed of *Alphaproteobacteria* (49,4%), *Bacteroidia* (21.8%) and *Gammaproteobacteria* (17.5%). Minor classes (<4%) were represented in ascending order by *Acidimicrobiia*, *Thermoplasmata*, *Verrucomicrobiae*, *Oxyphotobacteria*, *Planctomycetacia*, *Deltaproteobacteria*, *Pla3_lineage*, *Dadabacteria*, *Nitrospina*, *Campylobacteria*, OM190 and *Gracilibacteria*. As shown in **Figure 47**, the bacterial diversity dropped considerably after 24-h of incubation in all the conditions (control and EPS treatments). The number of OTUs dropped from 1044 initially to 242 OTUs on average. After 48 hours of incubation, *Gammaproteobacteria* OTUs dominated the other classes, accounting for about 95% of the total in all conditions. For the microcosms amended with *C. marina* EPS,

the number of OTUs dropped significantly between 24-h to 48-h for both native and degraded conditions ($F=9.65/p\text{-value}=0.036$ and $F=8.50/p\text{-value}=0.0435$, respectively) whereas the alpha diversity (Shannon index) increased slightly with degraded EPS. Regarding *V. alginolyticus* EPS, the number of OTUs remained the same at 24 and 48 hours but the Shannon index increased in both conditions ($F=739.2/p\text{-value}=1.36 \cdot 10^{-5}$ with native EPS, and $F=77.82/p\text{-value}=9.14 \cdot 10^{-4}$ with degraded EPS) (**Annexes III.5**).

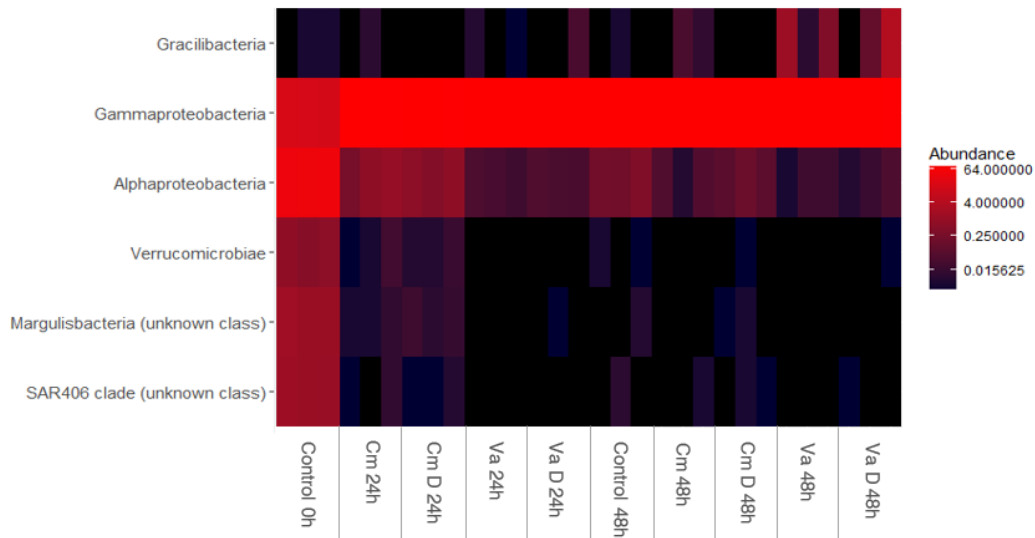


Figure 47: Heat-map showing bacterial class abundancies varying significantly between T0 and 24/48 hours of incubation ($p\text{-value}<0.05$). Cm: *Cobetia marina* native EPS; Cm D: *C. marina* EPS degraded by viruses; Va: *Vibrio alginolyticus* native EPS; Va D: *V. alginolyticus* EPS degraded by viruses

The Bray-Curtis NMDS analysis showed that the bacterial communities that developed following bacterial EPS amendments were different compared to the control (**Annexes III.6**). Interestingly, communities growing in microcosms amended with *C. marina* and *V. alginolyticus* EPS were significantly different (PERMANOVA, $F=3.71$ and $p\text{-value}=0.021$) (**Figure 48**). Specifically, *C. marina* EPS promoted the growth of the *Thalassotalea* genus, whereas EPS from *V. alginolyticus* allowed the growth of *Gracilibacteria* (**Annexes III.7**). Considering the dominance of *Gammaproteobacteria* in microcosms amended with bacterial EPS, the composition of this phylum was analysed in greater details after 48-h of incubation. Whereas control microcosms are represented by members of the genera *Vibrio* (85%), *Psychromonas* (5.8%), *Marinomonas* (2.8%) and *Alteromonas* (1.7%), microcosms amended by bacterial EPS are predominated by members of *Pseudoalteromonas*, *Vibrio* and *Colwellia* genera. Higher

proportion of *Colwellia* members appeared in microcosms amended with *C. marina* EPS (4.6%) compared to *V. alginolyticus* EPS (**Figure 49**). Interestingly, the proportion of *Pseudoalteromonas* OTUs was higher with degraded EPS. They represent 84% against 78.7% with *C. marina* EPS, and 83.3% against 52.9% with *V. alginolyticus* EPS.

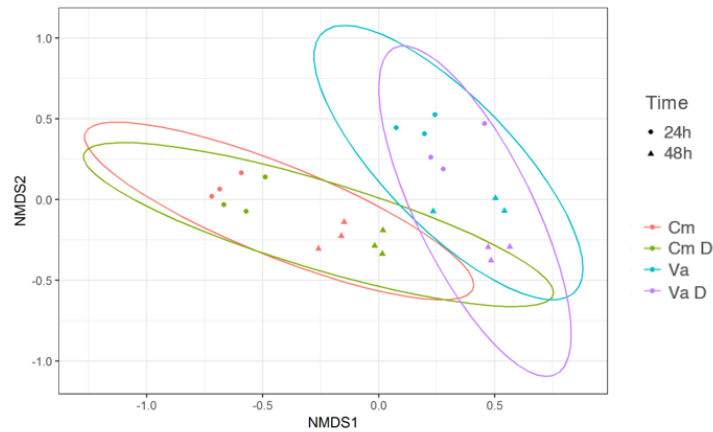


Figure 48: Bray-Curtis NDMS plot showing the modification of bacterial communities over time with the different EPS conditions. Ellipses represent a confidence interval of 95% and comprise the bacterial diversities of the different condition at 24h (circles) and 48h (triangles).

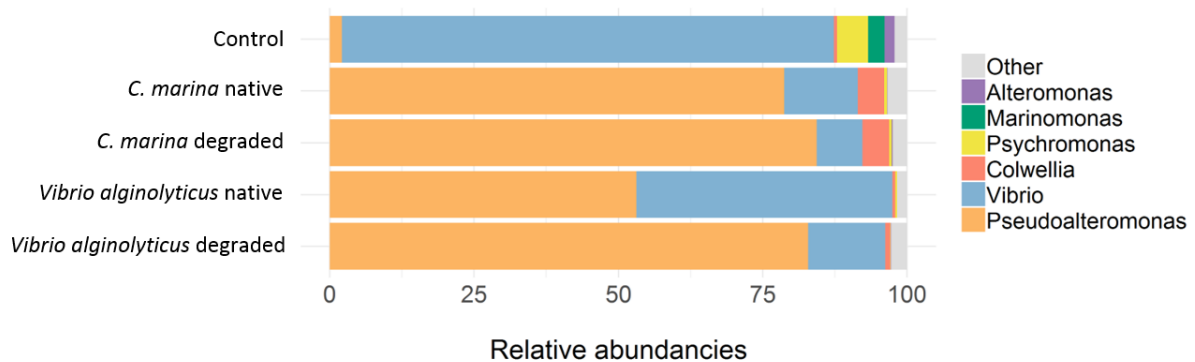


Figure 49: Relative abundancies of genus within Gammaproteobacteria (% of OTUs at 48 hours).

DISCUSSION

Most of the oceanic DOC (>95%) is refractory to microbial oxidation and persists in the ocean for extended periods of time. Even minor changes in the concentration of this refractory DOM could significantly affect carbon sequestration in the ocean with consequences for the climate (Jiao et al., 2010). Still, little is known about the mechanisms involved in the production of refractory DOM.

Marine bacteria constantly release dissolved EPS deriving from their capsule in marine waters and thereby are an important source of DOM. Laboratory experiments using natural bacterial assemblages suggested that these capsular compounds are partially accessible to microbes. The recent finding that marine viruses possess EPS depolymerases that efficiently degrade polysaccharides excreted by their host (F. Lelchat et al., 2019) led us to question whether viruses can modify the bioavailability of these compounds to the microbial communities. This study revealed that the depolymerisation of bacterial EPS by viruses reduced their utilisation by bacterioplankton and influences the composition of bacterial communities. Considering the predominance of viruses in the sea, this, so far, neglected process could generate considerable portion of refractory marine DOM and may have important biogeochemical implications.

Bioavailability of native bacterial EPS

Although the production of bacterial EPS was identified as a considerable source of marine DOM, only few studies (two to our knowledge) have explored their bioavailability (as a carbon source) to the microbial communities (Stoderegger and Herndl, 1998; Z. Zhang et al., 2015). Here, we investigated the utilization of EPS deriving from two ubiquitous marine *Gammaproteobacteria* known to be active EPS producers (Drouillard et al., 2018; Kokoulin et al., 2014; Lelchat et al., 2015; Maréchal et al., 2004). Both bacteria released complex heteropolysaccharides, containing high level of uronic acids, and thereby representative of bacterial EPS in marine systems. In contrast to previous studies reporting that bacterial EPS were barely accessible to the marine bacterioplankton, *C. marina* and *V. alginolyticus* EPS could substantially support bacterial growth within 48 hours of incubation. Theoretical calculations, using the bacterial abundance and an averaged cellular carbon content of 149 fg

of carbon per cell (Vrede et al., 2002), suggested that a small, yet significant, fraction of the EPS species (4.5% for *C. marina* and 6% for *V. alginolyticus*) was accessible to surface water bacterioplankton used in this study. The dynamics of total carbohydrate and extracellular glycoside activities indicated that, at least, part of the polysaccharide fraction was consumed by bacteria. The glycosidase activities measured liberated enough carbon to support bacterial growth with the N-acetyl glucosaminidase activity accounting for most of it. Regarding the microcosm experiment, longer incubation periods would have been required to examine whether the remaining EPS could be utilized by the community and in what time frame. The consumption of bacterial EPS would also need to be examined with a supplement of inorganic nutrients as these may influence the bioavailability of bacterial derived DOM (Stoderegger and Herndl, 1998; Z. Zhang et al., 2015).

The analysis of the bacterial community structure indicated that EPS produced by *Cobetia marina* and *Vibrio alginolyticus* selected distinct bacterial populations at the genus level resolution. *Alphaproteobacteria*, *Bacteroidia* and *Gammaproteobacteria* dominated the initial community, but the first two groups disappeared almost completely in favour of *Gammaproteobacteria* after 48h of incubation. *Alphaproteobacteria*, like SAR11 members, own a limited CAZyme repertoire (majority of exo-enzymes) that is unable to significantly degrade intact polymers. These bacteria rather act on terminal degradation products and are considered as DOM end users (Barbeyron et al., 2016). *Bacteroidia* also disappeared almost completely over time, while they are considered as pioneer micro-organisms, specialists in the use of macromolecules. Studies focused on the utilization of DOM derived from algae showed that *Bacteroidia* usually bloom first and provide substrates available for the growth of other clades of bacteria, including *Gammaproteobacteria* (Teeling et al., 2016, 2012). Our results, suggested that the amendment of bacterial EPS could have mimicked such a bacterial successive response. Among the *Gammaproteobacteria* promoted by bacterial EPS, two cosmopolitan and copiotrophic genera *Vibrio* and *Pseudoalteromonas* dominated. *Vibrios* comprised one of the most diversified genera. They usually display an opportunistic life-style likely related to their large enzymatic repertoire that enables the use a wide range of substrates (chitin, agar, alginate, laminarin, pectin, etc.). *Pseudoalteromonas* can also degrade complex polysaccharides and such capacities can be acquired by horizontal gene transfers (HGT) (Gobet et al., 2018; Hehemann et al., 2017). The predominance of these same genera

was also reported in microcosms containing labile DOM (glucose or EPS supplemented with glucose or amino acid (Elifantz et al., 2005)). The relative proportion of *Vibrio* and *Pseudoalteromonas* varied depending on the treatment. While *C. marina* EPS promoted the growth of *Pseudoalteromonas* and *Thalassotalea*, two genera of *Alteromonadales*; *V. alginolyticus* EPS favoured the presence of *Vibrio*. It is interesting to observe that the EPS produced by a bacterium are preferentially consumed by the genus to which this bacterium belongs. The EPS source also selected for specific genera. Members of the Gracilibacteria genus developed only on *V. alginolyticus* EPS while *Thalassotalea* genus specifically developed on *C. marina* EPS. Taken together, these results showed that bacterial EPS comprised variable proportion of refractory and labile DOM mostly up-taken by members of the *Gammaproteobacteria*. The origin of these compounds (and subsequently their chemical composition) are determinant for their bioavailability to the marine plankton (Li et al., 2016; Stoderegger and Herndl, 1998, 1999).

The impact of virally-mediated degradation of bacterial EPS for microbial communities

The rather recalcitrant nature of bacterial EPS was, so far, mainly explained by the chemical and structural diversity of EPS, which implies the use of specific set of enzymes for complete depolymerization. The finding that viruses own enzymes that efficiently depolymerize their host EPS led us speculate that they could modify the bioavailability of these compounds to the microbial community. Viral EPS depolymerases, including those of *Cobetia marina* viruses, are usually endo-active enzymes that cleave glycosidic bonds all along the polysaccharide chains and generate oligosaccharides (F. Lechat et al., 2019; Sutherland, 1999). We could thus expect that viruses provides “ready-to-consume” substrates to the microbial communities and stimulate their growth. As shown in a previous study, the virus Carin1 induced a nearly complete depolymerization of *C. marina* EPS into products of low molecular weight < 100 kDa. Likewise, Vigo-2 actively degraded *V. alginolyticus* EPS, except for a population that remained in the 100 kDa size range. Yet, our results indicated that the bacterial growth supported by the virally mediated hydrolysis products was 2 to 3-fold lower than their respective native EPS.

The analysis of EEA indicated a modification of the global carbohydrate metabolism. Overall, viral degradation induced a reduction in the hydrolysis rate of MUF-substrates (1/6

MUF-substrates considering *C. marina* EPS source, 4/6 with *V. alginolyticus* EPS). It is possible that viruses generated oligosaccharides that could be assimilated without further hydrolysis and sustained the development of taxons specialized in the uptake of low molecular weight substrates. Although no significant differences could be detected at the genus resolution, our diversity analysis showed that virally degraded EPS supported the growth of a higher proportion of *Pseudoaltermonas* members compared to native EPS. The remaining EPS substrates do not appear to be readily usable by the community, at least on the short-term. Because viruses were shown to act on the carbohydrate moiety of bacterial EPS, we mainly focused our analysis on the dynamics of carbohydrates and proxies of carbohydrate metabolism. It is, however, possible that viral degradation reduced the availability of other nutrients scavenged in bacterial EPS. A key role of polyanionic marine EPS, such as the EPS containing high levels of uronic acids, is in its potential role in binding iron (Fe^{3+}) and controlling the bioavailability of this vital trace metal (Gutierrez et al., 2008; C. S. Hassler et al., 2011). Interestingly, we observed that the EPS of *V. alginolyticus* that contained higher proportions of uronic acids compared to *C. marina* was more bioavailable. The EPS depolymerization by viruses may decrease the complexing properties of these polymers and limit the development of the microbial community. Another reason explaining the reduced bioavailability of degraded EPS could be that the hydrolysis products act as extracellular signals inhibiting the growth of congeners. Bacterial EPS are known to contain extracellular vesicles that can package a wide range molecules including DNA, RNA, enzymes, antibiotics and signal molecules (Biller et al., 2014; Irie et al., 2012; Kawaharada et al., 2015; Kuehn and Kesty, 2005). The release of these vesicles subsequent to the depolymerization of EPS matrix by the viruses may induce growth inhibition of conspecifics. Likewise, oligosaccharides deriving from the cell wall have been identified as defence elicitors in marine algae (Potin et al., 1999) yet this has never been documented in bacteria to our knowledge. Additional experiments are certainly required to understand why the hydrolysis products deriving from viral degradation comprise mainly refractory forms of DOM.

Biogeochemical and ecological implications

This modification of bacterial EPS structure and bioavailability induced by viral enzyme could be of a greater impact at larger scale. The degradation of bacterial EPS by viruses may contribute to the increase the semi-labile and refractory pool of DOM in the ocean.

Virus role on the modulation of the refractory carbon pool is likely not constrained to bacterial capsular and excreted EPS. It is commonly acknowledged that phytoplankton releases high amounts of EPS, especially during bloom events. Viruses are known for their ability to contain phytoplanktonic blooms thanks to their exponential replication when their host are of high abundance. Many CAZymes are reported in databases regarding viruses of cyanobacteria (*Synechococcus* and *Prochlorococcus*) and microalgae (*Chlorella* viruses, *Emiliania huxleyi* viruses and *Ostreococcus tauri* viruses) but never investigated. If viruses of phytoplankton are able to degrade EPS of their hosts, further work would be required to explore the bioavailability and the fate of those EPS after degradation by viral enzymes.

Knowing that the structure and composition of microbial EPS are highly complex we could expect an equal (if not larger) diversity of EPS degrading enzymes amongst viruses, which will be able to constantly degrade dissolved EPS in the water column. On a long time-scale, this enzymatic reservoir may also have a role in the formation/degradation of polymer aggregates (i.e. TEPs and bacterial biofilms). It is currently acknowledged that bacterial EPS are transiting between dissolved-, colloidal-, and gel-states (Decho and Gutierrez, 2017). Further studies may confirm whether viral enzymes could impact these transitions. Moreover, EPS aggregates act as ecological niches for bacteria. Degradation of those aggregates by viral enzymes may impact negatively the stability of such bacterial communities and further exploration on larger time-scales may provide interesting information regarding this process. Finally, in terms of bacterial aggregates, such viral enzymes could help the release of newly formed viruses, a mechanism that was not considered to this date (Bettarel et al., 2015).

CONCLUDING REMARKS.

Viruses are undoubtedly the most abundant biological entities in the ocean (Suttle, 2007). Any given marine bacterium is probably the host of one (but probably multiple) viruses (Breitbart et al., 2007). The detailed study of *Cobetia marina* and 5 associated viruses revealed that their ability degrade host EPS is a widespread character (F. Lechat et al., 2019). Although the structure and composition of microbial EPS are highly extensive, we could expect an equal (if not larger) diversity of EPS degrading enzymes among viruses. Of course, our results cannot be directly translated to *in situ* conditions but they suggest that viruses may depolymerase a significant fraction of the dissolved EPS derived from marine bacteria and contribute actively to the production of refractory DOC. In addition, bacterial EPS are known to transit between several states and may also take part in the formation of larger polymer aggregates (i.e. marine snow). It is likely that viral EPS depolymerases alter the reactivity of these molecules with important consequences for the ocean functioning. To date, the role of viruses has mainly been attributed to the mortality and the subsequent cell lysis that they impose on their host. Viral lysis induces the release of up to 150 Gt C per year, which mostly comprises labile DOM (Suttle, 2005). Our study suggests a dual role of viruses in the cycling of DOM in the sea. Viruses mediate the production of one of the largest flux of labile marine DOM through the lysis of their host, but they may also alter the size spectrum of the EPS produced by their host leading to the formation of refractory DOM. Considering the predominance of viruses in the sea, this, so far, unappreciated process could have important implications for the structure and the functioning of the ocean.

GENERAL DISCUSSION

MAIN RESULTS OF MY PHD PROJECT

The overarching objective of my PhD research was to characterize the polysaccharide depolymerases associated to marine viruses in order to understand their ecological role and their implication in biogeochemical cycles. During my master research (2016), I contributed to the detection of such enzymes in marine phages (Lelchat et al. 2019). This study provided, for the first time, experimental evidences of EPS degrading activities associated to marine phages and it demonstrated that these properties conferred advantages to phages to adsorb onto their hosts' membrane during intense EPS production (F. Lelchat et al., 2019). These findings led us to question the diversity of such enzymes in the marine environment, their enzymatic mechanisms, their role in the infection process, and their implication for the carbon recycling. To achieve this goal, we devised an integrative strategy, from the gene to the ecosystem. Our approach is divided into three major research areas, which corresponded to the three chapters of this thesis.

The chapter I aimed to identify the genes encoding this activity in cultured phages in order to determine their global distribution (Phylogeography) in the environment by interrogating viromes collected across the ocean. A combination of genomic and targeted activity screening using the *Cobetia* phage Carin1 and the *Vibrio* phage Vigo2 led to the identification of two new genes encoding polysaccharide depolymerases. The genes coding for these enzymes showed weak similarities to known sequences, and they appeared to be rare in marine environments. This study suggests that polysaccharide depolymerase associated to these phages probably constitute novel enzyme families and puts forward that marine viruses may represent an unappreciated reservoir of novel depolymerases.

The chapter II focused on the functional and structural characterization of the polysaccharide depolymerase associated to the phage Carin1 (Dpo31). Such analyses are essential to elucidate the mode of action of the enzymes and to predict the effect of environmental factors on enzymatic activity. This study showed that Dpo31 is a glycoside hydrolase, acting endolytically on a narrow range of substrate. This enzyme displayed a broad tolerance to temperature (inactivation at 60°C) and pH (5.5 – 9.5) with optimal activity at 45°C and pH 8.5. Although the depolymerisation occurred over a broad range of NaCl concentration (0 – 600

mM), increasing salt concentrations impacted negatively Dpo31 activity. The preliminary structure of Dpo31 was solved at 2.2 Å resolution and revealed a 236-Å-long homotrimer that was never reported in previous studies. Two structurally distinct domains were identified: a C-terminal region characterized by a β -helix and a N-terminal region with intertwined α -helices. The reconstruction of the C-terminal region shared structural features with tailspike proteins of terrestrial phages. We also predicted a potential catalytic module that may be involved in the polysaccharide depolymerization. The N-terminal region contains structural features reminiscent of phage needles and may have an anchoring role during the infection process.

Further studies will aim to elucidate the catalytic mechanisms of this enzyme as well as its role during host infection. Thus far, we do not know with certainty which domain interact with the substrate (i.e. *C. marina* EPS). Comparison with previous studies suggests a module in the C-terminal region to be the catalytic domain (Casjens and Molineux, 2012), but our findings showed also the presence of a putative sugar unit in a central region of the protein. The preparation of protein crystals with the substrate may help to identify the structural domain which interacts with the substrate; And may also give information regarding the cleavage site within the polysaccharide chain. Additionally, SAXS data should provide information on the actual behaviour of the enzyme structure in solution. These results are determinant to understand the enzymatic conformation in environmental conditions. Lastly, we plan to resolve the 3D structure of Carin1 particle by cryo-electron microscopy to elucidate the orientation of Dpo31 in the viral particle and identify the attachment module to the capsid. The cryo-EM reconstruction should also confirm the suspected function of the N-terminal region to act as “harpoons” anchoring the bacterial membrane before the injection of the DNA material.

The third chapter aimed to investigate the implications of polysaccharide depolymerases for the marine carbon cycle. The ability of different phages to depolymerize EPS excreted by their bacterial hosts led us to question about their implications for the recycling of these polymers in the environment. It is commonly accepted that heterotrophic marine bacteria enzymatically hydrolyze complex EPS to assimilate them. In Chapter III, we hypothesized that viral polysaccharide depolymerases participate in the degradation of dissolved polysaccharides, generating degradation products that are more easily assimilated by bacterial communities.

To test this hypothesis, we have cultured marine bacterial communities with two sources of bacterial EPS (those of *C. marina* or *V. alginolyticus*) native and degraded by their respective phage. The monitoring of various physicochemical parameters, bacterial growth and diversity showed that, in the short-term (48h), a small but significant fraction of EPS was accessible to bacterial communities. The EPS, as a sole carbon source, promoted the growth of *Gammaproteobacteria* yet the structure of bacterial communities (resolved at the genus level) was influenced by the source of the EPS. The viral degradation of EPS reduced their bioavailability to bacterial assemblages and modifies their composition with a relatively higher contribution of *Pseudoalteromonas* genus to the global community. Although the reasons explaining these results are still not clear, this study suggests an unsuspected role of marine viruses on the fate of bacterially derived DOM.

DISCUSSION AND RESEARCH PERSPECTIVES

From unknown genes to biological functions

Over the past decades, research on marine viruses has drastically moved away from bulk measurements of virally-mediated mortality to an intensive collection of viromes across the oceans (Coutinho et al., 2017; Hurwitz and Sullivan, 2013; Nishimura et al., 2017; Zeigler Allen et al., 2017). Metagenomics is an invaluable tool for the study of biological interactions, the functional diversity of microbial community, and the discovery of new molecules. Yet, our ability to study the viromes is limited by the number of unknown genes (up to 90%, (Krishnamurthy and Wang, 2017)) in these metadata. The characterization of this undescribed diversity is now a major challenge. In this context, my PhD project aimed to search and characterize specific protein encoding genes, the virion-associated EPS depolymerase, that were recurrently reported in phage that infect bacterial pathogens (Cornelissen et al., 2011; Hernandez-Morales et al., 2018; Kassa and Chhibber, 2012; Lin et al., 2014; Majkowska-Skrobek et al., 2016). As could be expected, the genes encoding EPS depolymerases in marine viruses were distantly related to known molecules with similar function. The identification and the characterization of these enzymes was a demanding and, at times, frustrating work; nevertheless, the payoff for this approach has been substantial. We provided a biochemical

annotation to genes that would have been otherwise overlooked and, thereby, contributed to enrich current reference databases, which is crucial to the interpretation of huge amount of protein encoding sequences generated by metagenomics.

We however realized that detecting EPS depolymerases associated to marine phage in the environment may constitute a substantial challenge. The chemical diversity of bacterial EPS is almost infinite (Laine, 1994) and because most bacteria are infected by at least one (but usually several) viruses, we expect that the EPS depolymerase diversity is similarly high or even greater. We are, of course, aware that bringing thousands of virus – host model systems into collection and characterize their associated depolymerases is presently impossible. Our results suggest that using the structure and the genetic environment of this protein may be more appropriate than using their amino acid sequence to deduce their biological function and study their distribution in complex environments. Advances in computer based structure prediction methods are currently attracting considerable interests especially for modeling “difficult” proteins that do not have close homologs in the reference (Baker et al., 2019; Emerson et al., 2018). The application of such tools may facilitate the detection of viral EPS polymerases in complex samples. The expression and functional assessment of the identified enzyme may confirm their functional activity in natural settings as reported recently (Emerson et al., 2018). Expanding the actual collection of virion-associated depolymerases and gaining basic knowledge about their reaction mechanisms by resolving their 3D structure is, however, a pre-requisite prior to more global analyses. We anticipate that such strategy should provide novel fundamental insights into the functional mechanisms of marine viruses and the extent of depolymerase gene transfer in the environments.

From functional assignments to ecological implications

The research carried out in the laboratory on the viral depolymerases suggests that EPS degrading activities are rather common in marine phages (Lelchat PhD thesis, 2014, Lelchat et al. 2019, this PhD thesis). Such ability to degrade host EPS is not surprising because it is long known that nearly all metabolically active bacteria in the ocean exhibit a capsule of polysaccharides that they constantly renew by releasing capsular compounds into the ambient water (Heissenberger et al., 1996; Stoderegger and Herndl, 1998). During my master, we showed that a given host (*Cobetia marina*) could be infected by phages that either displayed or not EPS depolymerase activity. Among five *Cobetia* phages, four had EPS degrading

properties (F. Lelchat et al., 2019). The ability to degrade the host was related to rapid phage adsorption during intense EPS production and may thus provide an ecological advantage to the phage during harsh environmental conditions. EPS depolymerases are also involved in the host specificities as evidenced for phage of pathogenic bacteria (Stummeyer et al., 2006; Thurow et al., 1974). During this PhD project, we found consistency between the host specificities of *Cobetia* phage Carin1 and the substrate specificities of Dpo31 but additional studies are required to demonstrate a direct link between these parameters.

In the ocean, EPS depolymerases are ubiquitous and there is no doubt that they degrade much of the marine DOM (Arnosti, 2011). It is commonly accepted that heterotrophic marine bacteria hydrolyze complex polysaccharide to assimilate them. The demonstration that phage can efficiently degrade bacterially derived EPS led us to question their contribution to marine DOM cycling. Our studies suggest that virally mediated degradation of bacterial EPS contributed to fuel the refractory pool of DOM, which in turn, influenced the structure of bacterial assemblages. This finding suggests a dual role of viruses in the cycling of DOM in the sea. Viruses mediate the production of one of the largest flux of labile marine DOM through the lysis of their host (Suttle, 2005), but they may also alter the size spectrum of the EPS produced by their host leading to the formation of refractory DOM species. Considering the predominance of viruses in the sea, this, so far, neglected process could have important implications for the structure and the functioning of the ocean.

To provide more robust evidence of this phenomenon, the experiment could be repeated with different bacterial assemblages (pre-bloom, post-bloom) and additional “EPS – virus systems”. The impact of viruses on the degradation of phytoplankton EPS would be particularly interesting. EPS depolymerases (including both glycoside hydrolases and polysaccharide lyases) were identified in the genome of several giant algal viruses (*Chlorella* viruses, *Aureococcus* viruses, see CAZY database) suggesting that these sophisticated microbes have evolved different manners to manipulate their host’s EPS. Considering the high amounts of EPS released by phytoplankton, especially during blooming events and their pivotal role in marine biogeochemistry (Biersmith and Benner, 1998), we anticipate that virally mediated degradation of algal EPS might profoundly impact the ecosystem functioning (Decho and Gutierrez, 2017).

In the actual context of global change, one major societal and environmental issue is to develop robust prediction of Earth climate. Therefore, we need to identify the mechanisms by which organic matter is produced and regenerated in the ocean and how these mechanisms are influenced by the changing environmental conditions. In that context, the discovered contribution of viruses with EPS degrading activities to the production of refractory forms of DOM is a breakthrough but the quantification of this process remains a challenge.

Virion-associated EPS depolymerase as novel research tools

To date, our understanding of DOM dynamics is bottlenecked by the lack of knowledge about the composition of this complex polymers mixture in the environment. Quantification of polysaccharides generally relies on the chemical hydrolysis of the polysaccharide chain into monosaccharides that can be detected colorimetrically (that infer total sugar concentration) or using improved high-performance anion-exchange chromatography with pulsed amperometric detection (HPAEC-PAD). This latter method is interesting as it allows simultaneous detection of several neutral sugars, alditols and anhydrosugars. Some species are however poorly resolved and the method is difficult to implement in the laboratory. EPS depolymerases were recently described as efficient tools to quantify specific polysaccharide species as exemplified in the recent study of Becker et al. (Becker et al., 2017). In this work, bacterial polysaccharide depolymerases were used to quantify laminarin in particulate organic matter in marine. Oligosaccharides released by the selective digestion of the algal β -glucan laminarin were quantified and used to infer accurate concentration of the initial compound. Likewise, the specificity of EPS depolymerases associated to marine phages could be used to quantify of a given polysaccharides species (EPS or CPS) in the dissolved or particulate fraction of the organic matter. Also, the polysaccharide depolymerase repertoire associated to the viral community (using viromes sequences) could provide a more thorough insight into EPS composition (EPS) in a given sample. Two studies published over the last months (Jin et al., 2019), (Emerson et al., 2018) have, for example, identified glycoside hydrolases associated to environmental viruses (soil, mangrove) and confirmed the functional activity of some of these enzymes. Likewise, Kappelman et al. (2018) used polysaccharide utilization loci of *Flavobacteria* to predict major phytoplankton polysaccharides (Kappelman et al., 2019). EPS depolymerases represent promising research tools to elucidate the

composition of polysaccharides and to advance our fundamental understanding of ocean biogeochemistry. Yet, the application of such approaches largely relies on the compilation of reference database. Expanding the collection of EPS depolymerases associated to marine viruses is thus essential.

We can also envision biotechnological applications of EPS depolymerases associated to marine phages. The ability of these enzyme to degrade biofilms has attracted substantial interest in the biomedical domain (Doolittle et al., 1996; Gutiérrez et al., 2015; Harper et al., 2014; Hughes et al., 1998; Kay et al., 2011; Lin et al., 2017; Mi et al., 2019; Sutherland et al., 2004; Tait et al., 2002). In the marine environment, the formation of biofilms by marine bacteria on immersed surfaces is also a major concern (Mieszkin et al., 2012; Salta et al., 2013). Biofouling indeed obstructs and degrades propellers, seawater pipes, or ship hulls (Salta et al., 2013). *Cobetia marina* is known for its ability to develop biofilms on immersed surface, and is often used model-organism biofilm/ biofouling related studies (Bauer et al., 2013; D'Souza et al., 2010; Ederth et al., 2011; Mieszkin et al., 2012). Preliminary analysis already demonstrated the biofilm-degradation properties of the phage Carin1 but a rapid regrowth of resistant hosts was observed (unpublished data, Lechat thesis, 2014, Annexes IV). Methods using immobilized enzymes (on membrane or polymers) to prevent the biofilm formation were developed recently (Asuri et al., 2007; Kim et al., 2011; Yao et al., 2014). The enzyme Dpo31, which is responsible for the degrading activity of *Cobetia* phage Carin1, could be use an alternative to prevent the development of *Cobetia* biofilms. Additionally, with the decreasing effectiveness of antibiotics against pathogenic or undesirable bacteria, natural biocontrol agents, such as phages, have emerged as efficient tools. The use of phages is also considered to prevent and/or limit the propagation of infectious diseases in aquaculture settings (Cohen et al., 2013b; Jacquemot et al., 2018; Martínez-Díaz and Hipólito-Morales, 2013; Oyster, 2018)). Over the past decade, promising *in vitro* and *in situ* trials of phage therapy have been reported for corals (Cohen et al., 2013a,b; Efrony et al., 2009, 2007; Jacquemot et al., 2018). The use of phage proteins with potential antimicrobial properties (i.e. endolysins, peptidoglycan hydrolases, and polysaccharidases, holins) are interesting alternatives to the application of whole bacteriophages for therapeutic purposes. (Lin et al., 2017, 2014; Liu et al., 2019; Mi et al., 2019). The characterization of EPS-depolymerase associated to marine

viruses may provide novel bioactive molecules applicable for the control targeted bacterial populations.

ANNEXES

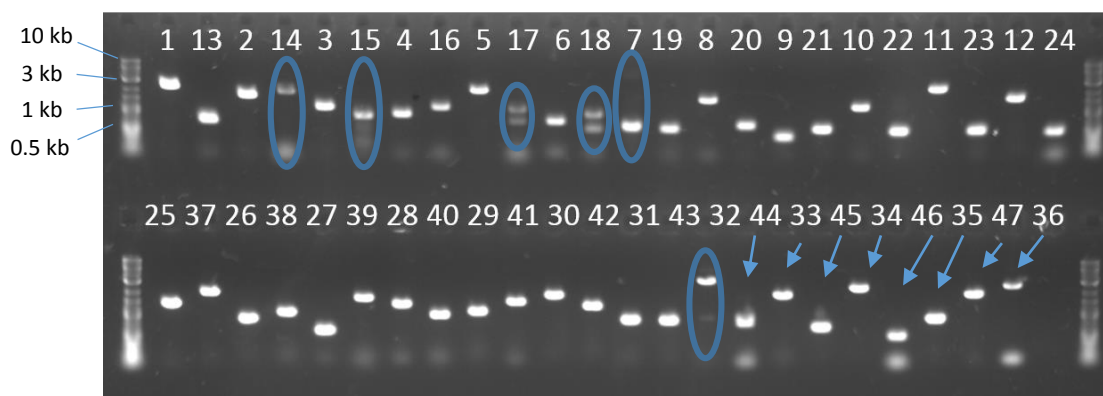
ANNEXES I



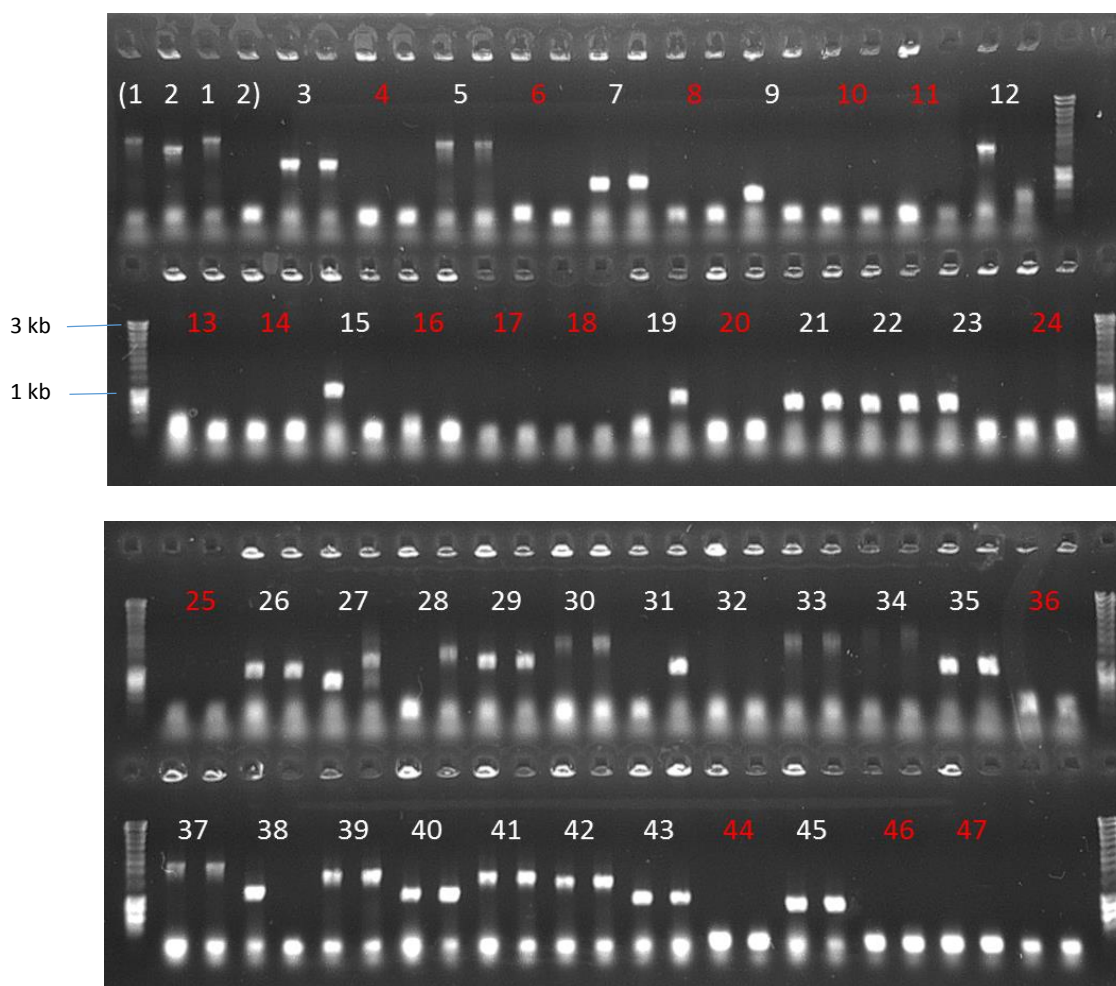
Annexes I.1: EPS obtained after all the production process. On the left, dense bacterial culture, and on the right, lyophilised EPS

Annexes I.2: Gene selected to be screened for encoding a polysaccharide depolymerase

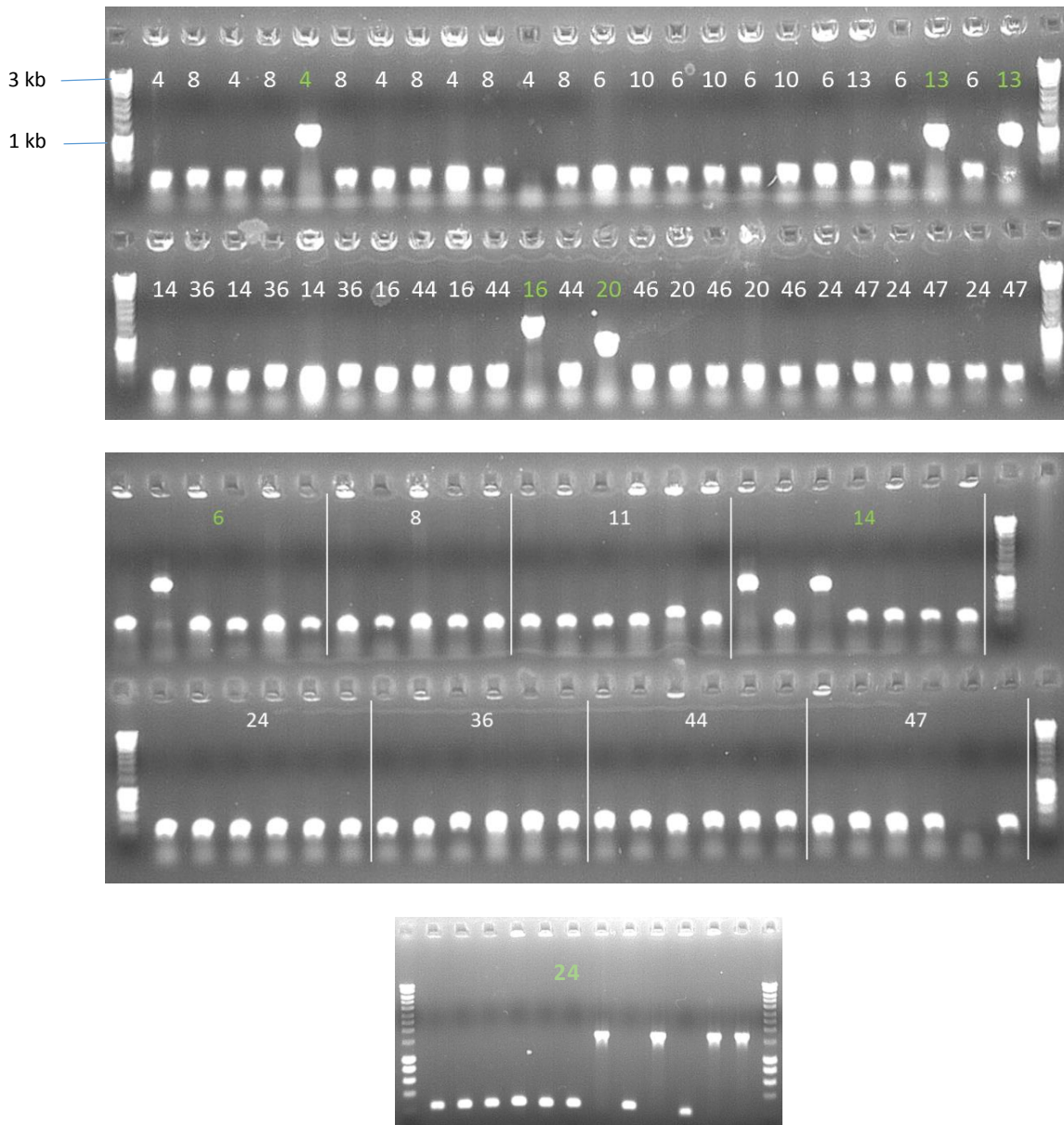
Phage	#	ORF	Annotation	Fragment	Size (kb)	Size (kDa)
Carin1	1	orf31	Polysaccharide depolymerase		2487	86.79
Carin1	2	orf31	Polysaccharide depolymerase	218-Cter	1836	62.73
Carin1	3	orf31	Polysaccharide depolymerase	417-Cter	1239	41.98
Carin1	4	orf31	Polysaccharide depolymerase	Nter-322	966	35
Carin1	5	orf31	Polysaccharide depolymerase	Nter-784	2352	82.43
Carin1	6	orf44	Peptidase [Halomonas xianhensis]		741	25.94
Carin1	7	orf44	Peptidase [Halomonas xianhensis]	31-Cter	651	22.8
Carin1	8	orf46	Phage tail protein		1671	62.15
Carin1	9	orf46	Phage tail protein	430-Cter	384	13.46
Carin1	10	orf46	Phage tail protein	Nter-425	1275	48.32
Carin1	11	orf36	Hypothetical protein [Halomonas xianhensis]		2493	91.23
Carin1	12	orf36	Hypothetical protein [Halomonas xianhensis]	269-Cter	1689	61.41
Carin1	13	prf36	Hypothetical protein [Halomonas xianhensis]	Nter-238	714	26.74
Carin1	14	orf33	Putative structural lysozyme [Pseudomonas phage AF]		2160	79.29
Carin1	15	orf33	Putative structural lysozyme [Pseudomonas phage AF]	417-Cter	912	33.93
Carin1	16	orf33	Putative structural lysozyme [Pseudomonas phage AF]	Nter-413	1239	45.11
Carin1	17	orf39	Hypothetical protein [Vibrio phage vB_VmeM-32]		1155	40.57
Carin1	18	orf39	Hypothetical protein [Vibrio phage vB_VmeM-32]	Nter-317	951	33.36
Carin1	19	orf28	N-acetylmuramoyl-L-alanine amidase [Halomonas phage QHHSV-1]		555	19.54
Carin1	20	orf16	Hypothetical protein [Pseudomonas phage KPP25]		639	23.72
Carin1	21	orf34	Hypothetical protein [Halomonas xianhensis]		558	18.84
Carin1	22	orf34	Hypothetical protein [Halomonas xianhensis]	21-Cter	537	16.9
Carin1	23	orf6	Hypothetical protein [Halomonas sp.]		501	18.54
Carin1	24	orf7	Hypothetical protein		471	17.48
Vigo2	25	orf20	Pectate lyase superfamily domain		1461	53.65
Vigo2	26	orf20	Pectate lyase superfamily domain	179-459	843	30.33
Vigo2	27	orf20	Pectate lyase superfamily domain	Nter-178	534	20.24
Vigo2	28	orf20	Pectate lyase superfamily domain	Nter-459	1377	50.62
Vigo2	29	orf32	Structural protein [Vibrio phage phiVC8]	289-Cter	1083	39.08
Vigo2	30	orf32	Structural protein [Vibrio phage phiVC8]		1947	71.5
Vigo2	31	orf32	Structural protein [Vibrio phage phiVC8]	Nter-288	864	32.43
Vigo2	32	orf2	Putative Tail fibre protein [Vibrio phages]		3504	126.67
Vigo2	33	orf2	Putative Tail fibre protein [Vibrio phages]	303-978	2028	74.4
Vigo2	34	orf2	Putative Tail fibre protein [Vibrio phages]	303-Cter	2598	94.62
Vigo2	35	orf2	Putative Tail fibre protein [Vibrio phages]	Nter-302	906	32.07
Vigo2	36	orf21	Structural protein [uncultured Mediterranean phage uvMED]		2865	106.36
Vigo2	37	orf21	orf00020_Putativestructuralprotein 250-Cter	250-Cter	2118	78.57
Vigo2	38	orf19	Hypothetical protein [Prokaryotic dsDNA virus sp.]		1035	36.51
Vigo2	39	orf28	Hypothetical protein		1695	61.93
Vigo2	40	orf28	Hypothetical protein	Nter-320	960	34.77
Vigo2	41	orf39	Head-tail connector protein [uncultured Mediterranean phage uvMED]		1542	58.43
Vigo2	42	orf39	Head-tail connector protein [uncultured Mediterranean phage uvMED]	60-Cter	1365	30.67
Vigo2	43	orf37	Hypothetical protein		849	30.67
Vigo2	44	orf5	Hypothetical protein		897	34.189
Vigo2	45	orf4	Hypothetical protein		672	25.2
Vigo2	46	orf16	Lysozyme [Vibrio phages]		483	17.72
Vigo2	47	orf34	Tail tubular protein B [uncultured Mediterranean phage uvMED]		2061	77.39



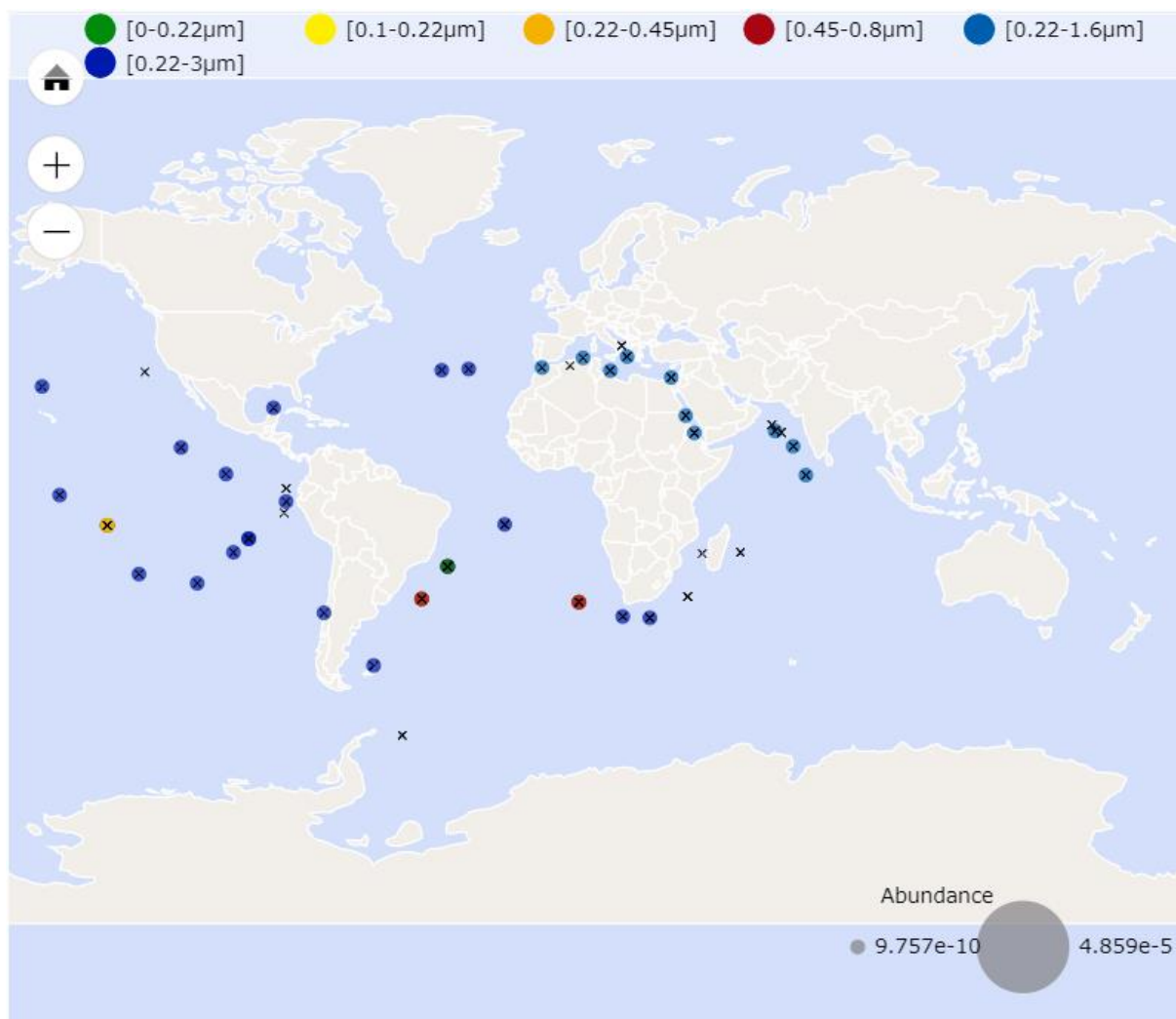
Annexes I.3: PCR control after amplification of selected genes. Genes surrounded got a new PCR amplification with more stringent condition to ensure a unique PCR product to be cloned.



Annexes I.4: Control after cloning in NEB5a *E. coli* strains. Clones in red did not contained a plasmid and/or with the right insert and other bacterial colonies were screened (Annexes I.5)

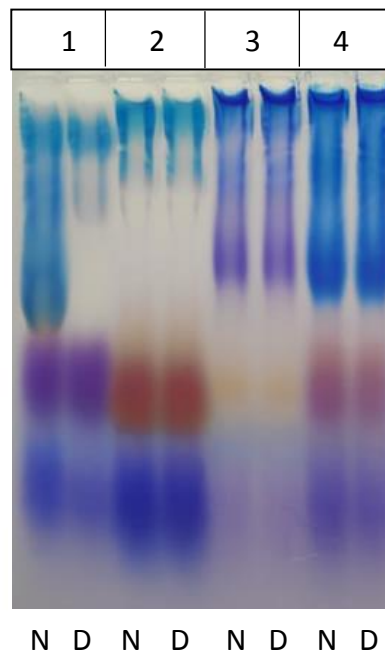
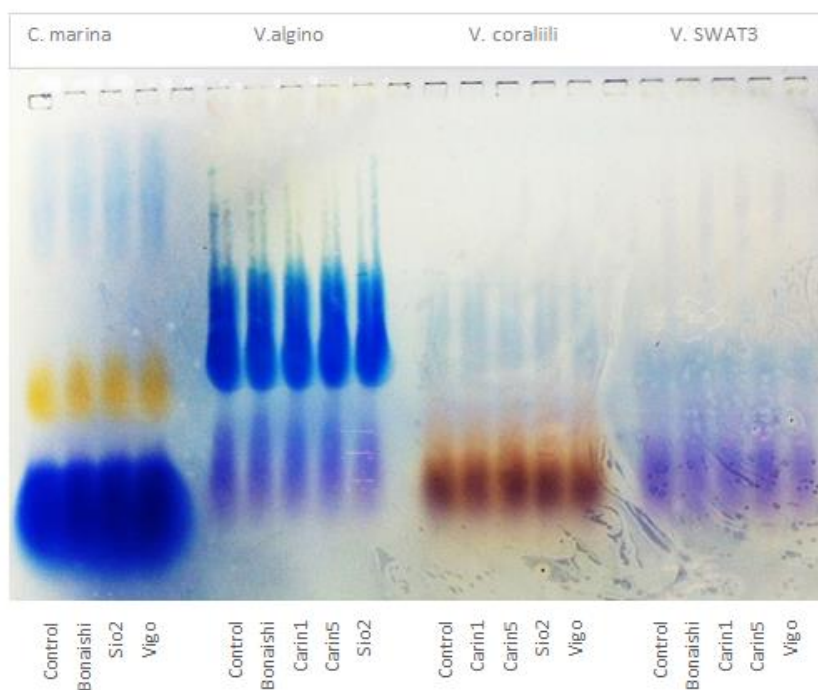
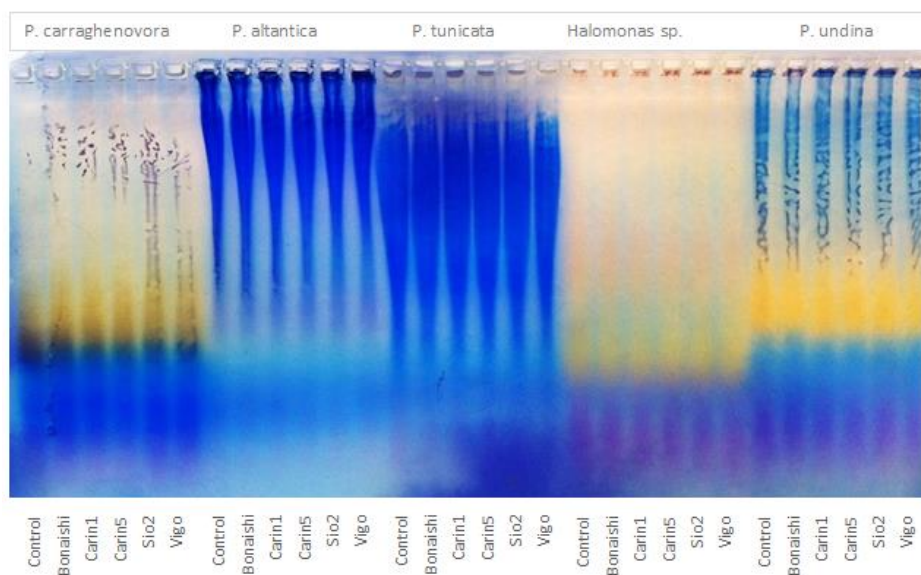


Annexes I.5: 2nd, 3rd, and 4th round control after cloning in NEB5a *E. coli* strains. Clones in green were validated, but those in white did not contained the right insert and were not pursue



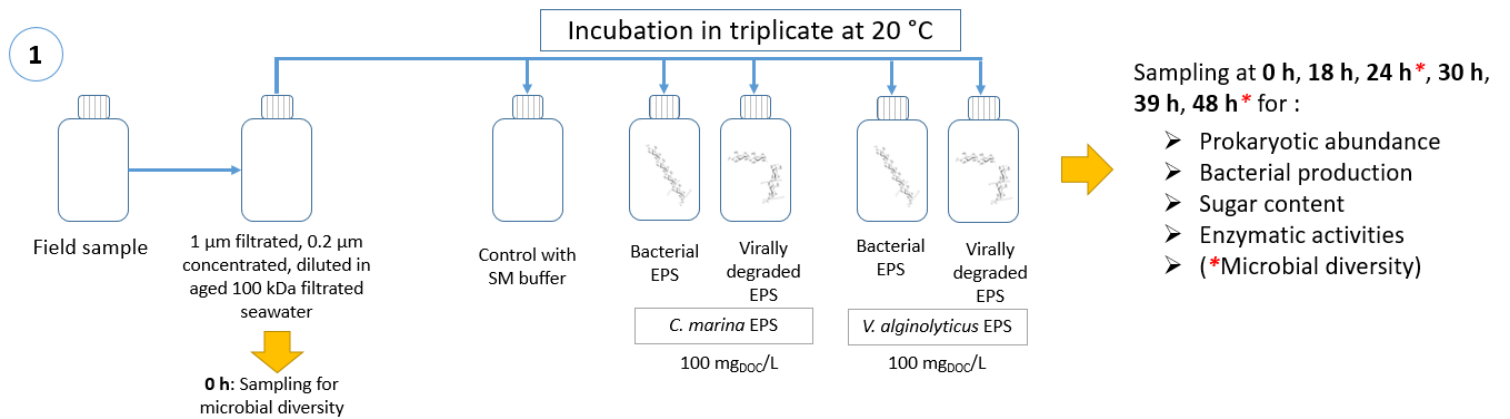
Annexes 1.6 : Distribution of the polysaccharide lyase of *Cellulophaga* phage *phi31* in Tara Ocean metagenomes, as seen with the Ocean Gene Atlas. A total of 56 similar sequences were recruited (e -value $< 1.10^{-35}$)

ANNEXES II

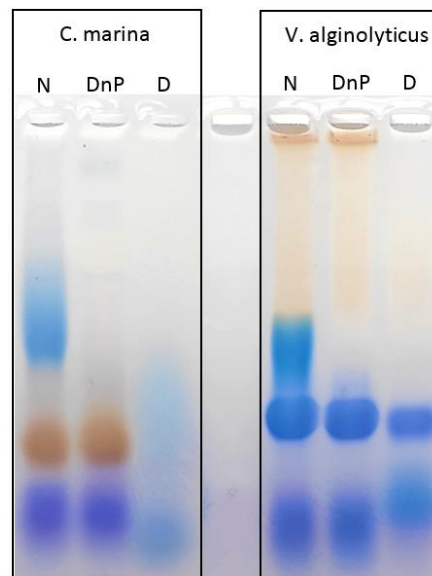


Annexes II.1: Depolymerization activity assays of Carin1 and Dpo31 with several bacterial EPS. EPS producers are listed on top of gels, and viruses or enzyme at the bottom. Regarding the gel at the bottom right corner; N: native EPS, D: Dpo31, 1: *C. marina* DSMZ 4741, 2: *C. marina* LMG2218t1, 3: *C. marina* LMG6798, 4: *C. marina* LMG6842

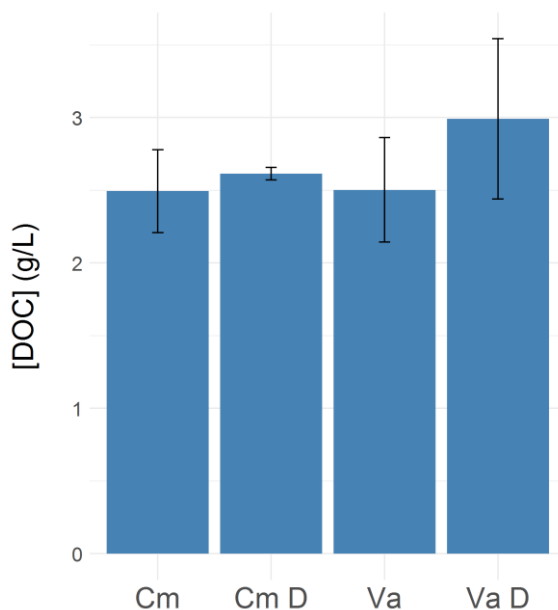
ANNEXES III



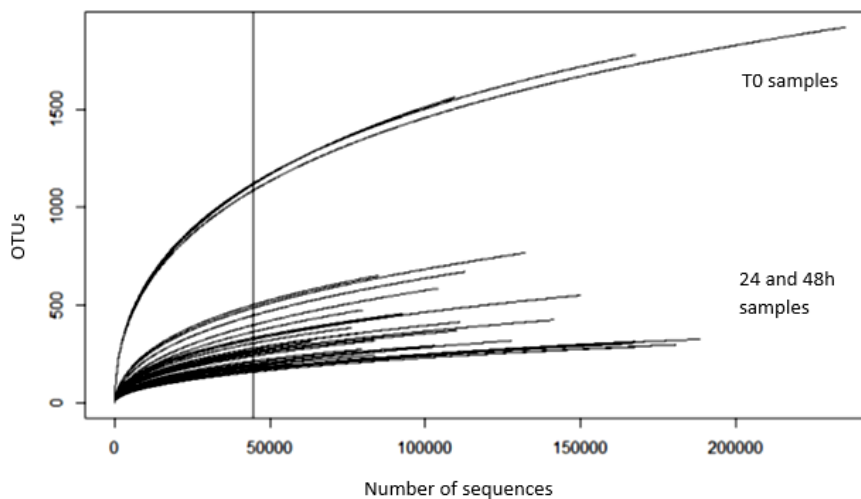
Annexes III.1: Schematic representation of the microcosm experiment performed in this study.



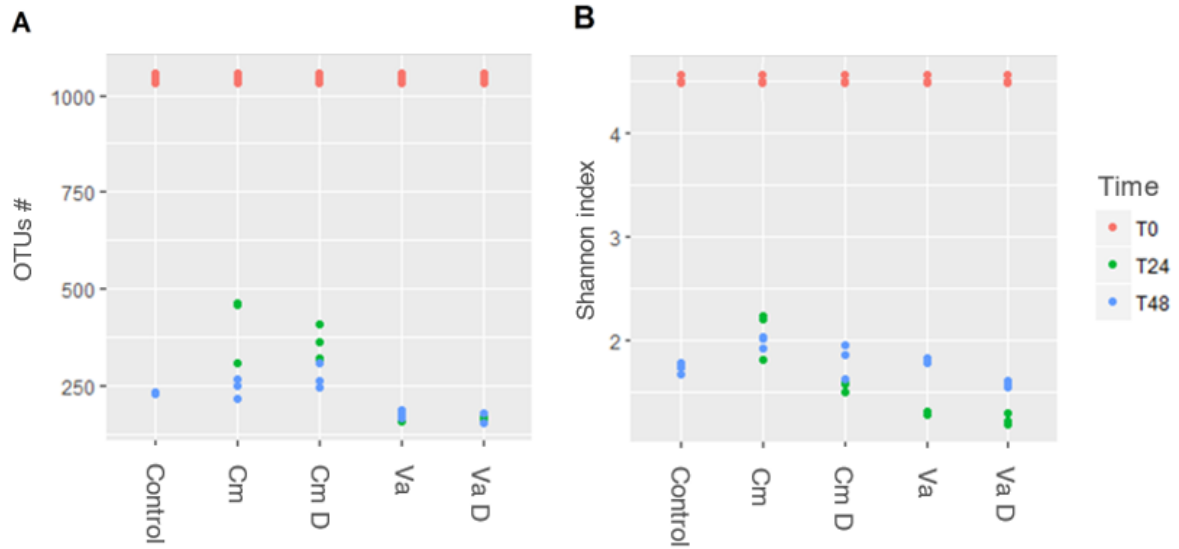
Annexes III.2: Agarose gel displaying EPS of *C. marina* (left) and *V. alginolyticus* (right) used in the study. EPS are stained with Stains-All solution (blue: polysaccharides, yellow: proteins). EPS where either natives (N) or degraded (D) by viruses. The step where EPS were degraded by viruses but not virus-purified are showed in-between (DnP).



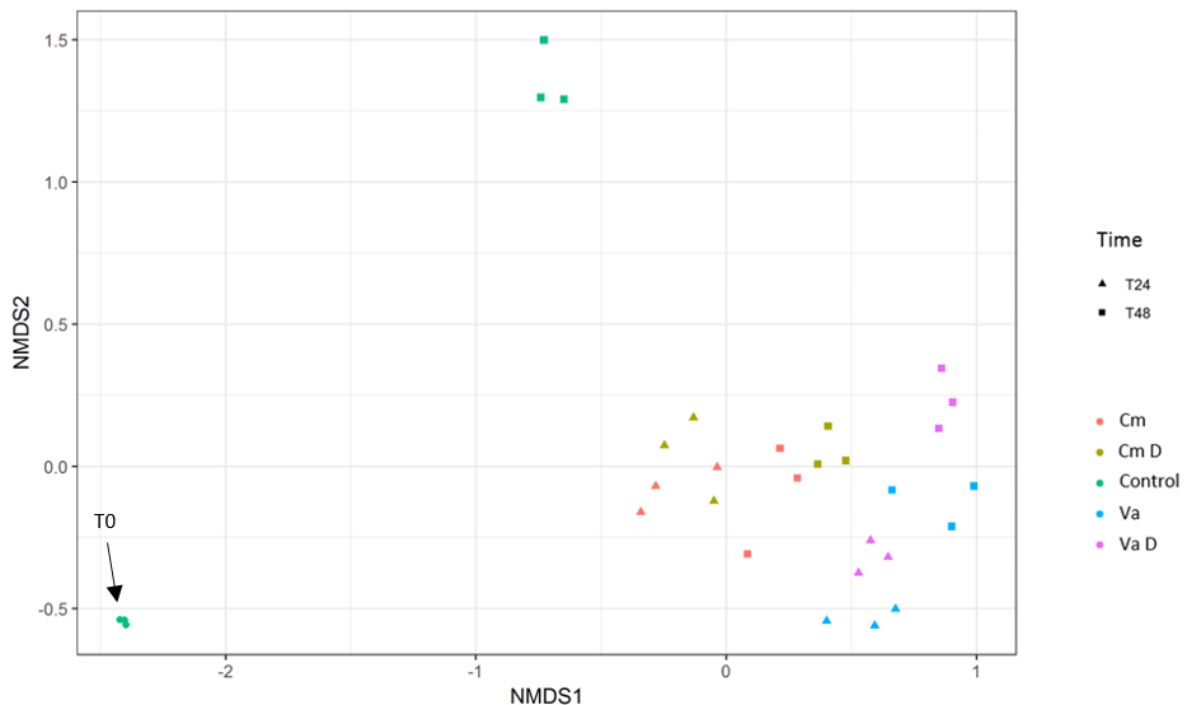
Annexes III.3: In this figure are shown the Dissolved Organic Carbon (DOC) concentrations of the dissolved EPS after purification and prior inoculation in microcosms. Cm: *Cobetia marina* native EPS; Cm D: *C. marina* EPS degraded by viruses; Va: *Vibrio alginolyticus* native EPS; Va D: *V. alginolyticus* EPS degraded by viruses.



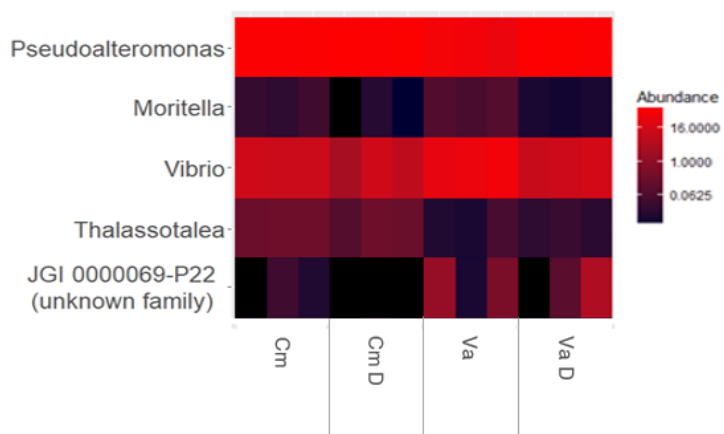
Annexes III.4: Rarefaction curve after sequencing of each sample. The total numbers of sequences are plotted against the unique operational taxonomic units (OTUs, Species) at T0 and after 24 and 48 hours of incubation for each treatment.



Annexes III.5: Alpha diversity of bacterial communities investigated with OTUs amount and the Shannon index for each condition over time. On A are shown the number of OTUs of each treatment at T0 (red), T24 (green) and T48 (blue). On B are displayed the Shannon indexes in a similar layout. Cm: *C. marina*, Va: *V. alginolyticus*, D: degraded EPS



Annexes III.6: Bray-Curtis NDMS plot showing the modification of bacterial communities over time by comparing control and the different conditions



Annexes III.7: Heat-map showing the abundancies of the bacterial genus varying significantly between the different treatments at 48-h. Cm: *C. marina*, Va: *V. alginolyticus*, D: degraded EPS

ANNEXES IV

Viral degradation of marine bacterial exopolysaccharides

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RESEARCH ARTICLE

Viral degradation of marine bacterial exopolysaccharides

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One sentence summary: Marine phages are able to passively degrade their hosts dissolved EPS.

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ABSTRACT

The identification of the mechanisms by which marine dissolved organic matter (DOM) is produced and regenerated is critical to develop robust prediction of ocean carbon cycling. Polysaccharides represent one of the main constituents of marine DOM and their degradation is mainly attributed to polysaccharidases derived from bacteria. Here, we report that marine viruses can depolymerize the exopolysaccharides (EPS) excreted by their hosts using five bacteriophages that infect the notable EPS producer, *Cobetia marina* DSMZ 4741. Degradation monitorings as assessed by gel electrophoresis and size exclusion chromatography showed that four out of five phages carry structural enzymes that depolymerize purified solution of *Cobetia marina* EPS. The depolymerization patterns suggest that these putative polysaccharidases are constitutive, endo-acting and functionally diverse. Viral adsorption kinetics indicate that the presence of these enzymes provides a significant advantage for phages to adsorb onto their hosts upon intense EPS production conditions. The experimental demonstration that marine phages can display polysaccharidases active on bacterial EPS lead us to question whether viruses could also contribute to the degradation of marine DOM and modify its bioavailability. Considering the prominence of phages in the ocean, such studies may unveil an important microbial process that affects the marine carbon cycle.

Keywords: marine phage; EPS; polysaccharidase; DOM; ocean

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INTRODUCTION

The dynamics of dissolved organic matter (DOM) in seawater plays a pivotal role in ocean biogeochemistry. Marine DOM is derived from different biological processes, including phytoplankton release particularly during bloom events (Carlson and Hansell 2014), excretion of capsular polysaccharides or exopolysaccharides (EPS) by heterotrophic bacteria (Stodereger and Herndl 1998, 1999, 2001), sloppy feeding by metazoan grazers (Møller 2005) or biodegradation of macroalgal biomass (Krause-Jensen and Duarte 2016). Viral-mediated lysis of microbes is also recognized as one of the largest source of DOM in the ocean (Middelboe, Jørgensen and Kroer 1996; Fuhrman 1999; Suttle 2005). Regardless of the source of DOM, extracellular enzymes encoded by microbial communities initiate the degradation of DOM in the sea (Arnosti 2011). These enzymes efficiently depolymerize high-molecular-weight organic matter into compounds of lower molecular weight. Part of these hydrolysis products is converted into bacterial biomass that supports higher trophic levels and inorganic nutrients that can be recycled by primary producers. Another part consists of refractory forms of organic matter that persist in the ocean for extended periods of time and contribute to carbon sequestration (Jiao et al. 2010; Krause-Jensen and Duarte 2016). Extracellular enzymes influence the fate of most of the organic matter and thereby, they play a pivotal role in marine carbon cycling (Arnosti 2011).

High molecular weight acidic polysaccharides represent one of the main constituents of marine DOM (McCarthy, Hedges and Benner 1993; Panagiotopoulos and Sempéré 2005). Despite their key-role in marine carbon cycling, the interactions between extracellular enzymes and marine DOM are not well understood. Studies, mostly conducted on terrestrial microorganisms, showed that Carbohydrate Active enZymes (CAZymes, Henrissat 1991; Davies and Henrissat 1995; Lombard, Golaconda and Drula 2014) are used to bind, modify and degrade these polymers for consumption. Among these CAZymes, the proteins that specifically catalyze the breakdown of polysaccharides are designated as polysaccharide depolymerases or polysaccharidases. These enzymes are either constitutive or induced; they act either on the terminal end of the polymers (exo-acting mode) or cleave the polymer mid-chain (endo-acting mode) (Sutherland 1995). They can be found attached to the cell or they can be released into the surrounding environments (Arnosti 2011). Studies of biological models from variable origins evidenced a notable diversity within these enzymes, which are currently classified among 304 protein families based on sequence homology in the CAZY database (Lombard, Golaconda and Drula 2014; <http://www.cazy.org/>). In marine environments, polysaccharidases are ubiquitous and there is no doubt that they degrade much of the DOM in ocean (Arnosti 2011a; Arnosti 2011b). It is commonly assumed that polysaccharidase activity derives mainly from bacteria. The recent advances in genomics clearly demonstrated that other microbes encode polysaccharidases. Among these, viruses, which usually outnumber bacteria by one order of magnitude in the ocean, can display such enzymes (Pires et al. 2016).

A wealth of biomedical studies reports that viruses of bacteria (bacteriophages or phages) use polysaccharidases to depolymerize their host capsule/ slime/ biofilms in order to gain access to their primary receptors (Bayer et al. 1979; Sutherland 1999; Sutherland et al. 2004). These enzymes, typically located on

phage tail, display a high level of functional and molecular diversity (Sutherland 1999; Scholl, Adhya and Merrill 2005; Cornelissen et al. 2011, 2012; Yele et al. 2012) and can influence biological traits of viruses such as host specificities (Leiman et al. 2007). To the best of our knowledge, there is no experimental evidence of polysaccharidase activity in marine phages. Yet, marine bacteria are active producers of polysaccharides or EPS that are either associated with the bacterial cell surface or excreted into the environment (Heissenberger, Leppard and Herndl 1996; Bier-smith and Benner 1998; Casillo et al. 2018). This led us to question as to whether marine phages also carry polysaccharidases to infect their host. The finding of polysaccharidases associated to marine viruses could provide novel fundamental knowledge about the ecology of these prominent microbes. Because polysaccharidases initiate the infection cycle, we could expect that their intrinsic properties (life-time, substrate specificity, activity velocity) and their ability to withstand environmental changes (salinity, temperature, pH, pressure) directly influence the rates of infection and subsequent viral induced mortality or even the life-time of a viral particle in the ocean. Importantly, the passive degradation of dissolved bacterial EPS by viruses could have important biogeochemical implications, such as affecting the size spectrum, the reactivity and the bioavailability of marine DOM.

In this study, we investigated the capacity of marine phages to depolymerize their host EPS and attempted to relate this to important biological traits of marine viruses. Therefore, we used five phages (*CobetiA marINa* phages: Carin-1 to 5) that infect the globally distributed γ -Proteobacteria *Cobetia marina* (DSMZ 4741, hereafter *C. marina*) known for its ability to produce EPS (Arahal et al. 2002; Yumoto et al. 2004; Ivanova et al. 2005; Lechat et al. 2015b) and its potential contribution to the carbon export (Guidi et al. 2016). Our study revealed that marine phages do possess enzymes active on bacterial dissolved EPS, which may influence virus ecology and the recycling of bacterially derived DOM in the ocean.

MATERIAL AND METHODS

Bacterial host

The marine gram-negative Gammaproteobacteria *Cobetia marina* DSMZ 4741 was used as reference host to isolate phages from seawater. This strain belongs to a halotolerant mesophilic, heterotrophic and aerobic genus routinely grown on liquid and agar Zobell medium (Baumann, Bowditch and Baumann 1983; Romanenko et al. 2013).

Phage isolation

Cobetia marina phages were isolated from surface water of the bay of Brest at the long-term monitoring station SOMLIT (4° 33' 07.19 W, 48° 21' 32.13 N) from June to September 2011. Seawater samples (20 L) were filtered through 0.7 μ m glassfiber filter (Whatmann®) and concentrated (100-fold) using a 100 kDa ultrafiltration cartridge (Pellicon, Sartorius®). Once concentrated, samples were filtered through a PES syringe filter (0.2 μ m cut-off, Sartorius®) and directly used in a plaque assay by spreading a mixture of the reference host and viruses in sterile molten Zobell agar (noble agar 0.6%, 5 g.L⁻¹ peptone, 1 g.L⁻¹ yeast extract dissolved in seawater) heated at 37°C on a layer Zobell agar (5 g.L⁻¹ peptone, 1 g.L⁻¹ yeast extract, 15 g.L⁻¹

agar dissolved in seawater). The plate was incubated at 20°C (Swanstrom and Adams 1951; Kropinski et al. 2009). After 24 h incubation, well-resolved plaques (PFU—plaque forming unit) were picked from the lawn of host cells, eluted in sterilized ultrafiltered seawater, and combined with host culture in a new plaque assay. This procedure was repeated two more times to ensure isolation of a clonal population for each phage. Clonal lysates of each viral strain was stored in SM buffer (NaCl: 100 mM; MgSO₄, 7H₂O: 8 mM; TrisHCl: 50 mM) at 4°C until further study.

Phage purification

Bacteriophages were purified according a protocol adapted from Bachrach and Friedmann (1971). Phages solution was purified on sucrose linear gradients (10% to 40% w/v). First, 500 µL of freshly prepared viral lysate was treated with 50% (v/v) chloroform for 1 h to remove organic contaminants such as vesicles which can contain enzymes including polysaccharidases (Li, Azam and Zhang 2016; Arntzen et al. 2017). Then, the aqueous phase was separated by low-speed centrifugation (3000 x g, 5 min at room temperature) and loaded on the top of the gradient before ultracentrifugation (134 000 g, 45 min, 4°C) using a SW 41 Ti rotor. The viral band was extracted using a sterile syringe needle. The viral particles were diluted in SM buffer or sterilized ultrafiltered seawater and they were pelleted by a second ultracentrifugation (2 h, 352 000 g, 4°C) using a 70.1 Ti rotor to separate them from sucrose. After resuspension of the viral pellet in SM buffer, bacteriophages were then filtered through a 0.2 µm PES filter (Sartorius®).

Transmission electronic microscopy

A drop (10 µL) of clonal phage suspension was applied to a copper EM grid (400 mesh size) with a nitrocellulose backed carbon surface (Ackermann 2007, 2009). The sample was adsorbed on the grid for 10 sec after which the grid was blotted with filter paper and stained with 2% (w/v) uranyl acetate for 45 sec, blotted again and allowed to air dry. Specimens were imaged using a JEOL® JEM 1400 transmission electron microscope operating at 100 keV at a magnification of 50 000X. Pictures were analysed using ImageJ software.

Host range

CobetiA marINA phages: Carin-1 to 5 host specificities were determined by plaque assay using three strains of *C. marina* and six strains of the related *Halomonas* genus. Dilution series of Carin-1 to 5 suspensions (10² to 10⁸ PFU.mL⁻¹) were incubated with the potential host cultures in exponential growth phase for 15 min. After incubation, samples were mixed with the molten agar, plated onto a lawn of Zobell agar and incubated at 20°C in darkness for 24 h.

EPS production

Cobetia marina EPS (hereafter referred to as EPS L₆) were produced as described previously (Lelchat et al. 2015b). Briefly, a pre-culture was grown in ZoBell medium (1 g.L⁻¹ yeast extract, 5 g.L⁻¹ peptone diluted in 80% filtered seawater and 20% MilliQ) supplemented with glucose (30 g.L⁻¹) at 25°C under agitation. After 10 h incubation, pre-culture was inoculated (10% v/v) in a 3 L fermenter (INFORS®) containing Zobell medium supplemented with Tris-base (1.5 g.L⁻¹) and glucose (30 g.L⁻¹). The pH

medium was adjusted to 7.6 and maintained at this value by addition of H₂SO₄ or NaOH. The culture was grown at 25°C for 33 h until the culture reached the late exponential phase. During the stationary growth phase, the temperature was decreased to 20°C for 48 h in order to enhance the EPS production. The oxygenation was monitored and regulated by stirring and air flux. The consumption of glucose was monitored with the enzymplus kit (St Gobain®, France). At the end of the fermentation, the culture medium was centrifuged (1 h, 14 000 g, 4°C) and the supernatant was filtered with a Buchner through a 0.45 µm glass filter (Whatman®). Sodium azide (NaN₃, 0.4 g.L⁻¹ final concentration) was added to the filtrate to prevent bacterial regrowth. The filtered culture supernatant containing the soluble EPS was then purified by tangential ultrafiltration with a 100 kDa cut-off cartridge (Millipore®) against MilliQ water. The purified EPS was then frozen, freeze dried and stored away from light and moisture.

Enzymatic screening

Enzyme assays were realized in triplicate. Purified viruses (10¹⁰ PFU.mL⁻¹) were mixed at 4°C with EPS L₆ (0.2% w/v final concentration) in incubation buffer (Tris-HCl 50 mM, NaCl 0.5 M, MgSO₄ 8 mM, pH 8 at 20°C), filtered through a 0.2 µm filter (Sartorius®) and incubated 24 h at 25°C under agitation (120 rpm). Controls containing viral suspension, *C. marina* lysate extract and EPS as well as EPS alone were processed and incubated under similar conditions to check for potential auto-hydrolysis phenomenon according Lelchat et al. (2015a).

The polysaccharide degradation was visualized using agarose gel electrophoresis according to the method of Lee and Cowman (1994). With L₆ being an acidic polysaccharide, runs were conducted at pH 8.5. Briefly, the agarose gel (1%) was prepared in Tris/acetic acid / EDTA (TAE) buffer (TAE 10X: Tris-Acetic acid 0.4 M, Na₂ EDTA 10 mM, pH 8.5). Samples (12 µL) were loaded on the gel and electrophoresis was run for 75 min at 100 V.

The gel was stained overnight in a Stains All solution (Lee and Cowman 1994; Volpi and Maccari 2002) (10 mL of 3,3'-diethyl-9-methyl-4,5,4',5'-dibenzothiacarbocyanine 0.1% (w/v) in *N,N*-dimethylformamide, 10 mL of *N,N*-dimethylformamide; 50 mL of isopropanol; 10 mL of 300 mM Tris HCl pH 8.8, completed to 200 mL with MilliQ water). The gel was then destained for 2 h under natural light in distilled water.

Polysaccharide degradation was also monitored using size exclusion chromatography (SEC) on an Akta Fast Protein Liquid Chromatography apparatus (GE Healthcare Life Sciences®) equipped with a Superdex 200 10/300 column (GE Healthcare 200®, optimum separation range: 1000–100 000 g.mol⁻¹ dextran equivalent) as described by (Lelchat et al. 2015a). Samples were recovered after incubation, then injected with a 200 µL loop, separated with the incubation buffer (Tris-HCl 50 mM, NaCl 0.5 M, MgSO₄ 8 mM, pH 8 at 20) at 0.5 mL.min⁻¹ and detected using UV and refractometry to discriminate signals that could be assigned to proteins or polypeptides, but not sugars.

Low molecular weight degradation (50kDa > x > 5 kDa) products were sampled, purified by centrifugal ultrafiltration (5 kDa cut-off, Amicon Ultra 15, Merckmillipore®) and analyzed by ¹H-NMR according (Lelchat et al. 2015b). About 10 mg of depolymerized L₆ fractions were analyzed by NMR after three exchanges/dehydration cycles in deuterated water (99.9%). EPS were resuspended in 700 µL D₂O. Spectra were recorded at room temperature in the Laboratory of Nuclear Magnetic Resonance

Spectroscopy (University of Western Brittany) on a 500 MHz Bruker® spectrometer.

Enzyme kinetics

The depolymerization kinetics of the EPS L_6 by Carin-1 and Carin-5 was monitored during a 32 h enzyme kinetic according Lelchat et al. 2015a. Purified bacteriophages (10^{10} PFU.mL⁻¹) were incubated with EPS L_6 (0.4% w/v) in incubation buffer (Tris-HCl 50 mM, NaCl 0.5 M, MgSO₄ 8 mM, pH 8 at 20°C) according the modified incubation conditions optimized previously but with addition of NaN₃ as bacteriostatic (6 mM final concentration). Samples were taken every hour from 1 to 17 h and at 23, 25, 29 and 32 h, flash frozen and stored at -80°C. Blanks containing virus solution alone, *C. marina* protein extract and EPS as well as EPS alone were incubated in the buffer to assess of the absence of auto-hydrolysis phenomenon. Depolymerization kinetics were visualized by 1% agarose gel electrophoresis and SEC through a superdex-200 column (GE Healthcare Life Sciences®) as described above.

Phage adsorption kinetics

The adsorption constant was determined for each phage with the host which was either induced or not induced to produce EPS following a protocol adapted from Hyman and Abedon (2009). A bacterial culture in exponential growth (5.10^8 bacteria.mL⁻¹) was incubated with each strain of bacteriophages at a multiplicity of infection of 1 in a total volume of 40 mL. Samples (1 mL) were taken every 3 min for 30 min, and immediately centrifuged (7000 rpm, 1 min, 20°C). A supernatant aliquot containing the non-adsorbed phages was fixed with glutaraldehyde (final concentration 0.5%) at 4°C for 15 min and flash-frozen in liquid N₂. Non-adsorbed viruses were enumerated by flow cytometry (FACs CANTO II, Beckton Dickinson®) upon SYBR-Green I staining (10 min, 80°C) according to Brussaard (2004). Blanks containing only viruses or bacteria were taken in parallel and processed as described above. Cytograms were analyzed using Flowing Software®. Adsorption coefficients (Cd) were calculated by dividing the regression coefficient of the natural logarithm of the curve describing the variation of viral abundance in 30 min ($x_{[virus]}$) by the average bacterial abundance [Bacterial abundance]: $Cd = -x_{[virus]} / [Bacterial\ abundance]$.

RESULTS AND DISCUSSION

In this study, five phages lytic to the EPS-producing bacteria *C. marina* DSMZ 4741 were isolated (referred to as Carin-1 to 5) from the coastal Atlantic Ocean. The transmission electronic microscopy examination of clonal suspensions of these phages indicated that they belong to the *Caudovirales* order characterized by an icosahedral head connected to a tail of variable morphology (Fig. 1). The strain Carin-1 exhibited a short tail and it was assigned to the *Podoviridae* family (head: $59_{\pm 3.2}$ nm, tail: $12.8_{\pm 2.1}$ nm) Carin-2 (head: $113.1_{\pm 15.4}$ nm, tail: $137.5_{\pm 18.9}$ nm), Carin-4 (head: $80_{\pm 3.4}$ nm, tail: $140.3_{\pm 6.8}$ nm) and Carin-5 (head: $69.7_{\pm 3}$ nm, tail: $130.7_{\pm 11.3}$ nm) displayed a contractile tail characteristic of the *Myoviridae* family while Carin-3 (head: $59_{\pm 3.2}$ nm, tail: $12.8_{\pm 2.1}$ nm) belonged to the *Siphoviridae* family with a long and non-contractile tail.

Evidence of marine phage mediated EPS depolymerization

Two phenotypic assays, that are typically used to screen for phages with biofilm disrupting abilities (Bessler et al. 1975), suggested that Carin phages possessed polysaccharidases active on their host EPS. All five phage isolates maintained their ability to form plaques on *C. marina* lawn even when the host EPS production was over stimulated using a glucose-enriched culture medium for growth. Furthermore, the five Carin phages formed clear plaques surrounded by an opaque halo zone that expanded in diameter over time (Fig. S1, Supporting Information). The increasing halo diameter is usually generated by phage-associated enzymes that depolymerize bacterial EPS as the phages spread out of the lysis zone by diffusion (Miyake et al. 1997; Glonti, Chanishvili and Taylor 2010; Cornelissen et al. 2011, 2012; Pleneteva et al. 2011).

To confirm the hypothesis that Carin phages possess EPS polysaccharidases, we incubated *Cobetia marina* EPS L_6 (hereafter L_6) with and without viruses and visualized the depolymerization pattern on agarose gel electrophoresis. The migration of native L_6 showed 2 EPS populations with distinct molecular weight (Fig. 2). The incubation of L_6 with purified Carin particles resulted in 3 degradation patterns. Carin-1 and -5 induced a nearly complete depolymerization of L_6 into products of low molecular weight. Carin-2 and -4 induced a partial degradation of the L_6 , while Carin-3 did not seem to affect L_6 migration profile. The ¹H-NMR analysis of degradation products (50kDa > x > 5 kDa) showed no difference of spectra compared to native L_6 (Fig. 3, Lelchat et al. 2015b), indicating that Carin-1 and 5 generated L_6 oligosaccharides.

Detailed degradation kinetics as monitored by agarose gel electrophoresis (Fig. 4) and SEC (Fig. S2, Supporting Information) suggest that even Carin-1 and Carin-5, which generated a nearly complete L_6 degradation, possess different polysaccharidases. The kinetics show that both phages produced the formation of intermediate molecular weight species during the course of L_6 depolymerization, which suggests that they possess endo-active polysaccharidases as consistently reported in literature for phages of biomedical interest (Sutherland 1995; 1999). Endo-active enzymes can cleave glycosidic bonds all along the polysaccharidic chain and thereby enable phages to efficiently penetrate the EPS layer, even for polymers of high viscosity (Sutherland 1999). Despite similar endo-active modes of action, comparison of the migration (or elution) profiles suggest that Carin-1 and -5 polysaccharidases act differently on L_6 . Carin-1 was able to significantly depolymerize the population of HMW species within 2 h while products of high molecular weight were still apparent for Carin-5 after this incubation period. Depolymerization occurs within a few hours and seems to start very fast even when the incubation mixture is still at 4°C. At T0, the high molecular weight spot is fainter for incubation with Carin-1 compared to incubation with Carin-5 and despite a similar EPS initial concentration and all the possible precautions during the kinetic preparation. It is likely that Carin-1 can degrade the EPS of its host at cold temperature in order to adsorb and initiate its lytic cycle. *C. marina* DSMZ 4741 can indeed grow at 5°C (Yumoto et al. 2004).

The results described above provide unequivocal evidence that marine phages can have the ability to degrade dissolved polysaccharides derived from their marine host. Because these assays were conducted with purified phage suspension, free of contaminants from the viral lysate, it is very likely that the

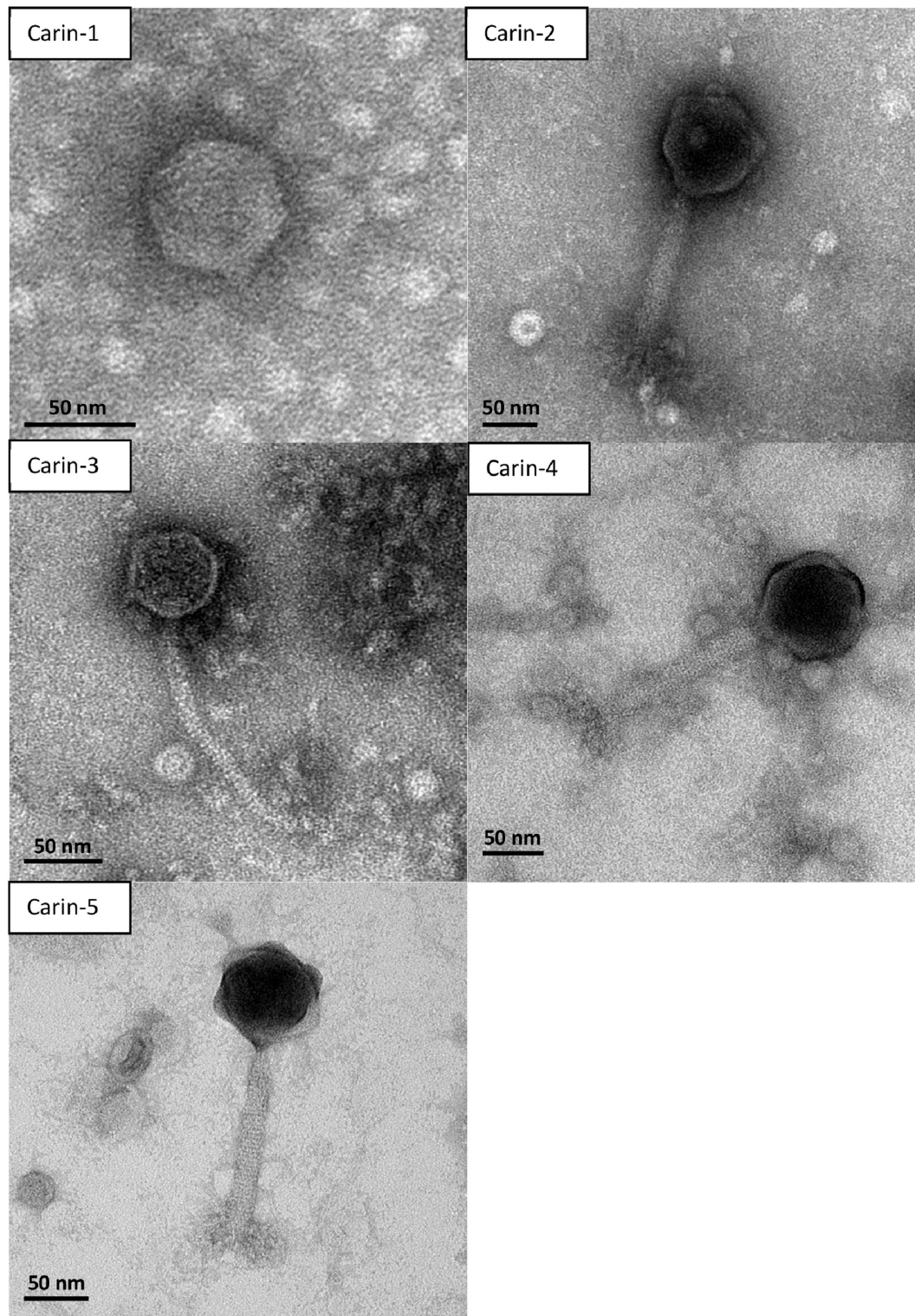


Figure 1. Transmission electron micrographs of the five bacteriophages that infect the marine bacterium *Cobetia marina* DSMZ 4741 (Carin phages).

polysaccharidases are bound to the virus particle. In the literature, the presence of structural polysaccharidases have often been described in *Podoviridae* (Miyake et al. 1997; Linnerborg et al. 2001; Jakobsson et al. 2007; Leiman et al. 2007; Shaburova et al. 2009; Glonti, Chanishvili and Taylor 2010; Pleneteva et al. 2011; Cornelissen et al. 2012; Roach et al. 2013) and *Myoviridae*

(Elsässer-Beile and Stirm 1981; Kwiatkowski et al. 1983; Nimnich et al. 1992; Shaburova et al. 2009; Cornelissen et al. 2011) that infect pathogenic bacteria but are also reported in *Siphoviridae* (Niemann et al. 1976, 1977; Rieger-Hug and Stirm 1981; Nimnich et al. 1992; Smith, Zamze and Hignett 1994; Miyake et al. 1997; Gutierrez et al. 2010, 2012; Chertkov et al. 2011; Roach et al.

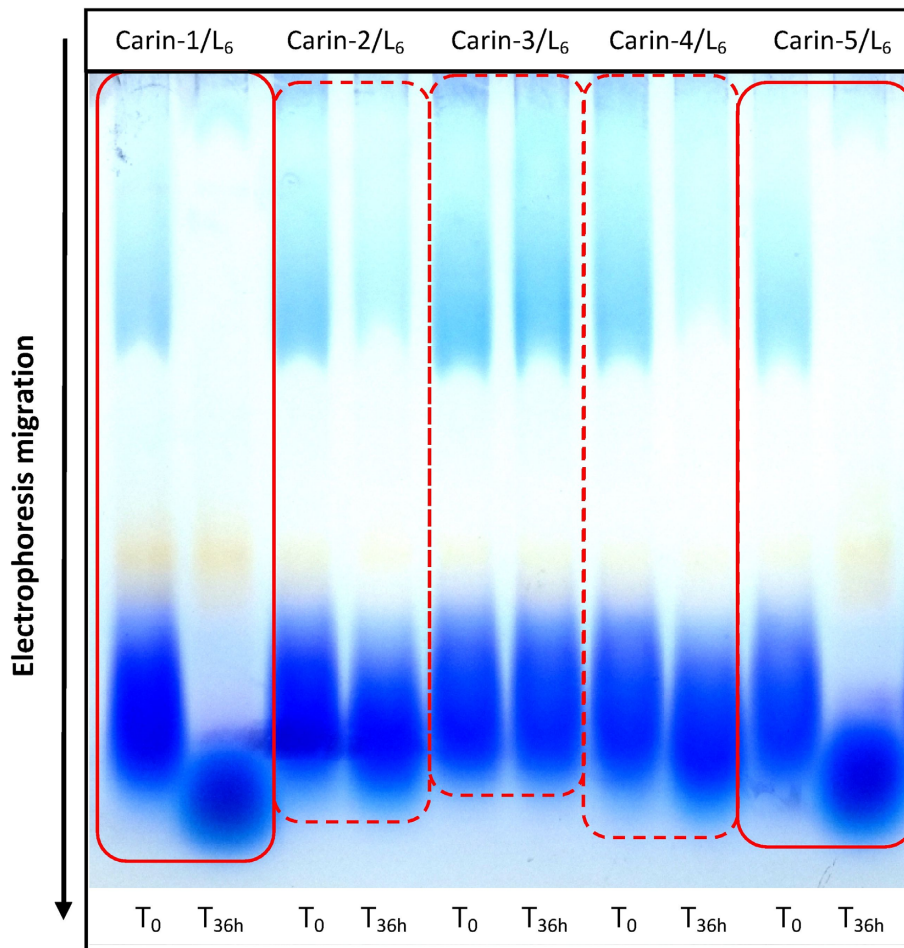


Figure 2. Migration pattern of *Cobetia marina* L₆ EPS (0.4 g.L⁻¹) with and without purified Carin phage suspensions (10⁹ PFU) as assessed by agarose gel electrophoresis after Stains all coloration (blue smear: polysaccharides, yellow smear: proteins). This assay indicate that Carin-1, -2, -4 and -5 were able to depolymerize, *C. marina* EPS into products of low molecular weight.

2013). Nevertheless, we cannot rule out that virion-free polysaccharidases are excreted during the course of Carin-3 infection cycle as suggested by the formation of plaques surrounded by halo zones. It is also possible that Carin-3 uses another type of enzyme, such as lysine murein hydrolase, which degrades the protein moieties of bacterial biofilm as reported previously (Fischetti 2008). The different degradation patterns observed for Carin phages suggest that their polysaccharidases are functionally diverse. This diversity does not appear to be related to the classification of the selected isolates. Indeed, Carin-2, -4 and -5 are all members of the *Myoviridae* family but display different L₆ degradation patterns. A detailed molecular and biochemical characterization would certainly help understanding whether this functional variability arises from the activity of different types of polysaccharidases, from a variable number of polysaccharidase copies or from a combination of different enzymes bound to phage particle as reported for the coliphage K1-5 (Leiman et al. 2007).

Implications of EPS polysaccharidase activity for phage ecology

In the literature, the characterization of phage-associated polysaccharidases is mostly dedicated to the discovery of novel enzymes active on the biofilm or polysaccharidic capsule formed

by pathogenic bacteria (Pires et al. 2016). By contrast, very few studies have investigated how EPS polysaccharidases influence phage biological traits and to a larger extent their ecology. The finding that four out of five of the Carin phages we isolated possessed EPS polysaccharidases suggests that this property may provide a competitive advantage in the natural environment.

Because the assumed primary role of phage-associated polysaccharidases is to degrade their host capsular/biofilm EPS in order to attain membrane receptors (Bayer, Thurow and Bayer 1979), it is reasonable to expect that the presence of these enzymes may affect the adsorption kinetics of Carin phages, particularly upon intense host EPS excretion. To address this question, we determined the adsorption constant (Cd) of Carin isolates (data missing for Carin-4) in culture conditions mimicking normal and stimulated EPS production (Table 1). The phage Cd in control conditions varied between 5.08×10^{-8} to 3.51×10^{-10} mL.min⁻¹ for Carin-2 and Carin-1, respectively, which ranged within the values reported in the literature (Fujimura and Kaesberg 1962; Olkkonen and Bamford 1989; Murray and Jackson 1992; Moldovan, Chapman-McQuiston and Wu 2007; Storms et al. 2010; Gallet, Kannoly and Wang 2011). The observed Cd variation between phage isolates may arise from differences in viral particle diameter, morphology, viral electric charge but

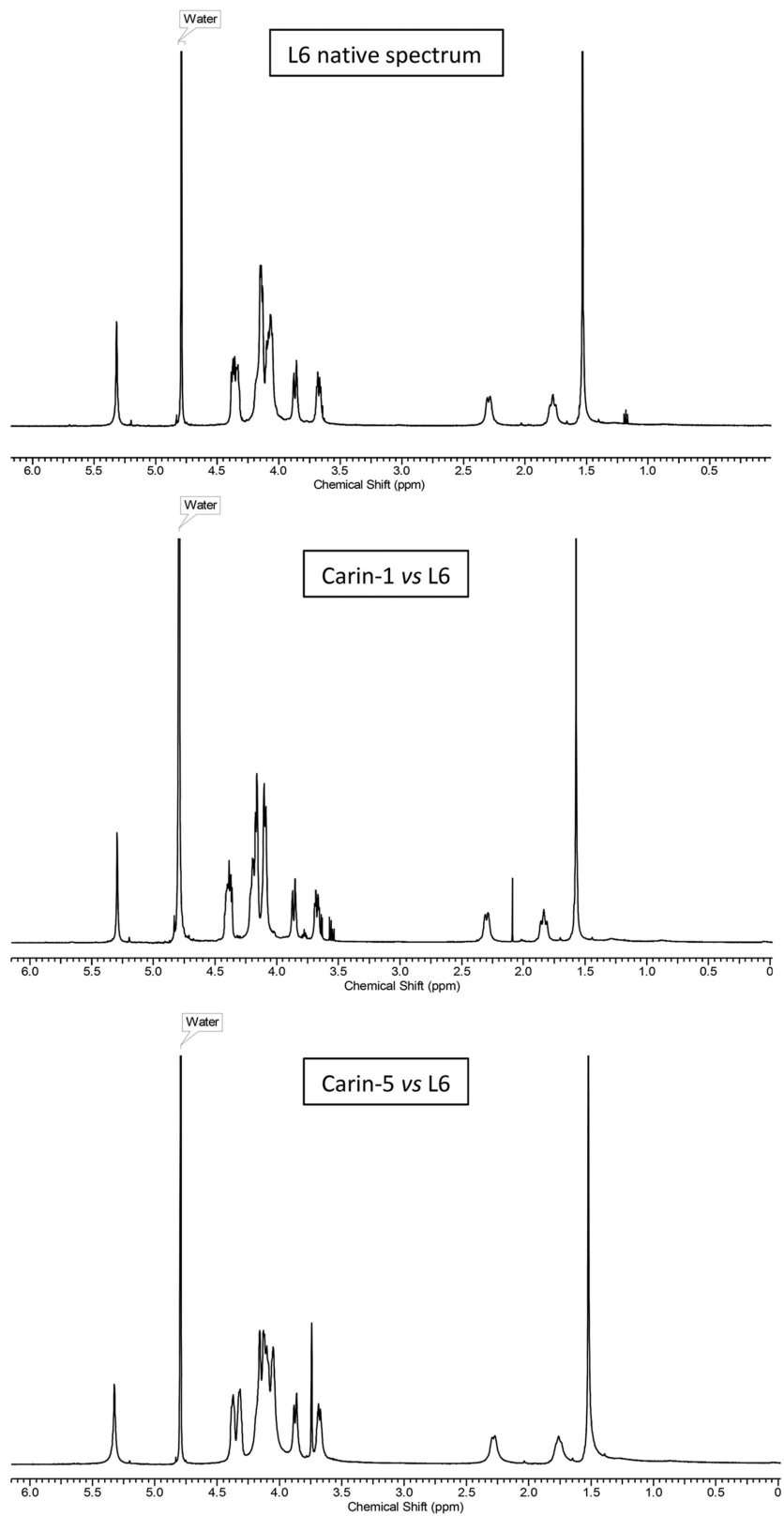


Figure 3. ¹H-NMR spectra of L₆ degradation products after Carin-1 and Carin-5 mediated depolymerization.

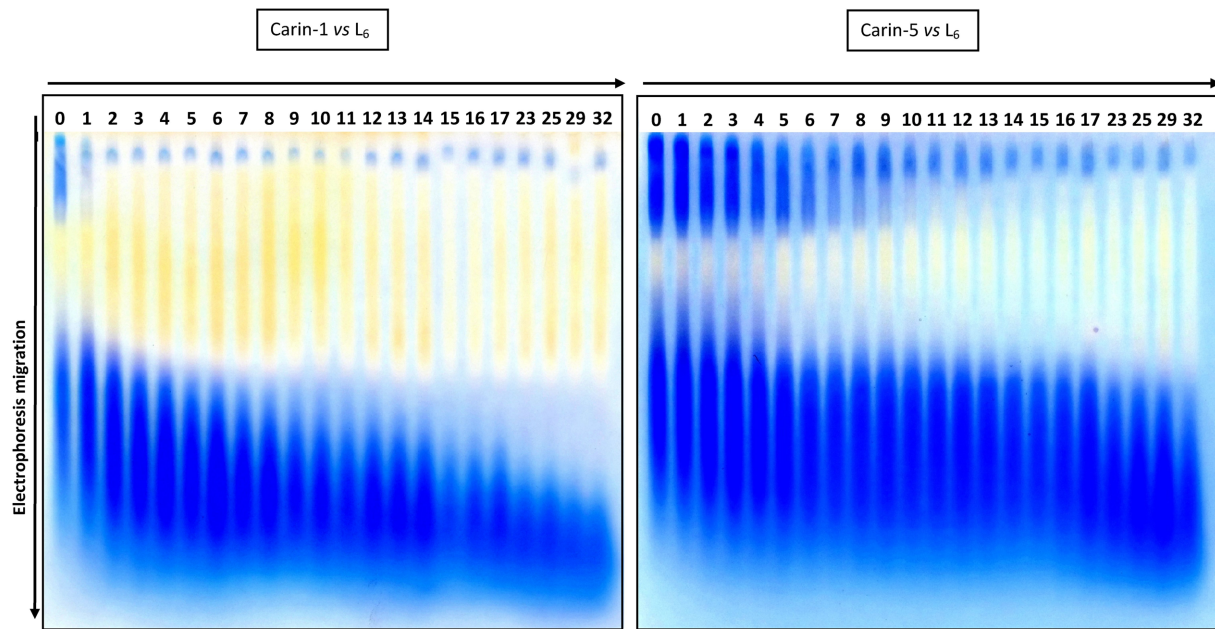


Figure 4. Degradation kinetics of the L₆ EPS (0.4 g.L⁻¹) by Carin-5 and Carin-1 (10⁹ PFU) as assessed by agarose gel electrophoresis (Stains all coloration, blue smear: polysaccharides, yellow smear: proteins). The migration profiles suggest that both phages induced the formation of intermediate molecular weight species during the course of L₆ depolymerization, yet, at different velocity.

Table 1. Adsorption coefficient of Carin-1, 2,3 and 5 under control and EPS induced host growing conditions.

Bacteriophage	Adsorption coefficient Cd (ml.min ⁻¹)		
	EPS synthesis non-induced	EPS synthesis induced	Ratio Cd _{non-ind} /Cd _{ind}
Carin-1	3,51.10 ⁻¹⁰	7,78.10 ⁻¹¹	4,51
Carin-2	5,08.10 ⁻⁸	3,32.10 ⁻⁹	15,30
Carin-3	1,75.10 ⁻⁸	3,32.10 ⁻¹¹	527
Carin-4	nd	nd	nd
Carin-5	1,56.10 ⁻⁹	2,07.10 ⁻¹⁰	7,53

also the number of viral receptors. Nonetheless, the stimulation of host EPS synthesis decreased Cd values (Table 1) regardless of the viral isolate, suggesting that intensive EPS production acts as a physical barrier for phage adsorption. Interestingly, the amplitude of the changes in Cd varied depending on the ability of Carin phages to degrade *C. marina* EPS. While the Cd value of Carin-1, -5 and -2, which encode EPS polysaccharidase, were increased upon induction of EPS synthesis 5, 7, and 15-fold respectively, that of the phage Carin-3 was drastically augmented with a calculated 527-fold increase. The resulting Cd values show that phages that display polysaccharidase activity generally adsorb faster on their host under conditions mimicking intense EPS production. In nature, the amount and the chemical composition of EPS excreted by bacteria can be influenced by environmental parameters such as temperature, nutrient, pH, but also by the bacterial lifestyle (planktonic versus biofilm) (Sutherland 1972; Kumar, Mody and Jha 2007). Under such conditions, the presence of polysaccharidases may provide a competitive advantage to efficiently propagate on their hosts. Likewise, the isolation of both EPS-polysaccharidase-encoding and EPS-polysaccharidase-lacking phages from the same geographical area could also reflect the dynamics or the variability of EPS production by *Cobetia marina* under natural settings.

Besides influencing phage adsorption kinetics, the presence and the diversity of polysaccharidase may influence host specificities as reported by Leiman et al. (2007). This previous study showed that the acquisition of several polysaccharidases by a given phage broaden its host range. In the light of this finding, we determined the host specificities of Carin phages using three *C. marina* strains and six strains of the genetically related genus *Halomonas* (Table S1, Supporting Information). This assay indicates that the five Carin phages have a narrow host range. In addition to *C. marina* DSMZ 4741, the phages Carin-1, 2, 3 and 4 could infect *C. marina* LMG 6798 whereas Carin-5 could not replicate on any alternate host. Hence, we could not relate the divergent EPS degrading abilities of Carin phages with their specificity pattern. The host spectrum assay was however conducted on a limited number of strains.

CONCLUDING REMARKS

Viruses are undoubtedly the most abundant biological entities in the ocean (Suttle 2007). So far, their role has mainly been attributed to the mortality and the subsequent cell lysis that they impose on their host. Viral lysis mediates the release of up to 150 Gt C per year, and as such, viruses are responsible for one of the main fluxes of DOM in the ocean (Suttle 2005). Although the ecological and biogeochemical implications

of marine viruses are acknowledged internationally, the regulation of viral activities are still poorly understood. Our study provides evidence that viruses of marine bacteria can display structural polysaccharidases that likely initiate the infection cycle. In the model system *C. marina*-specific viruses, these enzymes were detected in four out of five virus isolates, they appeared to be constitutive, endo-acting and functionally diverse. The detailed biochemical characterization of these molecules, that is the spectrum of substrates they can degrade, their regulation by environmental factors (i.e. temperature, pH, pressure, salinity), their life-time, or their structure, should provide novel insights into the functional mechanisms and the ecology of marine viruses.

More importantly, polysaccharidases are widely distributed in the ocean and they play an important role in biogeochemical cycles, digesting much of the marine DOM (Arnosti 2011). To date, it is broadly assumed that these enzymes mostly derive from bacteria. The finding that tailed marine phages, that are widespread and abundant in the ocean, can display constitutive polysaccharidases active on bacterial EPS lead us to question whether viruses could also contribute to the degradation of marine DOM, and, therefore, the marine carbon cycling in the ocean. Most marine bacteria indeed produce EPS, which potentially could fuel the pool of DOM in the ocean (Carlson and Hansell 2014). Yet, the fate of these compounds remains largely unknown (Zhang et al. 2015). Complex bacterial EPS are thought to be rather recalcitrant to microbial degradation suggesting that they have long resident time in the ocean (Jiao et al. 2010). The depolymerization of bacterial EPS by viral polysaccharidases could provide an unexpected process affecting size distribution and the recycling of these compounds even at cold temperature. Thus, viral polysaccharidases, according to their mode of action and polysaccharide specificities, could be unexpected additional players in the size-reactivity continuum model in DOM biogeochemistry (Benner and Amon 2015). Bacterial EPS, usually negatively charged, are also involved in the complexation of trace metals such as iron, which limits primary production in more than 40% of the ocean (Martin, Fitzwater and Gordon 1990; Falkowski et al. 1998; Boyd et al. 2000; Boyd and Ellwood 2010; Moore et al. 2013). Besides the possible direct impact on DOM recycling, virally mediated degradation could also modify the bioavailability of EPS-bound trace metals. Considering the numerical dominance of viruses in the ocean, future studies should focus on the extent of virally mediated EPS degradation in the sea, and the bioavailability of the degradation products. Over the past decades, the bacteria *C. marina* has been used as a model for studies on EPS production and biofilm remediation (Maréchal et al. 2004; Ivanova et al. 2005; Mieszkin et al. 2012; Salaün et al. 2012). Given the structural singularity of its EPS (Lelchat et al. 2015b) and the recent finding that members of the genus *Cobetia* may actively contribute to the carbon export in the global ocean (Guidi et al. 2016), this bacterium and its associated phages emerge as a model of considerable interest for such biogeochemical studies.

SUPPLEMENTARY DATA

Supplementary data are available at [FEMSEC](#) online.

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Résumé

Les virus constituent une force motrice pour le fonctionnement et l'évolution des écosystèmes marins. En infectant et tuant par lyse cellulaire une large fraction de la communauté planctonique, les virus influencent profondément la diversité microbienne et les cycles biogéochimiques à l'échelle globale. Il n'existe pourtant aucune information sur les mécanismes moléculaires qui conditionnent et qui régulent les interactions virales dans l'océan. Dans cette étude, je me suis intéressé à l'implication des polysaccharide dépolymérase (ou EPS dépolymérase) associées aux virus de bactéries (phages) dans la régulation des activités virales et leurs conséquences sur la biogéochimie océanique. Ces enzymes agissent comme « adjuvants » du cycle d'infection. Elles confèrent aux phages la faculté de dégrader les exopolysaccharides (EPS) excrétés par leurs hôtes avant d'atteindre leurs récepteurs membranaires. Les EPS dépolymérase font l'objet d'un grand nombre d'études biomédicales car elles offrent des perspectives prometteuses pour le bio-contrôle de bactéries pathogènes. A ce jour, ces enzymes sont inexploitées dans les environnements marins. Au cours de cette thèse, nous avons étudié de façon intégrative, du gène à l'écosystème, les EPS dépolymérase associées à 2 phages modèles (*Podoviridae*) qui infectent respectivement les bactéries marines *Cobetia marina* et *Vibrio alginolyticus*. Une combinaison d'approches bio-informatiques et protéomiques ont révélé que les gènes codant pour ces activités sont génétiquement éloignés des séquences connues avec des fonctions similaires. Une étude approfondie de l'une de ces enzymes (Dpo31, associée au phage de *C. marina*) suggère son appartenance à la classe des glycoside hydrolases et un mode de fonctionnement endolytique. Son activité de dépolymérisation est hautement spécifique, et ses paramètres biochimiques suggèrent une grande stabilité dans l'environnement marin. La surexpression et la cristallisation de Dpo31 nous ont permis de résoudre une majeure partie de sa structure. Cette dernière révèle une architecture moléculaire nouvelle avec certains domaines similaires à des protéines tailspike de phages connus. Dans l'océan, les EPS bactériens constituent un pool important de carbone organique dissout. La capacité des phages à dépolymériser les EPS de leurs hôtes suggère un possible impact sur l'utilisation de ces composés pour les communautés bactériennes. Une expérience en microcosme a montré que les dépolymérase virales réduisent la biodisponibilité des EPS et participent à la production de matière réfractaire dans le milieu naturel. Compte tenu de la prédominance des virus dans l'océan, ce processus jusqu'ici négligé pourrait avoir des implications biogéochimiques importantes. Au cours de cette thèse, l'identification et la caractérisation de l'EPS dépolymérase d'un phage marin a représenté un travail considérable mais les retombées sont significatives. Nous avons assigné une fonction biochimique à des gènes jusqu'alors inconnus et une structure 3D associée. Cette étude apporte de nouvelles connaissances fondamentales sur le processus d'infection des virus marins et, particulièrement intéressant, un rôle insoupçonné des virus sur la dégradation de la matière organique dissoute dans l'océan.

Abstract

Viruses represent a driving force for the functioning and evolution of marine ecosystems. Through the lysis of their hosts, viruses profoundly influence the diversity and biogeochemistry of the ocean. However, little is known on the mechanisms underlying virus-host interactions and how these mechanisms are regulated by changing environmental conditions. In this study, I investigated the implications of polysaccharide depolymerases (or EPS depolymerases) associated to bacterial viruses (phages) in the regulation of viral activities and their consequences on ocean biogeochemistry. These enzymes act as "adjuvants" of the infection cycle. They confer to phages the ability to degrade the exopolysaccharides (EPS) excreted by their hosts in order to access their membrane receptors. EPS depolymerases have been extensively studied in the biomedical domain because they offer promising prospects for the biocontrol of pathogenic bacteria. To date, these enzymes are unexplored in marine environments. In this thesis, we studied integratively, from gene to ecosystem, the EPS depolymerases associated to 2 model phages (*Podoviridae*) that infect marine bacteria *Cobetia marina* and *Vibrio alginolyticus*, respectively. A combination of bioinformatics and proteomic approaches revealed that the genes encoding these activities are genetically distant from known sequences with similar functions. An in-depth study showed that the enzymes Dpo31 (associated to *C. marina* phage) is a glycoside hydrolase with an endolytic mechanism. Its depolymerization activity is highly specific, and its biochemical parameters suggest a high stability of the enzyme in the marine environment. Also, the overexpression and crystallization of Dpo31 allowed us to solve a major part of its structure. The latter reveals a novel molecular architecture with some domains similar to tailspike proteins of known phages. In the ocean, bacterial EPS constitute a significant pool of dissolved organic carbon. The ability of phages to depolymerize the EPS of their hosts suggests a possible impact on the utilization of these compounds by bacterial communities. A microcosm experiment showed that viral depolymerases reduce the bioavailability of EPS and contribute to the production of refractory matter in the natural environment. Considering the predominance of viruses in the sea, this, so far, neglected process could have important implications for the functioning of the ocean. During this PhD research, the identification and the characterization of EPS depolymerase of marine phage represented a demanding work but the payoff was substantial. We assigned a biochemical function to genes that would have been otherwise overlooked, and an associated 3D structure. This study provided new insights into the infection process of marine viruses and, importantly, an unsuspected impact of viruses on the degradation of dissolved organic matter in the ocean.