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Rachid Aref El Aour Filho

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GRADUATE PROGRAM IN GENETICS



Doctoral Thesis

**Mechanisms of regulation of the host cell cycle by
*Staphylococcus aureus***

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Supervisors: Prof. Dr. Vasco Azevedo
Dr. Nadia Berkova
Co-supervisor: Dr. Thiago Castro
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Belo Horizonte

2016

Rachid Aref El Aouar Filho

**Mechanisms of regulation of the host cell cycle by
*Staphylococcus aureus***

PhD Thesis presented to the Graduate Program in Genetics of the Department of General Biology, at the Institute of Biological Sciences, Federal University of Minas Gerais, in partial fulfilment of the requirement for the degree of Doctor of Philosophy in Genetics.

**Supervisors: Prof. Dr. Vasco Azevedo
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Manuscript structure

This manuscript is subdivided into four major parts:

I. A general introduction to the following topics: (I) microbiological aspects of *Staphylococcus aureus* as well as diseases caused by this pathogen and associated pathogenic processes; (II) *S. aureus* phenol-soluble modulins (PSMs) and their role in pathogenesis; (III) the eukaryotic cell cycle and its primary regulators; (IV) the involvement of optineurin in different eukaryotic cell processes; (V) modulation of the eukaryotic cell cycle progression by different bacterial products (submitted as a review manuscript).

II. Chapter 1 reveals the pivotal role of PSMs in the *S. aureus*-induced G2/M transition delay during staphylococcal infection of human HeLa cells (presented as a published research article).

III. Chapter 2 presents our latest achievements in the analysis of molecular mechanisms of PSM-induced eukaryotic cell cycle alteration during *S. aureus* infection.

IV. General discussion, conclusions and perspectives of the work performed during PhD study.

Resumo

Staphylococcus aureus é uma bactéria Gram-positiva que coloniza a pele de animais e humanos saudáveis. Em condições específicas, incluindo o desequilíbrio da microbiota comensal, *S. aureus* pode interferir nas funções de defesa do organismo hospedeiro e causar diferentes doenças. Recentemente, nosso grupo de pesquisa mostrou que a linhagem MW2 (USA400) de *S. aureus*, resistente à meticilina (MRSA), causa atraso na transição da fase G2/M no ciclo de células HeLa. No presente trabalho, nós demonstramos que este atraso é provocado por componentes do sobrenadante de culturas de *S. aureus* MW2. Diferentes frações do sobrenadante destas culturas foram isoladas e avaliadas utilizando cromatografia por exclusão de tamanho e espectrometria de massa, o que nos permitiu identificar as modulinas solúveis em fenol alfa (PSM α) como sendo responsáveis pelo atraso observado no ciclo celular hospedeiro. Confirmando o papel destas modulinas, a linhagem MRSA LAC Δ psm α , mutante para os genes que codificam as PSM α 1 a 4, não foi capaz de impedir a progressão normal do ciclo em células HeLa. Além disso, o tratamento destas células com PSM α 1 e PSM α 3 sintéticas causou atraso na transição da fase G2/M associada a redução de genes de β -defensinas hospedeiras. Finalmente, nós demonstramos que a linhagem MW2, produtora de PSM α , causa a diminuição da produção de Optineurina e Optineurina fosforilada na ser 177 durante a infecção. A Optineurina, quando fosforilada na serina 177, é uma proteína cuja função está relacionada à transição da fase G2/M no ciclo celular eucariótico. O trabalho realizado nesta tese representa um grande avanço para a compreensão de como *S. aureus* modula o ciclo celular do hospedeiro, revelando um novo papel para as PSM α produzidas por esta bactéria.

Résumé

Staphylococcus aureus est une bactérie Gram positive qui colonise la peau des animaux et des humains sains. Dans certaines conditions, telles que la perturbation du microbiote, *S. aureus* peut induire différentes maladies en déjouant les fonctions de défenses de la cellule hôte. Récemment, notre équipe a montré que les *S. aureus* méthiciline-résistant (MRSA) souche MW2 (USA400) étaient capables d'induire un retard de la transition de phase G2/M des cellules HeLa. Dans ce travail, nous avons démontré que cette action est initiée par des composants du surnagent de culture de *S. aureus*. Différentes fractions de surnagents de culture de MW2 ont été obtenues par la chromatographie d'exclusion et analysées par la spectrométrie de masse. Ces techniques nous ont permis d'identifier les peptides phenol-soluble modulins alpha (PSM α) comme responsables du retard du cycle cellulaire des cellules hôtes. Confirmant l'implication de ces modulines, la souche LAC Δ psm α déficiente en PSM α 1 – 4, n'a pas affecté la progression normale du cycle cellulaire de cellules épithéliales HeLa. De plus, le traitement de ces cellules avec des PSM α 1 et PSM α 3 synthétiques a induit un retard de la transition de phase G2/M qui a été associé à la diminution de l'expression de gènes codant des défensines β . Enfin, nous avons démontré que la souche MW2 diminue le niveau d'optineurine et d'optineurine phosphorylée sur la sérine-177, une protéine hôte qui est impliquée dans la transition de phase G2/M. Ce travail représente une étape importante de la compréhension du mécanisme d'interférence de *S. aureus* avec le cycle cellulaire de la cellule hôte, révélant un nouveau rôle de PSM α , molécule produite par cette bactérie.

Abstract

Staphylococcus aureus is a Gram-positive bacterium that colonizes the skin of healthy animals and humans. In certain conditions, including the disruption of the commensal microbiota, *S. aureus* can cause different diseases by deviating the host defense functions. Recently, our group has shown that the methicillin-resistant *S. aureus* (MRSA) MW2 (USA400) strain causes delay in the transition of the G2/M phase of HeLa cells. In the present work, we demonstrated that this action is initiated by components of the supernatant of the *S. aureus* culture. Different supernatant fractions were obtained by size exclusion chromatography and were analyzed by mass spectrometry, which allowed to identify phenol-soluble modulins alpha (PSM α) as responsible for the host cell cycle delay. Confirming the involvement of these modulins in the delay, the MRSA LAC Δ *psma* strain, which is deficient in PSM α 1–4, did not affect the normal progression of the cycle in HeLa cells. In addition, the treatment of these cells with synthetic PSM α 1 and PSM α 3 caused delay in the transition of the G2/M phase associated with the decreased production of host β -defensins. Lastly, we demonstrated that the MW2 strain, which produce PSM α , decreases the level of optineurin and optineurin phosphorylated at serine 177, a host protein that is involved in the G2/M phase transition. The work conducted in this thesis represents an important achievement in the understanding of how *S. aureus* interferes with the host cell cycle, revealing a new role for PSM α produced by this bacterium.

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Abreviaturas/Abréviations

5' UTR = 5' untranslated region
aa = amino acids
ACT = Adenylate Cyclase Toxin
Agr = Accessory gene egulator
AIP = Auto-inducible peptide
APC/C = Anaphase-promoting complex/cyclosome
BHI = Brain Heart Infusion
CAK = CDK-activating kinase
CA-MRSA = Community-associated methicillin-resistant *Staphylococcus aureus*
Can = Collagen adhesion
CDC25 = Cell division cycle 25
CDK = Cyclins dependent kinase
Cdmn = Dulbecco's modified Eagle complete
CDT = Cytolethal Distending Toxin
CHIPS = Chemotaxis Inhibitory Protein of *Staphylococcus aureus*
Cif = Cycle Inhibiting Factor
Clfa and Clfb = Clumping factor (A and B)
CNF = Cytotoxic necrotizing factor
DTB = Double Block Thymidine
EDIN = Epidermal Cell Differentiation Inhibitor
ETA e ETB = Exfoliative Toxins (A and B)
FACS = Fluorescence-Activated Cell Sorting
Fg = Fibrinogen
FLIPr = Formyl Peptide Receptor-like 1 Inhibitor
Fn = Fibronectin
FnBPA and FnBPB = Fibronectin -Binding Protein (A and B)
FPR1 and FPR 2 = Formyl Peptide Receptor (1 and 2)
G1 and G2 = Gap (1 and 2)
HA-MRSA = Hospital-Associated Methicillin-Resistant *Staphylococcus aureus*
HIP7 = Huntingtin-Interacting Protein 7
HPLC = High-Performance Liquid Chromatography
Htt = Huntingtin protein
HYPL = Huntingtin yeast partner L
IKK = Inhibitor of Nuclear Factor Kappa-B Kinase
IL = Interleukin
IRF = Interferon Regulatory Factors
KD = Kinase Domain
LC3 = Light chain 3 receptor
M = Mitoses
MOI = Multiplicity of infection
MRSA - Methicillin-Resistant *Staphylococcus aureus*
MSCRAMM = Microbial Surface Components Recognizing Adhesive Matrix
MYPT1-PP1 β = Myosin Phosphatase complex
Neks = NIMA-related kinases
NEMO = NF κ B essential modulator
NES = Nuclear Export Signal
NRP = NEMO-related protein

OD = Optical Density
Optineurina - Optic neuropathy inducing
PAMPs = Pathogen-Associated Molecular Patterns
PBD = Polo Box Domain
PLKs = Polo-like kinases
PMNs = Polymorphonuclear Neutrophils
pRb = Retinoblastoma tumour suppressor gene
PSM = Phenol-Soluble Modulins
PVL = Pantan-Valentine Leucocidin
S = Synthesis
SCC = Staphylococcal Cassette Chromosome
SCCmec = Staphylococcal Cassette Chromosome-mec
SD = Standard Deviation
SE = Staphylococcal Enterotoxin
Ser177 = Serine 177
Ser473 = Serine 473
TFIIIA-INTP = Transcription factor IIIA-interacting protein
Thr14 = Treonina 14
Thr161 = Treonine 161
Thr210 = Threonine 210
TSST-1 = Toxic shock syndrome toxin 1
Tyr15 = Tyrosine 15
UBD = Ubiquitin binding domain
VacA = Vacuolating Cytotoxin
WB = Western Blotting

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Introduction Générale

Le genre *Staphylococcus* appartient à la famille des Micrococcaceae, composée de bactéries Gram-positives qui s'organisent en groupes de cocci. Les staphylocoques mesurent entre 0,5 et 1,5 μm , sont non motiles, ne forment pas de spores, et sont anaérobies facultatives. Ce groupe se caractérise par la pigmentation dorée des colonies, est catalase positif, peroxydase négatif et est capable de fermenter le mannitol (Lowy, 1998). *S. aureus* est un pathogène opportuniste qui peut se trouver dans le microbiote humain sans pour autant provoquer de maladie chez l'hôte. Cette bactérie colonise de préférence la région nasale et est persistente chez 20% de la population et intermittente pour 30% (Kuehnert et al., 2006). Lors d'un possible déséquilibre du système immunitaire et/ou du microbiote bactérien de l'hôte, *S. aureus* peut passer d'un état commensal à celui de pathogène (Lowy, 1998). Il peut ainsi provoquer un large panel d'infections telles que l'endocardite, la septicémie, la pneumonie, l'arthrite septique, l'ostéomyélite, la bactériémie, ainsi que des infections de la peau, principalement chez l'homme et chez les animaux à sang chaud (Feil et al., 2003; Ishii et al., 2006). Ce microorganisme est non seulement l'agent principal des maladies nosocomiales chez l'homme, mais aussi un des principaux agents étiologiques de la mammite, caractérisée par l'inflammation de la glande mammaire des petits ruminants et des bovins.

Le succès de *S. aureus* comme pathogène humain se doit à sa polyvalence et son adaptabilité, ce microorganisme étant capable de résister à l'action d'une grande variété d'antibiotiques. Le traitement le plus employé contre les infections à *S. aureus* est l'administration de pénicilline, un antibiotique bêta-lactame (Lowy, 1998). Cependant, en cas de résistance, d'autres classes d'antibiotiques sont utilisées, conjointement ou séparément (Lowy, 1998). C'est en 1942 que la résistance à la pénicilline de *S. aureus* a été enregistrée pour la première fois, un an après son introduction.

Les mécanismes de résistance de *S. aureus* aux bêta-lactamines ont été décrits durant cette même décennie (Rammelkamp and Maxon, 1942). Dans les années 50, un relevé, basé sur diverses publications, a montré que de nombreuses souches de *S. aureus* présentes dans de grands centres hospitaliers étaient résistantes à la pénicilline (Finland, 1955; Panlilio et al., 1992). Au fil des années, *S. aureus* a développé une résistance à d'autres antibiotiques et, en 1959, la méticilline a été introduite pour contourner l'inefficacité de la pénicilline (Stryjewski and Corey, 2014). Toutefois, en l'espace de

deux ans après l'introduction de la méticilline, des souches résistantes ont été isolés d'échantillons prélevés chez des humains infectés. On a nommé ces souches *Staphylococcus aureus* résistants à la méthicilline. (SARM) (Jevons, 1961). Le mécanisme de résistance à la méticilline, identifié en 1981, est lié à l'expression d'une transpeptidase codée par le gène *mec* localisé dans la cassette chromosomique staphylococcique (SCC*mec*). La SCC*mec* peut présenter des variations de structure et coder différents types de transpeptidases (types I à V) capables de diminuer l'action de tous les β -lactames (Hanssen and Ericson Sollid, 2006). La résistance de *S. aureus* à des antibiotiques comme la pénicilline, la métacilline et la vancomicine a diminué le nombre de médicaments efficaces contre ce microorganisme (Levy and Marshall, 2004). La base de la colonisation par *S. aureus* est complexe et encore mal comprise car impliquant la production de facteurs de virulence qui facilitent l'adhésion et l'invasion des cellules-hôtes, l'interaction cellule-cellule, l'évasion de la réponse immunitaire et la lésion des tissus (Gordon and Lowy, 2008; Holden et al., 2004).

Le répertoire des facteurs de virulence de *S. aureus* est étendu et composé de produits sécrétés et de protéines de la membrane cellulaire, qui dirigent le processus de la pathogénèse (Gordon and Lowy, 2008; Lowy, 1998). Parmi les facteurs de virulence produits par ce microorganisme, nous pouvons citer les Molécules Matricielles Adhésives de Reconnaissance d'un Composant Superficiel Microbien (MSCRAMMs), ainsi que les leucocidines et des enzymes comme la coagulase et la β -lactamase. Tous ces facteurs peuvent jouer un rôle important dans les étapes de la pathogénèse comme l'adhésion, l'invasion, la persistance, l'évasion, et la mort de cellules-hôtes (Gordon and Lowy, 2008; Labandeira-Rey et al., 2007; Otto, 2014a).

Récemment, un nouveau groupe de facteurs de virulence a été décrit pour *Staphylococcus epidermidis*, englobant les modulines phénol-solubles (PSMs). Ces facteurs ont été isolés à partir du surnageant de cultures de *S. aureus* par chromatographie d'exclusion de taille en utilisant un solvant contenant du phénol. Les PSMs de *S. epidermidis* sont capables d'induire une activité pro-inflammatoire dans des lignées de cellules humaines transfectées avec le virus HIV-1, menant à l'activation de NF- κ B et de cytokines qui modulent la réponse immunitaire de l'hôte (Mehlin et al., 1999).

Les PSMs font partie d'une famille de peptides amphipathiques, disposés en forme d' α -hélice et ont été rapportés dans la littérature comme étant important dans la virulence de *S. aureus*. Les PSMs peuvent être classées en fonction de la longueur de leur chaîne d'acides aminés (aa). La longueur de celles du type α (1-4) se situe entre 20-25 aa, la taille de celles de type β (1-2) est environ le double (entre 43-45 aa), et celles du type δ mesurent entre 25-26 aa (Cheung et al., 2014, Otto, 2014). Ces peptides sont codés dans trois régions différentes du génome; (i) les PSM α 1-4 sont codés par l'opéron *psma*, (ii) les PSM β 1-2 par l'opéron *psm β* , et la (iii) toxine de type δ par le gène *hdi* (Cheung et al., 2014; Peschel and Otto, 2013). Divers pathogènes présentent une variété de stratégies pour manipuler les fonctions des cellules-hôtes, probablement pour leur propre bénéfice (Bhavsar et al., 2007). La capacité qu'ont les bactéries à adhérer aux cellules-hôtes, à internaliser et se multiplier dans ces dernières se doit au développement d'un arsenal de mécanismes pour bloquer ou contrôler les processus normaux des cellules-hôtes (Bhavsar et al., 2007). L'interférence dans les processus cellulaires de l'hôte implique normalement la production et l'exportation de différentes toxines par les systèmes de sécrétion les plus variés, ce qui interfère avec des processus tels que l'organisation du cytosquelette et le contrôle de l'apoptose (Knodler et al., 2001). La participation des toxines bactériennes dans la modulation du cycle cellulaire de l'hôte implique aussi divers mécanismes, comme la diminution de la prolifération/activation des cellules du système immunitaire, et la diminution de la prolifération des cellules des barrières épithéliales. Ces processus conduisent à une augmentation de la survie bactérienne pendant le processus infectieux (Nougayrède et al., 2005; Oswald et al., 2005). Les toxines bactériennes liées à la modulation du cycle cellulaire ont été largement étudiées (Nougayrède et al., 2005; Oswald et al., 2005), il n'existe cependant que très peu d'information concernant l'implication des toxines modulatrices produites par *S. aureus* dans le retard du cycle cellulaire des cellules eucaryotes. Le premier travail sur ces toxines, présenté dans la littérature, décrit la participation de la souche Cowan I de *S. aureus*, inactivée par la formaldéhyde, altérant la différenciation et l'activation des cellules B (Jelinek and Lipsky, 1985). Au début des années 90, un facteur appelé Inhibiteur de la Différenciation Cellulaire Épidermique (EDIN, Epidermal cell Differentiation Inhibitor), produit par *S. aureus*, a été décrit comme induisant l'hyperplasie de cellules épithéliales humaines et murines (Sugai et al., 1992). La participation de l' α -toxine, un des deux facteurs principaux de la virulence de *S. aureus*, a démontré l'augmentation du nombre de cellules eucaryotes

dans la phase S+G2/M (Haugwitz et al., 2006). L'analyse de transcrits des cellules épithéliales exposées à *S. aureus* a démontré une altération de l'expression de gènes impliqués dans la régulation du cycle cellulaire, comme *plk1* et *cdc25c* (Heimer et al., 2010). Notre groupe de recherche a démontré que la souche MRSA MW2 induit le retard de la phase G2/M du cycle cellulaire des cellules HeLa (Alekseeva et al., 2013). Postérieurement, une autre étude a rapporté qu'un composant de la leucocidine de Panton-Valentine (PVL) de *S. aureus* conduit à l'apoptose, dû au retard dans la phase G0/G1 du cycle cellulaire (Bu et al., 2013). Récemment, une étude effectuée par l'équipe de Centre National de Référence des Staphylocoques (Lyon, France) et notre groupe de recherche a analysé la capacité d'entérotoxines SEA, SEG, SEI, SEIM, SEIN et SEIO à altérer le cycle cellulaire des cellules hôte et a démontré que la *Staphylococcal Enterotoxin O* (SEIO) induit un retard de la phase G0/G1 sans provoquer la nécrose ou l'apoptose des cellules-hôtes (Hodille et al., 2016).

Le cycle cellulaire est un processus complexe responsable de la coordination de la prolifération cellulaire et la réparation de l'ADN, parmi d'autres processus cellulaires. Les altérations dans la progression normale du cycle cellulaire peuvent provoquer des maladies comme le cancer (Vermeulen et al., 2003). La régulation du cycle cellulaire est déterminée par l'action conjointe d'un groupe de protéines variées qui culmine dans la mitose et la génération subséquente de deux cellules filles identiques (Schafer, 1998). Elles coordonnent les phases de progression du cycle cellulaire qui se divisent en interphase (G₁, S, G₂) et en mitose (M). La mitose est subdivisée en prophase, métaphase, anaphase et télophase (Vermeulen et al., 2003).

La régulation du cycle cellulaire se réalise par un complexe formé par des *cyclin-dependent kinases* (CDKs) et des cyclines. Les CDKs sont des protéines sérine/tréonines kynases qui jouent un rôle critique dans l'activation et l'inactivation de points spécifiques du cycle cellulaire. Par exemple, dans la mitose, l'inactivation des CDKs empêche la progression du cycle cellulaire (Satyanarayana and Kaldis, 2009). Les CDKs s'expriment constamment au contraire des cyclines, leurs protéines activatrices, qui varient durant le cycle en augmentant ou diminuant en fonction de différents *checkpoints* (Vermeulen et al., 2003). Par ailleurs, d'autres protéines kynases, comme les *Polo-like kinases* (PLKs), *Aurora* et *NIMA-related kinases* (Neks) ont des fonctions essentielles dans le contrôle du cycle cellulaire. De plus, la protéine *multifunctional Optic neuropathy inducing* (optineurin), est associée à plusieurs

pathologies humaines et participe à la régulation de la transition G2/M dans les cellules eucaryotes (Kachaner et al., 2012a).

Introdução Geral

O gênero *Staphylococcus* pertence à família Micrococcaceae, que é composta por bactérias Gram-positivas que se organizam em grupos de cocos. Os estafilococos medem entre 0,5 e 1,5 μm , são imóveis, não formam esporos e são anaeróbicos facultativos. Este grupo é caracterizado pela pigmentação dourada das colônias, sendo positivo para catalase, negativo para oxidase e capaz de fermentar manitol (Lowy, 1998). *S. aureus* é um micro-organismo oportunista que pode fazer parte da microbiota humana sem causar enfermidades no hospedeiro. Esta bactéria coloniza preferencialmente a região nasal, sendo persistente em 20% e intermitente em 30% da população (Kuehnert et al., 2006). Durante possíveis desequilíbrios no sistema imune e na microbiota bacteriana do hospedeiro, *S. aureus* é capaz de passar de organismo oportunista para patogênico (Lowy, 1998). Assim, pode ocasionar doenças tais como endocardite, septicemia, pneumonia, artrite séptica, osteomielite, bacteremia e infecções de pele principalmente em humanos e bovinos (Feil et al., 2003; Ishii et al., 2006). Este micro-organismo é o principal agente causador de doenças nosocomiais em humanos, além de ser um dos principais agentes etiológicos da mastite, que é caracterizada pela inflamação da glândula mamária em pequenos ruminantes e bovinos.

O sucesso de *S. aureus* como patógeno humano se deve à versatilidade desse micro-organismo, que é capaz de resistir à ação de uma grande variedade de antibióticos. O tratamento mais empregado contra infecções por *S. aureus* é a administração de penicilina, um antibiótico beta-lactâmico (Lowy, 1998). Entretanto, em casos de resistência a este antibiótico, são utilizados antibióticos de outras classes em combinação ou separadamente (Lowy, 1998). Em *S. aureus*, a resistência à penicilina foi primeiramente registrada em 1942, um ano depois da utilização deste antibiótico. Nesta mesma década, os mecanismos de resistência de *S. aureus* aos beta-lactâmicos foram descritos (Rammelkamp and Maxon, 1942). Na década de 50, um levantamento feito com base em diversas publicações apresentou que muitas linhagens de *S. aureus* presentes em grandes centros hospitalares mostraram-se resistentes à penicilina (Finland, 1955; Panlilio et al., 1992). Com o passar dos anos, *S. aureus* adquiriu resistência a diversos outros antibióticos; e em 1959 a meticilina foi introduzida para contornar a ineficácia da penicilina (Stryjewski and Corey, 2014). Entretanto, em apenas dois anos após o início da utilização da meticilina, as linhagens

resistentes a este antibiótico foram isoladas de amostras de humanos infectados, às quais foram designadas *Staphylococcus aureus* meticilina resistentes (MRSA) (Jevons, 1961). O mecanismo de resistência à meticilina, identificado em 1981, está relacionado à expressão de uma transpeptidase, codificada pelo gene *mec* localizado no cassete estafilocócico cromossômico (SCC*mec*). O SCC*mec* pode apresentar variações em sua estrutura e codificar diferentes tipos de transpeptidases (tipos I a V) capazes de diminuir a ação de todos os β -lactâmicos (Hanssen and Ericson Sollid, 2006). A resistência de *S. aureus* a antibióticos como penicilina, meticilina e vancomicina tem diminuído o número de drogas efetivas contra este micro-organismo (Levy and Marshall, 2004). A base da colonização por *S. aureus* é complexa e pouco compreendida, envolvendo a produção de fatores de virulência que facilitam a adesão e a invasão das células hospedeiras, a interação célula-célula, a evasão da resposta imune e o dano tecidual (Gordon and Lowy, 2008; Holden et al., 2004).

O repertório de fatores de virulência de *S. aureus* é extenso e composto por produtos secretados e estruturais, como as proteínas de membrana celular, que direcionam o processo de patogênese (Gordon and Lowy, 2008; Lowy, 1998). Dentre os fatores de virulência produzidos por este micro-organismo podemos citar as chamadas *Microbial Surface Components Recognizing Adhesive Matrix Molecules* (MSCRAMMs), além das leucocidinas e das enzimas como a coagulase e β -lactamase. Todos esses fatores podem desempenhar um papel importante nas etapas de patogênese, incluindo adesão, invasão, persistência, evasão e morte de células hospedeiras (Gordon and Lowy, 2008; Labandeira-Rey et al., 2007; Otto, 2014a).

Recentemente, um novo grupo de fatores de virulência foi descrito para *Staphylococcus epidermidis*, englobando as chamadas Modulinas Solúveis em fenol (PSMs). Esses fatores foram isolados a partir do sobrenadante de culturas desta bactéria por cromatografia de exclusão de tamanho com o uso de um solvente contendo fenol. As PSMs de *S. epidermidis* são capazes de induzir atividade pró-inflamatória em linhagens de células humanas transfectadas com o vírus HIV-1, levando à ativação de NF- κ B e citocinas que modulam a resposta imune do hospedeiro (Mehlin et al., 1999).

As PSMs fazem parte de uma família de peptídeos anfipáticos, dispostos em forma de α -hélice, e têm sido relatadas na literatura por serem importantes na virulência de *S. aureus*. As PSMs podem ser classificadas de acordo com o comprimento da cadeia de aminoácidos (aa), sendo que as do tipo α (1-4) possuem comprimento entre 20-25aa, as do tipo β (1-2) possuem aproximadamente o dobro do tamanho (entre 43-45aa), e as

do tipo δ possuem entre 25-26aa (Cheung et al., 2014; Otto, 2014a). Estes peptídeos são codificados em três regiões diferentes do genoma (i) as PSM α 1-4 codificadas pelo operon *psma*, (ii) as PSM β 1-2 codificadas pelo operon *psm β* , e a (iii) toxina do tipo δ codificada pelo gene *hdl* (Cheung et al., 2014; Peschel and Otto, 2013).

Diversos patógenos apresentam uma variedade de estratégias para manipular as funções das células hospedeiras, provavelmente para seu próprio benefício (Bhavsar et al., 2007). A habilidade das bactérias de aderir às células hospedeiras, internalizar e multiplicar se deve ao desenvolvimento de um arsenal de mecanismos para bloquear ou controlar processos normais das células hospedeiras (Bhavsar et al., 2007). A interferência nos processos celulares dos hospedeiros normalmente envolve a produção e exportação de diferentes toxinas pelos mais variados sistemas de secreção, as quais interferem em processos como o montagem do citoesqueleto e controle de apoptose (Knodler et al., 2001). A participação de toxinas bacterianas na modulação do ciclo celular hospedeiro também envolve diversos mecanismos, como diminuição da proliferação/ativação de células do sistema imune, diminuição da proliferação das células das barreiras epiteliais. Esses processos levam ao aumento do sucesso bacteriano durante o processo infeccioso (Nougayrède et al., 2005; Oswald et al., 2005). As toxinas bacterianas relacionadas à modulação do ciclo celular têm sido amplamente estudadas (Nougayrède et al., 2005; Oswald et al., 2005), porém não existem muitas informações sobre o envolvimento das toxinas modulatórias, produzidas por *S. aureus*, no atraso do ciclo celular de células eucarióticas. O primeiro trabalho apresentado na literatura sobre essas toxinas descreve a participação da linhagem Cowan I de *S. aureus*, inativada com formalina, alterando a diferenciação e ativação de células B (Jelinek and Lipsky, 1985). No início da década de 90, um fator chamado *Epidermal Cell Differentiation Inhibitor* (EDIN), produzido por *S. aureus*, induz hiperplasia de células epiteliais humanas e murinas (Sugai et al., 1992). A participação da α -toxin, um dos principais fatores de virulência de *S. aureus*, demonstrou aumentar o número de células eucarióticas na fase S+G2/M (Haugwitz et al., 2006). As análises do transcriptoma das células epiteliais expostas a *S. aureus* demonstraram uma alteração na expressão de genes regulatórios do ciclo celular, como *plk1* e *cdc25c* (Heimer et al., 2010). O nosso grupo de pesquisa demonstrou que linhagens MRSA foram capazes de induzir o atraso na fase G2/M do ciclo celular de células HeLa (Alekseeva et al., 2013). Posteriormente, outro estudo relatou que um componente da Leucocidina de *Panton-Valentine* (PVL) de *S. aureus* leva a apoptose devido ao atraso na fase G0/G1 do ciclo celular (Bu et al., 2013).

Recentemente, um estudo realizado pela equipe do Centro Nacional de Referência a Estafilococos (Lyon, France) e nosso grupo de pesquisa, analisou a capacidade das enterotoxinas SEA, SEG, SEI, SEIM, SEIN e SEIO alterarem o ciclo celular de células hospedeiras, e demonstrou que a enterotoxina estafilocócica O (SEIO) leva ao atraso na fase G0/G1 sem causar necrose ou apoptose das células hospedeiras (Hodille et al., 2016).

O ciclo celular é um processo complexo responsável por coordenar a proliferação celular e o reparo do DNA, dentre outros processos celulares. As alterações na progressão normal do ciclo celular podem levar a doenças como o câncer (Vermeulen et al., 2003). A regulação do ciclo celular é determinada pela ação conjunta de um variado grupo de proteínas que culmina na mitose e a geração subsequente de duas células filhas idênticas (Schafer, 1998). Elas coordenam as fases de progressão do ciclo celular que são divididas em intérfase (G₁, S, G₂) e mitose (M). A mitose é subdividida em prófase, metáfase, anáfase e telófase (Vermeulen et al., 2003).

A regulação do ciclo celular é realizada por um complexo formado por quinases dependentes de ciclina (CDKs) e ciclinas. As CDKs são proteínas serina/treoninas quinases que assumem um papel crítico na ativação e inativação de pontos específicos do ciclo celular. Por exemplo, na mitose a inativação das CDKs previne a progressão do ciclo celular (Satyanarayana and Kaldis, 2009). As CDKs são expressas constantemente ao contrário das suas proteínas ativadoras, as ciclinas, que variam constantemente durante o ciclo, aumentando e diminuindo de acordo com determinados *checkpoints* (Vermeulen et al., 2003). Em adição as CDKs, outras proteínas quinases, como as *Polo-like kinases* (PLKs), *Aurora* e *NIMA-related kinases* (Neks) possuem papéis essenciais na participação do controle do ciclo celular (Salaün et al., 2008). Além disso, a proteína *multifuncional Optic neuropathy inducing* (Optineurin), está associada a diversas patologias humanas e participa da regulação da transição G2/M em células eucarióticas (Kachaner et al., 2012a).

1 - Introdução/ Introduction

Parte 1

1 - Staphylococcus aureus

O gênero *Staphylococcus* pertence à família Micrococcaceae, que é composta por bactérias Gram-positivas que se organizam em grupos de cocos. Os estafilococos medem entre 0,5 e 1,5 μm , são imóveis, não formam esporos e são anaeróbicos facultativos. Este grupo é caracterizado pela pigmentação dourada das colônias, sendo positivo para catalase, negativo para oxidase e capaz de fermentar manitol (Lowy, 1998). *S. aureus* é um micro-organismo oportunista que pode fazer parte da microbiota humana sem causar enfermidades no hospedeiro. Esta bactéria coloniza preferencialmente a região nasal, sendo persistente em 20% e intermitente em 30% da população (Kuehnert et al., 2006). Durante possíveis desequilíbrios no sistema imune e na microbiota bacteriana do hospedeiro, *S. aureus* é capaz de passar de organismo comensal para patogênico (Lowy, 1998). Assim, *S. aureus* pode causar várias doenças em seus hospedeiros, tais como endocardite, septicemia, pneumonia, artrite séptica, osteomielite, bacteremia e infecções de pele (Feil et al., 2003; Ishii et al., 2006). Este microrganismo é o principal agente causador de doenças nosocomiais em humanos, além de ser um dos principais agentes etiológicos da mastite, que é caracterizada pela inflamação da glândula mamária em pequenos ruminantes e bovinos.

1.1 - Staphylococcus aureus resistentes a meticilina

A base da colonização por *S. aureus* é complexa e pouco compreendida, envolvendo a produção de fatores de virulência que facilitam a adesão e a invasão das células hospedeiras, a interação célula-célula, a evasão da resposta imune e o dano tecidual (Gordon e Lowy, 2008; Holden et al., 2004). O sucesso de *S. aureus* como patógeno humano se deve à versatilidade desse micro-organismo, que é capaz de resistir à ação de uma grande variedade de antibióticos. O tratamento mais empregado contra infecções por *S. aureus* é a administração de penicilina, um antibiótico beta-lactâmico

(Lowy, 1998). Entretanto, em casos de resistência a este antibiótico, são utilizados antibióticos de outras classes, em combinação ou separadamente (Lowy, 1998).

Em *S. aureus*, a resistência à penicilina foi primeiramente registrada em 1942, um ano depois do início da utilização deste antibiótico. Nesta mesma década, os mecanismos de resistência de *S. aureus* aos beta-lactâmicos foram descritos (Rammelkamp e Maxon, 1942). Na década de 1950, uma revisão, tomando como base publicações anteriores, relatou que muitas linhagens de *S. aureus* presentes em grandes centros hospitalares mostraram-se resistentes à penicilina (Finland, 1955). Em 1959, a meticilina foi introduzida para contornar a ineficácia da penicilina (Stryjewski e Corey, 2014). Porém, apenas dois anos após o início da utilização da meticilina, as primeiras linhagens resistentes a esse antibiótico foram isoladas de amostras de humanos infectados, recebendo a denominação MRSA (do inglês, *Methicillin-resistant Staphylococcus aureus*) (Jevons, 1961). A resistência de *S. aureus* a antibióticos como penicilina, meticilina e vancomicina tem contribuído para uma diminuição no número de drogas efetivas contra este micro-organismo (Levy e Marshall, 2004).

O mecanismo de resistência à meticilina, identificado em 1981, está relacionado à expressão de uma transpeptidase, codificada pelo gene *mec*, localizado no cassete estafilocócico cromossômico (SCC*mec*, do inglês, *Staphylococcal Cassette Chromosome mec*). O SCC*mec* pode apresentar variações em sua estrutura e codificar diferentes tipos (I a V) de transpeptidases capazes de diminuir a ação de todos os β -lactâmicos (Hanssen e Ericson Sollid, 2006). A emergência de linhagens pertencentes ao grupo MRSA tem sido historicamente associada aos procedimentos de cuidados à saúde, os quais constituem uma das causas mais comuns de infecções nosocomiais associadas à resistência a várias drogas. Um fato importante é que mais de 40% das infecções por *S. aureus* se devem a linhagens do grupo MRSA, as quais podem infectar a corrente sanguínea, trato respiratório, trato urinário e pele (File, 2008).

Atualmente, este grupo de bactérias é dividido em *S. aureus* resistentes à meticilina e associadas a hospitais (HA-MRSA, do inglês, *Hospital-associated methicillin-resistant Staphylococcus aureus*) e *S. aureus* resistentes à meticilina e associadas à comunidade (CA-MRSA, do inglês, *Community-associated methicillin-resistant Staphylococcus aureus*). As HA-MRSA estão normalmente relacionadas a pacientes hospitalizados por pelo menos 48-72h, a pacientes que receberam alta hospitalar há poucos dias e a pacientes com infecções originadas em unidades de tratamento intensivo. As CA-MRSA estão associadas a infecções que não envolvem

centros de tratamento de saúde, podendo ocorrer na ausência de fatores de risco envolvendo procedimentos hospitalares (Millar et al., 2007).

Diferenças genótípicas, fenotípicas e epidemiológicas são encontradas entre HA-MRSA e CA-MRSA. As linhagens CA-MRSA são mais comumente susceptíveis aos antimicrobianos não β -lactâmicos do que as linhagens HA-MRSA. Os isolados de HA-MRSA possuem, predominantemente, o gene *mec* dos tipos I a III. Além disso, o *mec* tipo IV e V são mais comumente encontrado nas CA-MRSA, ao passo que esses dois últimos *mec* são de menor tamanho (Daum et al., 2002; File, 2008). No entanto, o tamanho inferior dos *mec* encontrado nas CA-MRSA pode facilitar a transferência horizontal de genes de resistência entre bactérias, caracterizando um fator alarmante para a disseminação do grupo CA-MRSA. A produção de fatores de virulência e a capacidade de causar doenças são maiores em linhagens CA-MRSA, as quais frequentemente possuem genes responsáveis pela produção da leucocidina de Panton-Valentine (PVL, do inglês, *Panton-Valentine Leucocidin*). A PVL é uma citotoxina que causa destruição de células do sistema imune e necrose tecidual. Entretanto, os dois grupos de MRSA são responsáveis por diversas infecções e problemas de saúde na população humana (File, 2008).

1.2 - Fatores de virulência e patogenicidade

Devido ao impacto gerado por *S. aureus* na saúde humana, bem como ao fato desta bactéria estar associada à comunidade sadia e ao ambiente hospitalar, tem-se intensificado o estudo da estrutura e função de seus fatores de virulência, da sua interação com o hospedeiro e dos mecanismos moleculares envolvidos na resistência a antibióticos (Hecker et al., 2010; Holden et al., 2004). O repertório de fatores de virulência de *S. aureus* é extenso e composto por produtos secretados e estruturais, incluindo proteínas de membrana que direcionam o processo de patogênese (Gordon e Lowy, 2008). Dentre os fatores de virulência produzidos por este micro-organismo, podemos citar os componentes de superfície microbiana reconhecedores de moléculas adesivas da matriz (MSCRAMMs, do inglês, *Microbial Surface Components Recognizing Adhesive Mmatrix Molecules*), as leucocidinas, toxinas, enzimas e um novo grupo de fatores englobando as modulinas solúveis em fenol (PSM, do inglês, *Phenol-Soluble Modulins*). Esses fatores podem desempenhar um papel importante nas diferentes etapas da patogênese, incluindo adesão, invasão, persistência, evasão e morte

de células hospedeiras (Gordon and Lowy, 2008; Labandeira-Rey et al., 2007; Otto, 2014b).

As MSCRAMMs formam um grupo de moléculas que se ligam aos componentes da matriz extracelular, como o colágeno, a fibronectina (Fn) e o fibrinogênio (Fg). A ligação a estes componentes facilita a internalização celular bacteriana e promove o dano tecidual (Wann et al., 2000). As proteínas ligantes a fibronectinas FnBPA e FnBPB (do inglês, *fibronectin-binding protein*) de *S. aureus* são responsáveis pelo processo de adesão a Fn da célula hospedeira (Foster and Höök, 1998). Embora *S. aureus* apresente a capacidade de se ligar mais comumente a fibronectina, existem receptores associados ao fibrinogênio e ao colágeno. A ligação ao fibrinogênio é mediada principalmente pelas proteínas ClfA e ClfB (do inglês, *Clumping factor A e B*), que permitem a adesão da bactéria em plasma sanguíneo e a biomateriais expostos a sangue humano ou animal (Foster and Höök, 1998). Os anticorpos que reagem contra a proteína adesina do colágeno (Cna, do inglês, *collagen adhesin*) de *S. aureus* são suficientes para inibir a aderência dessa bactéria ao colágeno, sendo o mutante para o gene codificador desta proteína menos virulento que as linhagens selvagens (Foster and Höök, 1998; Vaudaux et al., 1995).

A presença de suturas em pacientes e a utilização de instrumentos para aplicações intravenosas elevam o risco de infecções por induzirem Fn e outras proteínas relacionadas à adesão celular, facilitando a infecção por *S. aureus* pelo aumento dessas proteínas da matriz. (Foster and Höök, 1998; Lowy, 1998). Essas habilidades de aderência e invasão são de grande relevância para a infecção de células do epitélio intestinal e outras mucosas (Lowy, 1998). Diferentes linhagens de *S. aureus* apresentam os mesmos receptores para ligação aos componentes da matriz extracelular, facilitando a infecção de diversos tipos de células, como as epiteliais e as imunológicas (Sinha e Herrmann, 2005).

Após o estabelecimento da infecção, muitas bactérias diminuem a expressão de proteínas de superfície associadas à adesão e internalização, para produzir toxinas e outros fatores de virulência responsáveis pela dispersão bacteriana e, conseqüentemente, os sintomas das infecções (Novick, 2003). O gene regulador acessório estafilocócico (Agr, do inglês, *accessory gene regulator*) é formado por um sistema de dois componentes, que é responsável pela regulação de vários fatores de virulência secretados e associados aos processos de infecção (Yarwood and Schlievert, 2003). O operon *agr* é composto por quatro genes *agrA*, *agrB*, *agrC* e *agrD* que produzem

respectivamente os AgrA, AgrB, AgrC e o peptídeo auto-indutível (AIP). O AIP, que é processado e secretado pelo AgrB, se liga ao receptor de membrana formado pelo AgrC, que fosforila ou desfosforila o AgrA. O AgrA funciona como um transdutor de sinal da ligação do AIP ao receptor ArgC, ativando a expressão do operon *agr* e do gene *rnaIII*. Esse último expressa o RNAIII, um regulador de diversos genes relacionados a virulência de *S. aureus* (Novick, 2003) (Figura 1). Diversas linhagens de *S. aureus* secretam citotoxinas e enzimas que normalmente convertem as moléculas do hospedeiro em nutrientes para o crescimento bacteriano, além de clivarem moléculas de antibióticos (Otto, 2014b). Dentre as enzimas, incluímos hemolisinas (α , β , γ e δ), nucleases, proteases, lipases, hialuronidase e colagenase, as quais podem fornecer nutrientes para a bactéria a partir de material hospedeiro. Além disso, enzimas como as β -lactamases, são responsáveis por inibir a atividade dos antibióticos derivados de anéis β -lactâmico. As linhagens virulentas de *S. aureus* produzem um grupo de exoproteínas com ação tóxica, sendo as principais: a toxina-1 da síndrome do choque tóxico (TSST-1, do inglês, *Toxic shock syndrome toxin 1*), as enterotoxinas estafilocócicas (SEA, SEB, SECn, SED, SEE, SEG, SEH, e SEI, do inglês, *Staphylococcal Enterotoxin*), as toxinas esfoliativas (ETA e ETB, do inglês, *Exfoliative Toxins*), a leucocidina e as PSMs. Essas exoproteínas possuem funções variadas desde a morte celular até a regulação do sistema imune hospedeiro (Dinges et al., 2000; Otto, 2014b).

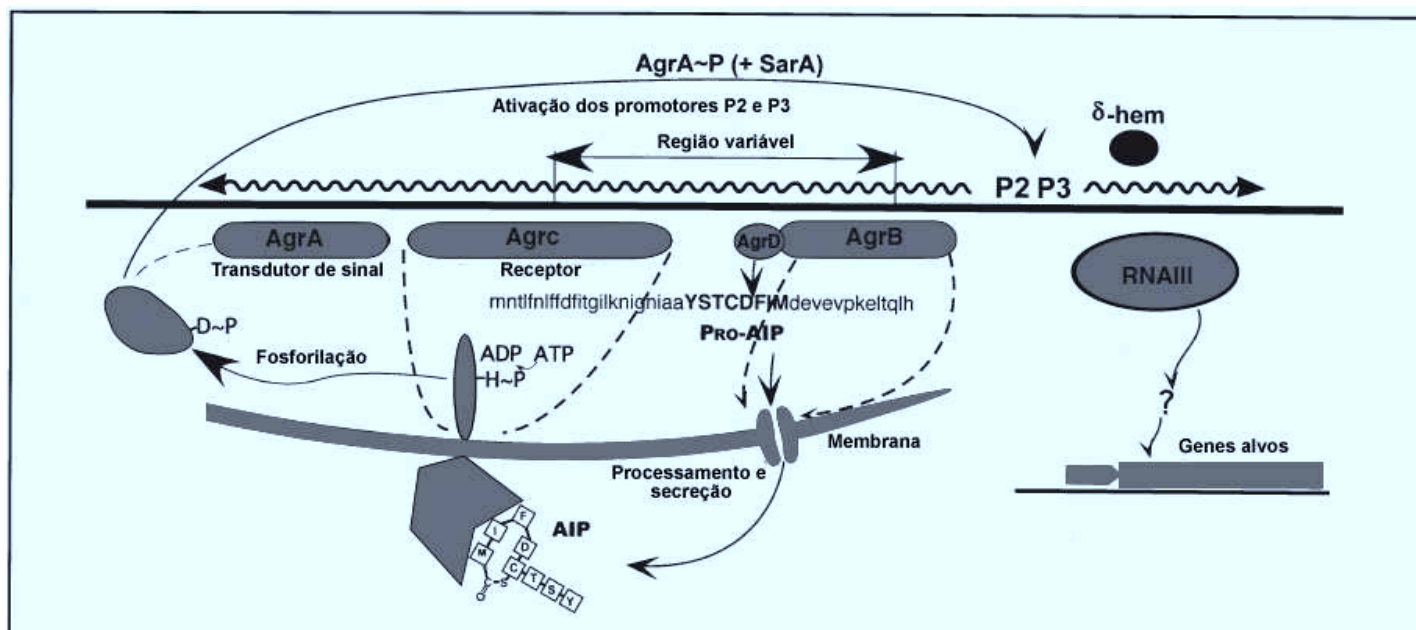


Figura 1 – Regulação do Gene regulador acessório (Agr). AgrD é processado e secretado pelo AgrB. AgrD processado (AIP), liga-se ao AgrC (receptor) e ativa o AgrA. AgrA ativado é hábil a ativar o promotor do Agr (P2) e o promotor do RNAIII (P3). ArgA e RNAIII são responsáveis por regular genes de virulência (Fonte: Adaptado, Novick, 2003).

1.2.1 - Modulinas solúveis em fenol (PSM)

Em 1999, um grupo de moléculas contidos no sobrenadante de *Staphylococcus epidermidis*, foram isoladas por cromatografia de exclusão de tamanho com o uso de um solvente contendo fenol, sendo assim chamados de modulinas solúveis em fenol (PSM) (Mehlin et al., 1999). As PSMs de *S. epidermidis* foram descritas como responsáveis por induzir uma atividade pró-inflamatória em linhagens de células humanas transfectadas com o vírus HIV-1 (Mehlin et al., 1999).

As PSMs fazem parte de uma família de peptídeos anfipáticos, dispostos em forma de α -hélice e que apresenta grande importância na literatura devido à sua grande participação no processo de virulência de *S. aureus*. As PSMs podem ser classificadas de acordo com o comprimento da cadeia de aminoácidos (aa), sendo que as do tipo α (1-4) possuem comprimento entre 20-25aa, e as do tipo β (1-2) possuem aproximadamente o dobro do tamanho (entre 43-45aa), e as do tipo δ possuem entre 25-26aa (Cheung et al., 2014; Otto, 2014a). Esses peptídeos são codificados em três regiões diferentes do genoma: (i) as PSM α 1-4 codificadas pelo operon *psma*, (ii) as PSM β 1-2 codificadas pelo operon *psm β* , e a (iii) toxina do tipo δ codificada pelo gene *hdl*. As PSMs são reguladas pelo *agr* através do AgrA, e não pelo RNAlII, que modula vários outros genes de virulência em *S. aureus* (Cheung et al., 2014; Peschel and Otto, 2013).

Em estudos com linhagens CA-MRSA (LACWT e MW2), as PSMs mostraram participar da virulência de *S. aureus*, causando infecções na pele e bacteremia em camundongos (Wang et al., 2007). Especificamente, as PSM α apresentam maior participação na indução do processo inflamatório por *S. aureus*, já o grupo PSM β e a δ -toxina não influenciou significativamente o processo de inflamação. Dessa forma, o grupo de PSM α aumentou a quantidade de neutrófilos e a quimiotaxia dos monócitos, além de elevar a morte desses leucócitos *in vitro* (Wang et al., 2007). Além disso, a capacidade de disseminação de *S. aureus* e *S. epidermidis*, associada à formação de biofilme, está diretamente relacionada à produção de PSMs (Tsompanidou et al., 2013). De fato, as PSMs têm participação em diversos processos patogênicos relacionados a lise celular, a formação de biofilme, aos efeitos antimicrobianos e a função imunomoduladora (Figura 2) (Cheung et al., 2014).

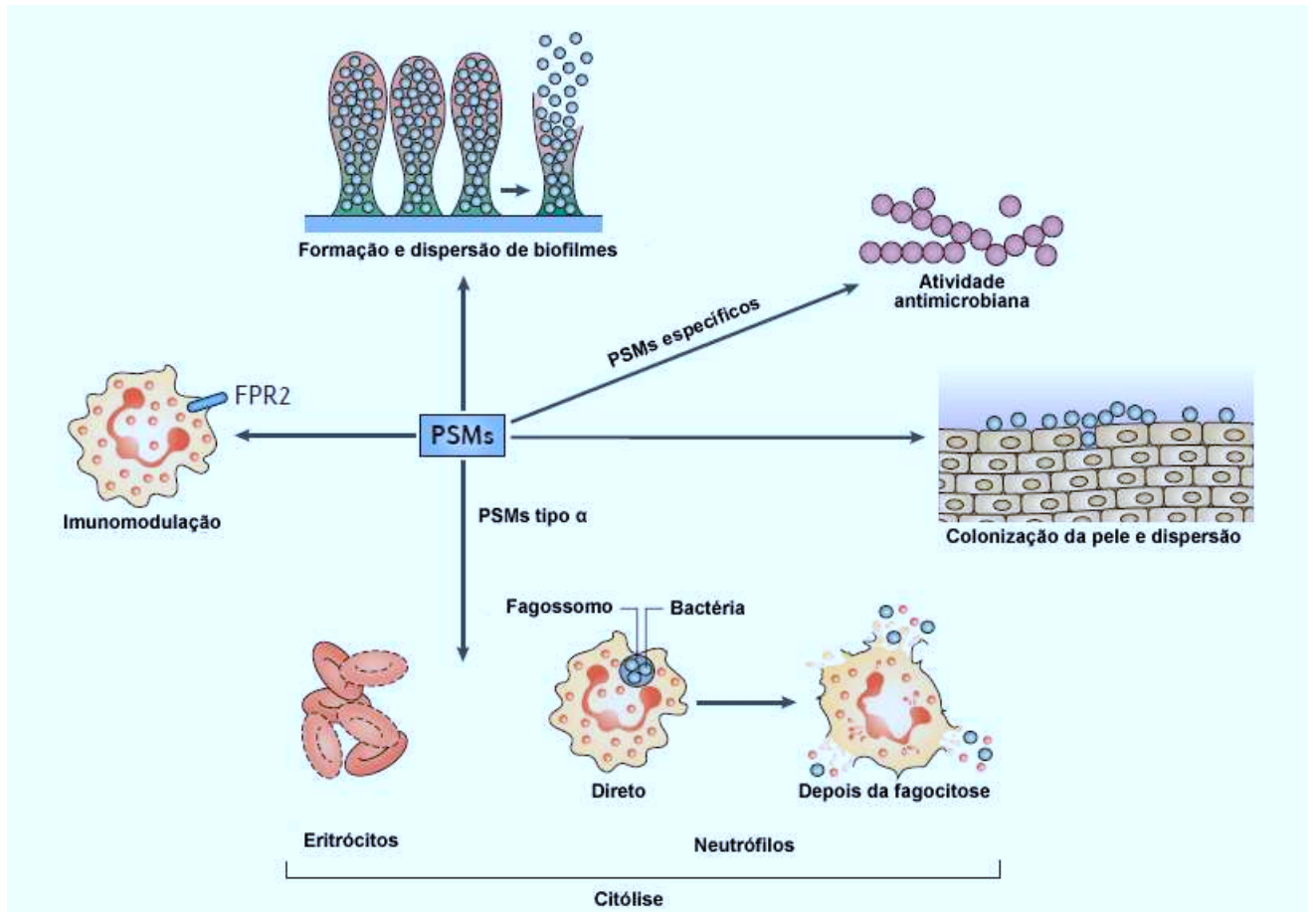


Figura 2 - As PSMs e suas funções em *Staphylococcus aureus*. As PSMs participam da formação dos biofilmes, induz lise celular, produção de citocinas através do receptor FPR2, servem como agente antimicrobiano, além de participar no processo de dispersão do *S. aureus*. (Fonte: Adaptado, Peschel & Otto, 2013)

1.2.1.1 - Lise celular mediada por PSMs

A lise celular por PSM é a característica mais relevante dessas modulinas (Cheung et al., 2014). A ação das PSMs não é específica e é independente de receptores, atingindo praticamente todas as membranas eucarióticas e provavelmente a lise celular é devido a uma perturbação da membrana plasmática (Cheung et al., 2012; Kretschmer et al., 2010). Ao contrário, outras toxinas de *S. aureus* como a α -toxina ou PVL, são responsáveis por lisar especificamente neutrófilos polimorfonucleares (PMNs, do inglês, *polymorphonuclear neutrophils*) (Löffler et al., 2010). Apesar desse grupo de peptídeos estarem ligados ao processo de lise celular, somente uma parcela dos PSMs é responsável por essa atividade (Cheung et al., 2014). Em adição, as PSM α 3 de *S. aureus* e a PSM δ de *S. epidermidis* apresentam as atividades citolíticas mais evidentes dentre as PSMs, enquanto que as PSM α 1 e PSM α 2 são moderadamente citolíticas (Cheung et al., 2010; Wang et al., 2007).

Diferentemente da HA-MRSA, as linhagens CA-MRSA são altamente virulentas, sendo capazes de escapar do sistema imune do hospedeiro e causar a lise de neutrófilos infectados. Quando as linhagens HA-MRSA foram complementadas com o operon *psm α* , mostraram ser capazes de produzir quantidades de PSM α comparáveis as linhagens CA-MRSA, assim como promover a lise de neutrófilos (Wang et al., 2007). Além disso, a expressão do operon *psm α* de *S. aureus* linhagem MW2 foi demonstrada em neutrófilos após a fagocitose, o que poderia indicar um mecanismo de escape após a lise do fagossomo (Surewaard et al., 2012).

1.2.1.2 - Formação de biofilme por PSMs

Os biofilmes são formados por comunidades de bactérias fixadas a uma superfície e cobertas por uma matriz extracelular esponjosa (O'Toole et al., 2000). Esses biofilmes constituem uma rede de canais que são responsáveis pela distribuição de nutrientes para as camadas mais profundas, possibilitando a manutenção de toda a comunidade bacteriana (O'Toole et al., 2000). Além disso, os biofilmes permitem que as bactérias colonizem uma ampla variedade de superfícies bióticas ou abióticas, protegendo esses micro-organismos da ação direta de antibióticos (O'Toole et al., 2000). Em bactérias como *Pseudomonas aeruginosa* e *Bacillus subtilis*, moléculas com propriedades surfactantes foram identificadas como participantes da formação e

estruturção do biofilme (Angelini et al., 2009; Boles et al., 2005). Em *S. aureus* e *S. epidermidis*, as PSMs são moléculas surfactantes responsáveis pela formação e estruturção dos biofilmes (Periasamy et al., 2012; Wang et al., 2011). Em *S. aureus*, tanto as PSM α e as PSM β estão envolvidas na maturação dos biofilmes, enquanto que em *S. epidermidis* a PSM β é o peptídeo mais importante envolvido nessa maturação (Periasamy et al., 2012).

1.2.1.3 - Atividades antimicrobianas das PSMs

Algumas espécies bacterianas, como *Streptomyces* sp, *Agrobacterium radiobacter* e *Lactococcus lactis*, possuem a capacidade de produzir substâncias que inibem o crescimento de outros micro-organismos (Hibbing et al., 2010). Um sistema bem conhecido é denominado toxina-antitoxina, que tem como funções o controle populacional e a diminuição do número de bactérias que disputam nutrientes no meio (Gerdes et al., 2005). Em *S. epidermidis*, as PSM δ e δ -toxina (PSM γ) apresentaram atividades antimicrobianas contra *Streptococcus pyogenes* e *S. aureus*, devido à capacidade de formar poros na membrana dessas bactérias (Cogen et al., 2010). De acordo com Joo e colaboradores (2011), análises em bancos de dados genômicos mostram que *S. aureus* possui regiões codificantes para antibióticos do grupo dos Lantibióticos. Em adição, as PSMs obtidas através de cromatografia inibiram a proliferação de *Micrococcus luteus* e *S. pyogenis* em teste de difusão em ágar. Além disso, foi atribuído a capacidade antimicrobiana as PSM α 1 e PSM α 2 após sofrerem proteólises, a qual foi confirmada pelo sequenciamento da porção N-terminal desses peptídeos (Joo et al., 2011).

1.2.1.4 - Imunomodulação por PSMs

As PSMs, em concentrações micromolares, são responsáveis por causar a lise celular de neutrófilos, e em concentrações nanomolares essas modulinas são responsáveis por estimular leucócitos, que induzem uma resposta anti-inflamatória (Wang et al., 2007). Além disso, as PSMs induzem quimiotaxia, produção de radicais de oxigênio, influxo intracelular de cálcio e liberação de citocinas pró-inflamatórias como Interleucina 8 (IL-8) (Wang et al., 2007). A capacidade das PSMs induzirem uma resposta inflamatória as classificam como PAMPs (do inglês, *Pathogen-associated*

Molecular Patterns), que são um grupo de moléculas microbianas que alertam os organismos a respeito da infecção por patógenos (Bianchi, 2007). Kretschmer e colaboradores (2010) observaram que a indução de uma resposta inflamatória por PSMs é mediada pela ação do receptor FPR2 (do inglês, *Formyl Peptide Receptor 2*), sendo capaz de reconhecer a porção N-terminal das PSMs que apresentam grupos formil. Essas modulinas são agrupadas entre os mais potentes agonistas do receptor FPR2 e essa atividade é compartilhada por todas as PSMs (Kretschmer et al., 2010). Entretanto, as PSMs diferentemente da indução de uma resposta inflamatória iniciada pela ação dos receptores FPR2, mostraram ser capazes de diminuir a expressão de citocinas pró-inflamatórias como IL-32, IL-6 e IL8 (Deplanche et al., 2016). Em adição, as PSMs induzem uma resposta não inflamatória em células dendríticas, as quais também apresentam receptores FPR2. As células dendríticas na presença de PSMs apresentam um perfil de secreção de IL-10, inibindo a produção de citocinas pró-inflamatórias e assim diminuem a ativação de células Th1, o que permite que essas bactérias escapem do sistema imune hospedeiro (Schreiner et al., 2013).

O FPR2 pode agir como um sensor para detectar o nível de virulência de diferentes linhagens ou espécies de estafilococos (Kretschmer et al., 2010). Em linhagens altamente virulentas, como é o caso do grupo CA-MRSA, a resposta desencadeada por esse receptor é mais forte do que das linhagens do grupo HA-MRSA, que possuem uma resposta imune moderada (Kretschmer et al., 2010). Em linhagens de *S. aureus* não patogênicas ou *knockout* para PSMs, a estimulação dos receptores FPR2 é quase inexistente (Kretschmer et al., 2010, 2012; Peschel and Otto, 2013)

S. aureus desenvolveu uma estratégia para “driblar” o recrutamento de leucócitos para o sítio de infecção (Cheung et al., 2014), produzindo inibidores de quimiotaxia, como as proteínas CHIPS (do inglês, *Chemotaxis inhibitory protein of S. aureus*) e a FPR-like1 (FLIPr, do inglês, *FPR-like 1 inhibitory protein*). Essas proteínas são responsáveis por inibirem a atividade dos receptores FPR1 e FPR2, impossibilitando o recrutamento de células do sistema imune realizado pelas PSMs (Haas et al., 2004; Stemerding et al., 2013). As linhagens CA-MRSA não produzem somente CHIPS e FLIPr, mas também as PSMs que estão sobre regulação do sistema Agr (Cheung et al., 2014). Durante a fase inicial de crescimento dessas bactérias, em que há concentrações baixas de PSMs, as CHIPS e FLIPr impedem a ativação de uma resposta inflamatória, devido a competição pelos receptores FPR (Kretschmer et al., 2012) Assim, nos estágios mais tardios do crescimento bacteriano, o sistema Agr induz

uma grande produção das PSMs que levam a indução da resposta pró-inflamatória via receptores FPR2 (Cheung et al., 2014; Kretschmer et al., 2010, 2012). Além disso, o exato papel biológico da interferência das PSMs na resposta imune do hospedeiro ainda não foi bem elucidado.

1.3 - *S. aureus* e o ciclo celular eucariótico

A produção de toxinas bacterianas que participam dos processos de modulação do ciclo eucariótico hospedeiro já é bem conhecida (Nougayrède et al., 2005; Oswald et al., 2005). Entretanto, não existem muitos trabalhos envolvendo *S. aureus* no processo de modulação do ciclo celular hospedeiro. Entre as décadas de 80 e 90, dois trabalhos mostraram a participação de *S. aureus* alterando o ciclo celular de células eucarióticas. O primeiro descreveu o processo de diferenciação e ativação de células B expostas a linhagem Cowan I de *S. aureus* tratadas com formalina (Jelinek and Lipsky, 1985). O segundo mostrou que um fator chamado Inibidor de Diferenciação de Células Epidérmicas (EDIN, do inglês, *Epidermal Cell Differentiation Inhibitor*) induzia hiperplasia em células epiteliais humanas e murinas (Sugai et al., 1992). Outro estudo, publicado nos anos 2000 demonstrou a participação da α -toxina no aumento do número de células eucarióticas na fase S+G2/M depois de 26h de exposição a essa toxina (Haugwitz et al., 2006). Em adição, a análise do transcriptoma de células epiteliais infectadas com linhagens patogênicas de *S. aureus* apresentaram diferentes perfis de expressão de genes reguladores do ciclo celular como *plk1* e *cdc25c* (Heimer et al., 2010).

Recentemente, o nosso grupo de pesquisa observou a capacidade de MRSA induzir o atraso na fase G2/M do ciclo celular em células HeLa, através da técnica de bloqueio duplo com timidina (DTB, do inglês, *double thymidine block*) (Figura 3) (Alekseeva et al., 2013). Além disso, foi demonstrado que há diminuição no número de células HeLa em mitose quando há infecção por *S. aureus* (Alekseeva et al., 2013). Esta bactéria alterou várias características associadas ao ciclo celular das células HeLa, causando (i) o aumento da CDK inativada pela fosforilação na tirosina 15; (ii) a formação de células gigantes e núcleos aumentados, característica de células que duplicaram o material genético sem divisão celular; e (iii) a redução da histona H3 fosforilada na serina 10, um marcador de condensação cromossômica (Alekseeva et al., 2013). Nesse mesmo ano, outro trabalho foi publicado mostrando que o componente S

(LukS-PV) da PVL levou ao atraso celular associado a apoptose na fase G0/G1, através da inibição da ação da ciclina D1 (Bu et al., 2013). Por último, um trabalho envolvendo as enterotoxinas estafilocócicas SEA, SEG, SEI, SEIM, SEIN e SEIO demonstrou que a SEIO levou ao atraso na fase G0/G1 de linhagens tumorais humanas por intermédio da *cullin-3*, que é uma proteína associada a regulação da fase G1/S pela modulação da ciclina E e CDK2 (Hodille et al., 2016).

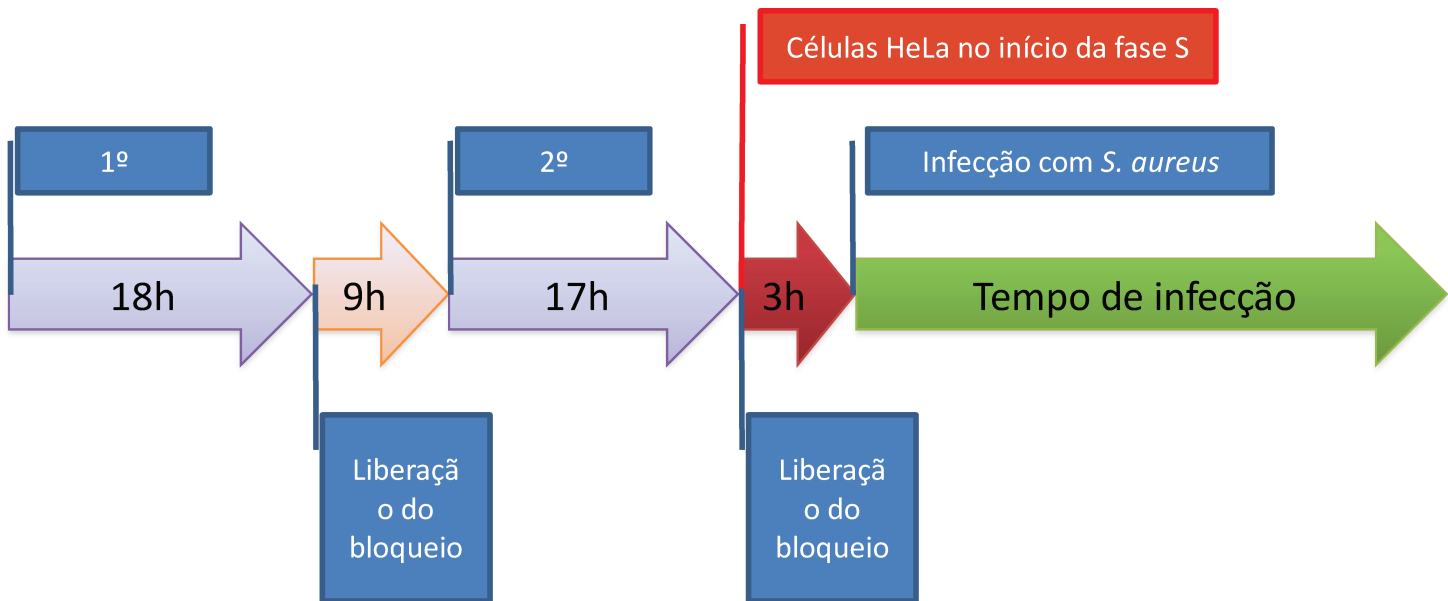


Figura 3 - Esquema de sincronização pela técnica de duplo bloqueio com timidina.

A técnica é realizada através do uso da timidina, um composto que atrasa o ciclo celular na fase S através do impedimento da duplicação do material genético. O duplo bloqueio ocorre pela exposição das células HeLa à timidina primeiramente por 18h e posteriormente por 17h (roxo), intercalando entre esses dois um período de 9h (laranja) em que as células são liberadas do primeiro bloqueio. Após o segundo bloqueio as células são novamente liberadas da exposição à timidina, sendo que neste momento todas as células se encontram na fase S do ciclo celular. Após 3h da segunda liberação (vermelho) as células são infectadas com a linhagem bacteriana *S. aureus* (verde), o qual atrasa o ciclo na fase G2/M.

2 - Ciclo celular eucariótico

O ciclo celular é um processo complexo responsável por coordenar a proliferação celular e o reparo do DNA (Schafer, 1998). Além disso, a alteração na progressão normal do ciclo celular pode desencadear doenças cancerígenas e doenças degenerativas como o Alzheimer (Moh et al., 2011; Schafer, 1998). O ciclo celular é dividido em Interfase, que é subdividido nas fases Gap1 (G_1), Síntese (S), Gap2 (G_2), e na Mitose (M), subdividido em prófase, metáfase, anáfase e telófase (Vermeulen et al., 2003). A ordem de progressão do ciclo celular parte da fase G_1 para a S e desse último para a G_2 , que em seguida entra na fase M, assim, completando o ciclo celular e voltando para a fase G_1 (Vermeulen et al., 2003). Durante a fase G_1 a célula está se preparando para iniciar a síntese do DNA, sendo comum encontrar células em aneuploidia. A fase G_2 é representada pela preparação da célula, através da expressão de genes específicos para entrar fase M, o qual gera duas células filhas idênticas. As células que entraram em um estado de inatividade no ciclo celular, normalmente sendo apresentadas como células totalmente diferenciadas, estão em uma fase conhecida como G_0 . (Vermeulen et al., 2003).

A regulação das fases do ciclo celular é determinada pela ação conjunta de um variado grupo de proteínas. Entretanto, as principais proteínas reguladoras do ciclo celular são as quinases dependente de ciclinas (CDKs, do inglês, *cyclin-dependent kinases*), que são serina/treoninas quinases, e suas ciclinas (Figura 3) (Schafer, 1998). As CDKs são expressas constantemente, diferentes das suas ciclinas ativadoras que variam durante o ciclo celular, ativando e desativando as CDKs de acordo com os *checkpoints* específicos (Vermeulen et al., 2003). A fase G_1 é modulada pela ação dos complexos CDK4/ciclinaD e CDK6/ciclinaD e a transição entre as fases G_1 e S é modulada pelo complexo CDK2/ciclinaE (Vermeulen et al., 2003). A fase S está sob a regulação do complexo CDK2/ciclinaA, a fase G_2 está sob a regulação do complexo CDK1/ciclinaA e a transição entre G_2 e M estão sob a regulação do complexo CDK1/ciclinaB, respectivamente. Esses dois últimos complexos possuem domínios para ubiquitina que são ubiquitinados durante o fim da fase M, encerrando o ciclo celular eucarioto.

O principal alvo regulador dos complexos CDK4-6/ciclinaD é o gene supressor de tumor retinoblastoma (*pRb*, do inglês, *retinoblastoma tumour supressor*) que ativa os fatores de transcrição E2F-1 e DP-1 responsáveis por transcrever genes que coordenam

a fase G1 como ciclinas da fase G1, além das ciclinas A e B que regulam as fases subsequentes S e G2, e a fosfatase CDC25 que regula a transição entre G2 e M. O complexo CDK2/ciclinaE participa de processos como a fosforilação da histona H1, que é requerida para a replicação do DNA na fase S (Porter and Donoghue, 2003; Schafer, 1998; Vermeulen et al., 2003). A CDK1/ciclinaA e a CDK1/ciclinaB modula a progressão da fase G2 e M, alterando proteínas estruturais do citoesqueleto como as lâminas nucleares e os microtúbulos, ambos necessárias para a correta divisão da célula (Porter and Donoghue, 2003; Schafer, 1998; Vermeulen et al., 2003). Além dos complexos CDK/ciclinas, outras proteínas quinases, como a Polo-Like Kinases (PLKs), Aurora, Neks e NIMA-related kinases, participam da ativação de vias de sinalização modulatórias do ciclo celular (Salaün et al., 2008). Recentemente, a proteína multifuncional Optineurina (do inglês, *Optic neuropathy inducing protein*), que está relacionada à doença de Huntington, foi associada à regulação do ciclo celular eucariótico na fase G2/M (Kachaner et al., 2012a).

A regulação da transição da fase G2 para M ocorre através da ativação da CDK1 pela sua ciclina B ligante. O complexo CDK1/ciclinaB é regulado pela fosforilação e desfosforilação de três resíduos de aminoácidos da sua estrutura protéica da CDK1, treonina 161 (Thr161), tirosina 15 (Tyr15) e treonina 14 (Thr14) (Schafer, 1998). A ativação do complexo CDK1/ciclina B ocorre através da fosforilação da Thr161 e a inativação ocorre pela fosforilação da Thr14 e Tyr15. A fosforilação da Thr161 é realizada pela quinase ativadora de CDK (CAK, do inglês, *CDK-activating kinase*) e essa fosforilação é mantida até o final da mitose (Schafer, 1998). Os sítios regulatórios Thr14 e Tyr15 são fosforilados por quinases sítio específicas chamadas de Myt1 e Weel/Mik1, os quais inativam CDK1/ciclinaB, entretanto, durante a mitose a Myt1 e Weel/Mik1 são mantidas inativadas (Vermeulen et al., 2003). A desfosforilação das Thr14 e Tyr15 da CDK1/ciclinaB é realizado por membros da família das CDC25 no final da fase G2. A CDC25, durante a interfase, está associada com proteínas repressoras da família 14-3-3, os quais previnem a entrada prematura das células na fase M. A dissociação da CDC25 da 14-3-3 ocorre pela fosforilação daquela em sua porção N-terminal, que possui um sítio de exportação nuclear (NES, do inglês, *nuclear export signal*), pela PLK1 (Porter and Donoghue, 2003). Além disso, PLK1 fosforila a proteína ubíqua Optineurina, que media a conexão entre CDK1/ciclinaB e o complexo miosina fosfatase (MYPT1/PP1 β , do inglês, *Myosin Phosphatase*). Juntas no núcleo,

Optineurina-CDK1/ciclinaB-MYPT1/PP1 β inativam a PLK1, encerrando a fase M do ciclo celular (Kachaner et al., 2012b).

A Optineurina é uma proteína de 67 kDa que funciona como um dímero, sendo inicialmente chamada de *14.7K-interacting protein-2* por se ligar à proteína adenoviral E3-14.7k (Li et al., 1998). Posteriormente, o nome *Optineurina* foi formulado devido à associação dessa proteína com o glaucoma primário de ângulo aberto (Rezaie, 2002). A Optineurina também possui outros nomes, incluindo *NEMO-related protein* (NRP), *Huntingtin-interacting protein 7* (HIP7), *Huntingtin yeast partner L* (HYPL) e *transcription factor IIIA-interacting protein* (TFIIIA-INTP) (Rezaie, 2002). O alinhamento da sequência das proteínas Optineurina e NEMO, mostrou uma similaridade de 53% entre elas. A proteína NEMO, diferentemente da Optineurina, não possui uma porção de 166 aminoácidos incluídos entre a porção N-terminal e uma região provável para uma porção estrutural conhecida como zíper de leucina. Entretanto, a presença de um domínio ligante de ubiquitina (UBD, do inglês, *Ubiquitin Binding Domain*), duas hélices superenroladas e um C-terminal *zinc finger* ubiquitina ligante são encontrados tanto na Optineurina quanto em NEMO (Kachaner et al., 2012a). A Optineurina, assim como NEMO, é identificada normalmente em complexos moleculares pelas interações com um grande número de proteínas e por participar de diversos processos celulares fundamentais, como transporte, secreção de vesículas, organização do complexo de Golgi, sinalização e defesa do hospedeiro (Kachaner et al., 2012a). A participação dessa proteína na modulação do ciclo celular foi atribuída recentemente pela sua capacidade de regular indiretamente a PLK1 (Kachaner et al., 2012b).

A PLK1 é um membro da família das serino/treoninas quinases, que são conservadas desde o reino Fungi (Hamanaka et al., 1994). Essa proteína tem um conservado domínio polo-box (PBD, do inglês, *Polo Box Domain*), composto pelos motivos polo-box 1 (PBD1) e polo-box 2 (PBD2) na porção C-terminal, e o domínio catalítico (KD, do inglês, *kinase domain*) na porção N-terminal (Glover et al., 1998). O PBD coordena o processo de distribuição da PLK1, através da interação dessa região com os seus substratos (Park et al., 2010). Por exemplo, PLK1 pode se ligar através do PBD a mais de 600 proteínas mitótico-específicas em pontos definidos do ciclo celular (Lowery et al., 2007). Além disso, PLK1 também pode se ligar a proteínas associadas ao controle da transcrição e ao transporte de vesículas (Lowery et al., 2007). O KD de PLK1 assemelha-se ao KD da Aurora quinase, sendo ambos ativados pela fosforilação

do seu T-loop na treonina 210 (Archambault et al., 2015). Entretanto, outros sítios da PLK1, em seu KD, permitem que essa quinase fosforile diferentes proteínas (Archambault et al., 2015). A principal função da PLK1 está relacionada com a progressão da fase G2 e M e seu envolvimento no início da mitose, organização do centrossomo, montagem do fuso, segregação dos cromossomos e citocinese (Weerd and Medema, 2006; Zitouni et al., 2014).

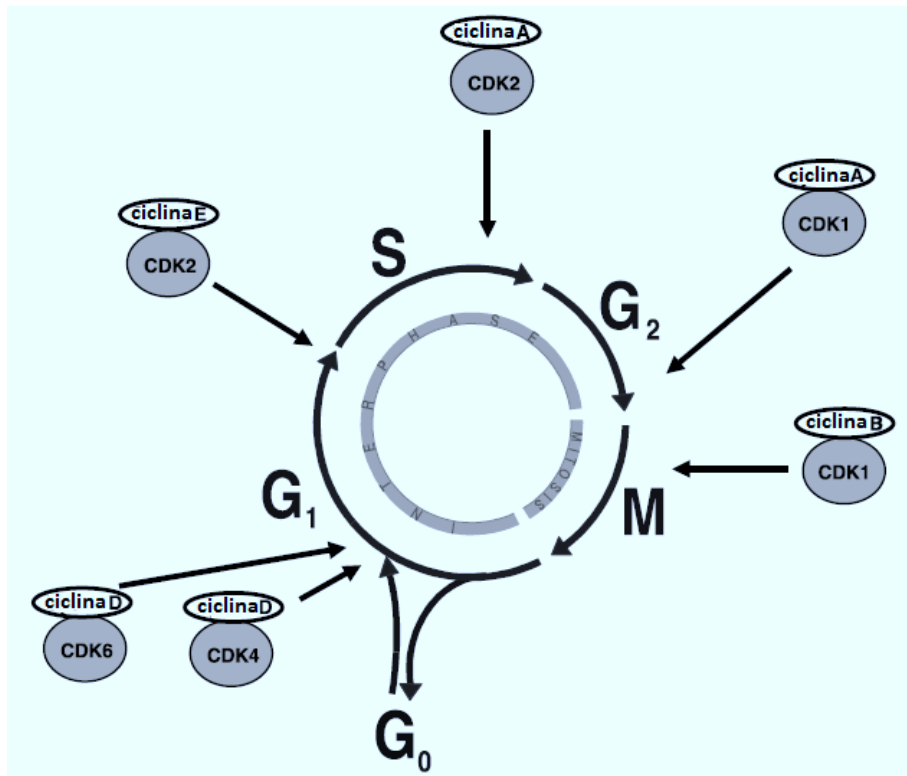


Figura 4 - Representação esquemática do ciclo celular de células eucarióticas e os principais complexos ciclina-CDK para cada fase. (Fonte: Adaptado, Vermeulen et al., 2003)

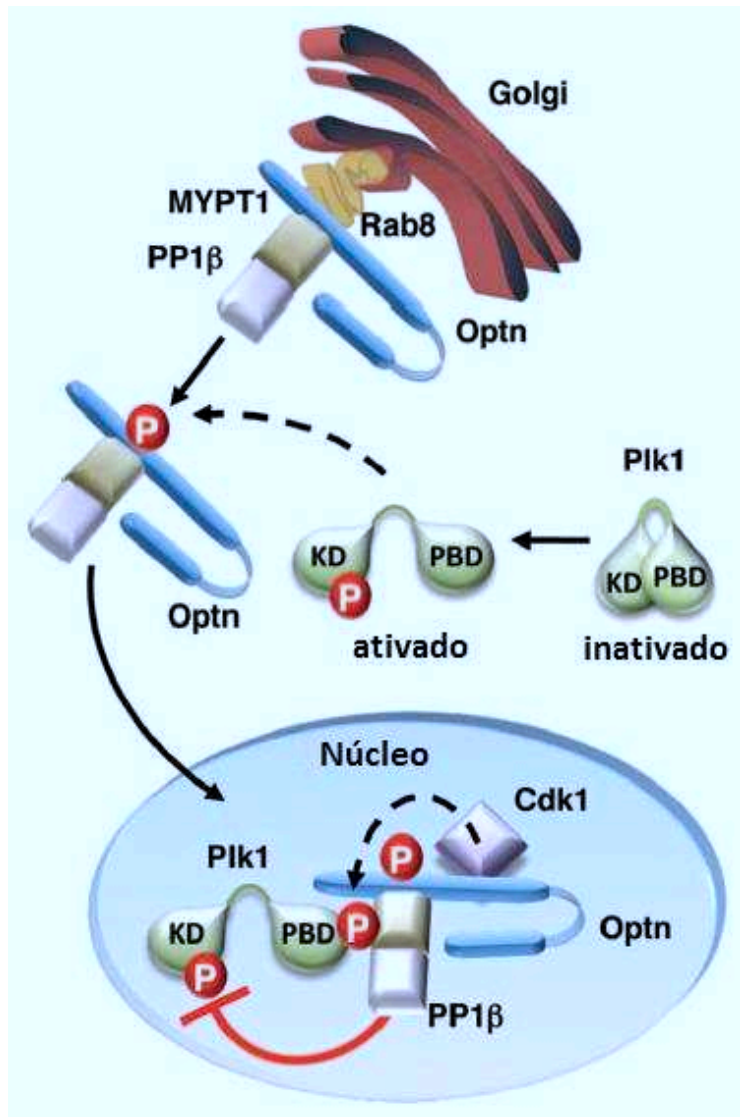


Figura 5 - Participação da Optineurina na regulação do ciclo celular. A Optineurina regula a ativação da PLK1 por permitir a desfosforilação da PLK1 ativada. (Fonte: Adaptado, Kachaner et al., 2012).

Parte 2 – Artigo de Revisão/ Partie 2 – Article de revue

1 - Présentation

Le cycle cellulaire des cellules hôtes peut être altéré par certaines toxines bactériennes, qui démontrent une polyvalence et une capacité à affecter les points de contrôle (*checkpoints*) spécifiques du cycle cellulaire. L'analyse de mécanisme d'action des différents effecteurs bactériens qui modifient le cycle cellulaire des cellules hôtes (cyclomodulines), ainsi que la connaissance des conséquences de ces modifications facilitera la classification des cyclomodulines. Nous avons effectué une révision bibliographique, sous le format d'article, abordant les principales toxines qui altèrent/régulent la prolifération cellulaire et/ou la progression normale du cycle cellulaire de l'hôte.

Cet article présente les cyclomodulines les plus importantes déjà décrites dans la littérature. Les cyclomodulines abordées dans cet article sont les toxines de distension *cytoléthale* (CDT), le facteur d'inhibition de cycle (Cif), la cytotoxine vacuolisante (VacA), la mycolactone, le facteur cytotoxique nécrosant (CNF), la toxine adénylate cyclase (ACT), la shiga-toxine, la subtilase toxine, la γ -glutamyltranspeptidase et la moduline phénol-soluble (PSM). L'article discute leurs rôles, leurs mécanismes d'action et les bénéfices qu'elles fournissent aux bactéries durant la colonisation de l'hôte.

1 - Apresentação

O ciclo celular do hospedeiro pode ser regulado através de diferentes mecanismos por algumas toxinas bacterianas, as quais demonstram versatilidade e capacidade para interferir em *checkpoints* celulares específicos. Além disso, a participação das toxinas no aumento do *fitness* bacteriano demonstra o seu envolvimento na patogenicidade dessas bactérias. A análise do mecanismo de ação de diferentes efetores bacterianos que modificam o ciclo celular das células hospedeiras (cyclomodulinas), permitiria a classificação dessas cyclomodulinas pela suas consequências e modificações no hospedeiro. Assim, o desenvolvimento dessa tese de doutorado permitiu fazer um levantamento literário de efetores bacterianos que interferem no ciclo celular eucariótico. Dessa forma, foi possível direcionar o conhecimento obtido na redação de uma revisão bibliográfica em formato de artigo, abordando as principais toxinas que alteram/regulam a proliferação celular e/ou progressão normal do ciclo celular hospedeiro.

Esta revisão apresenta as ciclomodulinas mais relevantes já descritas na literatura, que pertencem a uma família de efetores bacterianos que alteram o ciclo celular hospedeiro. As ciclomodulinas são as toxinas responsáveis pelo atraso no ciclo celular observado em infecções por diferentes bactérias patogênicas como *Shigella dysenteriae* e *Helicobacter pylori*. Algumas das ciclomodulinas abordadas na revisão são, a toxina distensora citoletal (CDT), fator inibidor do ciclo (Cif), citotoxina vacuolizante (VacA), micolactona, fator necrosante citotóxico (CNF), toxina adenilato ciclase (ACT), toxina shiga, toxina subtilase, γ -glutamyltranspeptidase e modulinas solúveis em fenol (PSM). No artigo são discutidos os papéis dessas ciclomodulinas, seus mecanismos de ação e os benefícios que elas provêm às bactérias durante a colonização do hospedeiro.

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1 Review

2 How and Why Bacterial Toxins Modulate the 3 Eukaryotic Cell Cycle?

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19 **Abstract:** Bacterial pathogens modulate various signaling pathways of host cells in order to subvert a host
20 response for their own benefit, leading to the successful colonization and invasion of the host. Pathogenic
21 bacteria produce multiple compounds that play a pivotal role in the enhancement of bacterial fitness during
22 infection of eukaryotic hosts. The toxins excreted by bacteria alter diverse cellular transduction pathways, thus
23 providing bacteria with a noteworthy fitness advantage against a eukaryotic host as well as against competing
24 bacteria. Many bacterial toxins can alter the cell cycle progression of host cells, impairing essential cellular
25 functions and impeding host cell division. This review summarizes current knowledge regarding
26 cyclomodulins, the family of bacterial effectors that induce eukaryotic cell cycle alterations. We discuss the
27 mechanisms of the action of cyclomodulins according to their biochemical properties, providing examples of
28 various cyclomodulins such as cytolethal distending toxins, cycle inhibiting factor, vacuolating cytotoxin,
29 mycolactone, cytotoxic necrotizing factor, adenylate cyclase toxins, shiga toxin, subtilase toxin, γ -
30 glutamyltranspeptidase of *H. pylori*, leucocidin and phenol soluble modulins. Special attention is paid to the
31 benefit provided by cyclomodulins to bacteria during colonization of the host.

32 **Keywords:** host cell cycle alteration; bacterial toxins; AB toxins; adenylate cyclase toxins; pore-forming toxins;
33 cyclomodulin; bacterial fitness; infective efficiency; reduced host response.

34 PACS: J0101

35

36 1. Microbial pathogens hijack host defense response.

37 The host-pathogen interaction is an area of research that covers multiple fields of investigation,
38 including immunological responses of infected hosts, characterization of virulence factors of various

39 pathogens, physiopathology of infectious diseases, gene expression and epigenetic studies using *in*
40 *vivo* and *in vitro* models of infections.

41 Many microorganisms employ sophisticated mechanisms to interfere with signal transduction
42 pathways of eukaryotic cells, subverting a host response for their own benefit, thus contributing to
43 the colonization and invasion of the host. Pathogens can precisely target specific host cell activities
44 such as cytoskeletal organization, cell cycle progression, vesicular trafficking and apoptosis using
45 different microbial effector delivery pathways. Due to a unique virulence mechanism, bacterial
46 effectors may be injected into the host cytoplasm, thus bypassing the extracellular milieu and the
47 membrane barrier through Type III or Type IV Secretion Systems [1]. Microbial pathogens also
48 adhere to and invade host cells through the interaction with cell membrane components or with the
49 cytoskeleton, usually by modulating factors involved in the assembly of actin filaments, as was
50 shown for the Rho family of GTPases [2,3]. Various microbial pathogens hijack host response despite
51 the negative pressure of the host immune system. The inhibition of NF- κ B activation, the major player
52 of the host immune response, is a strategy commonly used by pathogens to invade the host.
53 Moreover, some bacterial effectors target evolutionarily conserved ubiquitylation machinery that
54 regulates multiple cellular processes, including development, transcription, replication, cell signaling
55 and immune function [4,5].

56 There is a growing body of knowledge on how and where bacterial pathogens modulate
57 chromatin, which maintains the stability and the accessibility of the host genome to the
58 transcriptional machinery [6,7]. It is evident that, on the one hand, pathogens target chromatin in
59 order to control major cell functions such as the cell cycle and, on the other hand, that pathogens
60 injure chromatin in order to induce epigenetic modifications in the host cells that shape the host
61 immune response [8]. Many other mechanisms targeted by pathogens were examined during the past
62 decade.

63 Despite the fact that various biological processes are linked to the cell cycle and that its alteration can
64 disrupt cellular homeostasis, until recently, not much attention was paid to the investigation of the
65 capacity of bacteria to alter the host cell cycle or to the analysis of the input of its modulation on the
66 outcome of the infection.

67

68 2. The cell cycle of eukaryotic cells and cell cycle regulation.

69 The eukaryotic cell cycle is a ubiquitous and complex process involving DNA replication,
70 chromosome segregation and cell division. The cell cycle consists of different phases: the gap phase,
71 G1, characterized by cell growth; the S-phase characterized by DNA replication; the gap phase, G2,
72 in which cells are prepared for division; and the M (mitosis) phase, which culminates in cell division.
73 Cells can also exit the cell cycle and enter a quiescent state, the G0 phase (Fig. 1A) [9].

74

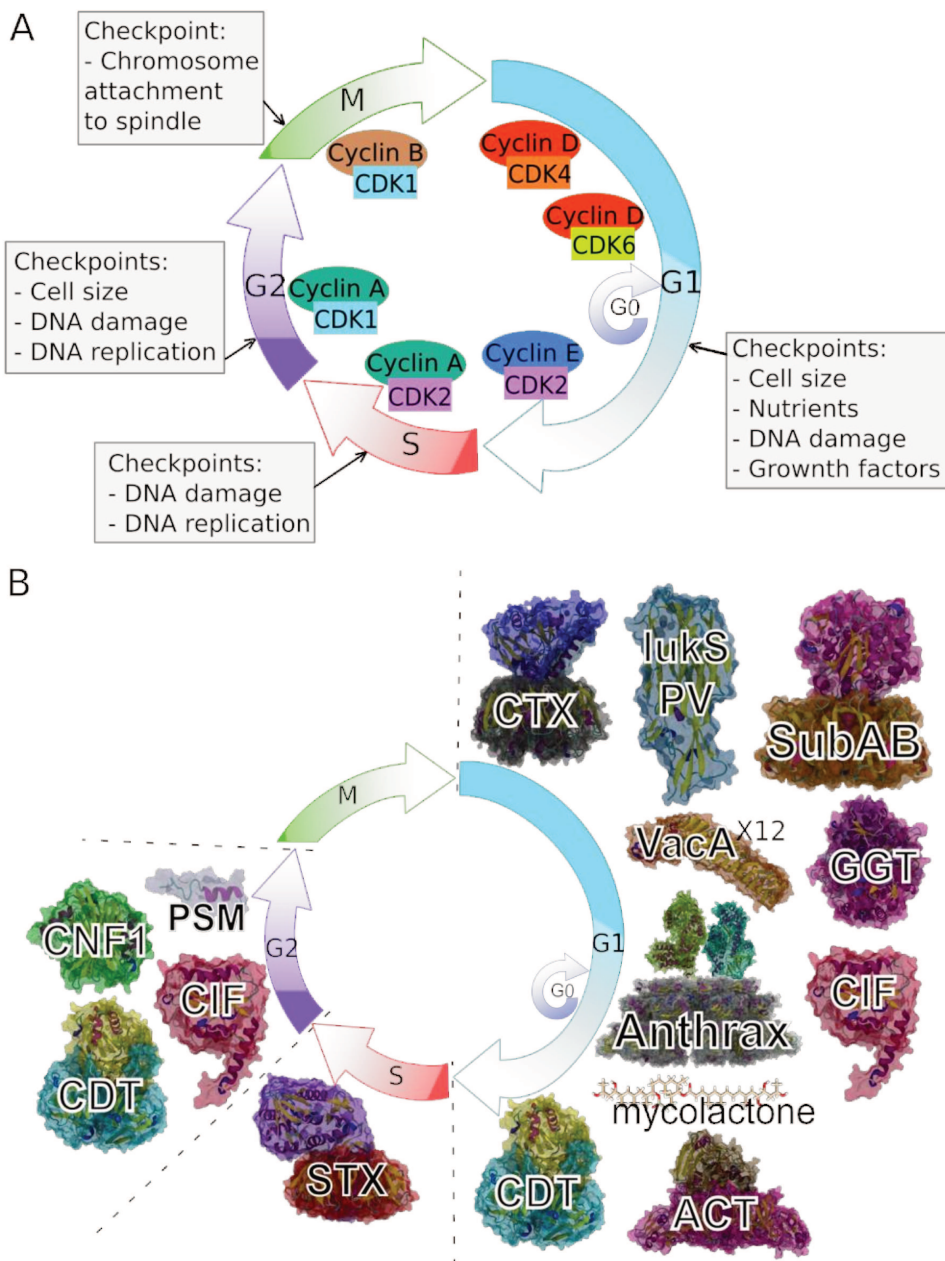
75 **Figure 1 A. Schematic presentation of the eukaryotic cell cycle and its regulation.**

76 The cell cycle consists of a number of events that result in cellular division. The
77 eukaryotic cell cycle consists of two gap phases, the G1 and the G2 phase, the S-phase
78 characterized by DNA replication, and the M (mitosis) phase. Cells can also enter a
79 quiescent state, the G0 phase. Cell cycle phases are indicated by colored arrows. The cell
80 cycle is regulated by complexes that are composed of cyclins, which are bound to cyclin-
81 dependent protein kinases (CDKs). Cyclin-CDK complexes are positioned in the front

82 of the arrow that designates the corresponding cell cycle phase. Cyclin-CDK complexes
 83 are controlled via checkpoint pathways whose role is to prevent the cell from
 84 progressing to the next stage when it is not allowed. Multiple stimuli that exert the
 85 checkpoint control are indicated in an appropriate text insert.

86 **B. Bacterial cyclomodulins operate at different points in the eukaryotic cell cycle.**

87 Three-dimensional structures of cyclomodulins were retrieved from the deposited
 88 Protein Data Bank when available (ACT: 1PRT; CDT: 2F2F; CTX: 1XTC; CIF: 3EFY;
 89 CNF1: 1HQ0; Anthrax: 1ACC, 1K8T, 4XM6; GGT: 2DBU; STX: 1R4P; SubAB: 4BWG;
 90 VacA: 2QV3). Structures were visualized with VMD.



93
 94

95 Cell cycle progression is controlled by the activities of complexes that consist of cyclins (A, B, D,
96 E) bound to cyclin-dependent protein kinases (CDKs). The D-type cyclins activate the CDK4 and
97 CDK6, which are required for an entry and a progression of cells into the G1-phase. To progress from
98 the G1 to the S phase, cyclin E associates with CDK2. Cyclin A associated with CDK2 allows
99 progression through the S phase. In the G2 phase, cyclin A associated with CDK1 enables entry into
100 the M-phase. Subsequently, cyclin B activates the CDK1 and promotes the M-phase of the cell cycle
101 [10].

102 The formation and activity of cyclin-CDK complexes are regulated by the synthesis of cyclins
103 and their degradation during a cell cycle progression, in addition to the inhibition of CDK
104 phosphorylation, or by the binding of inhibitory proteins to the complexes [10]. The combined effects
105 of these pathways control the cell cycle progression in response to external stimuli as well as to the
106 internal cell environments, e.g., through the checkpoint pathways. In addition to the control of cell
107 cycle arrest, checkpoint pathways control DNA repair pathways, activation of transcriptional
108 programs and stimulation of apoptosis in the case of persistent damage [11]. Checkpoint arrests occur
109 at different stages of the cell cycle: the G1/S transition (the G1 checkpoint), the S phase progression
110 (the intra-S phase checkpoint), the G2/M boundary (the G2/M checkpoint) and the spindle checkpoint
111 at the transition from metaphase to anaphase during mitosis (Fig. 1). The deregulation of complex
112 cell cycle progression machinery results in the disruption of essential cellular functions, with
113 dramatic consequences for cell survival.

114 Bacterial pathogens may damage the host from a distance through the action of secreted toxins
115 that use multiple sophisticated approaches to target main host functions, including the cell cycle. This
116 review is designed to summarize current knowledge regarding cyclomodulins, bacterial effectors
117 that induce eukaryotic cell cycle alterations (Fig. 1B) [12,13]. At present, there is no precise
118 classification of bacterial cyclomodulins. We will therefore discuss the mechanisms of the action of
119 cyclomodulins according to their biochemical properties. Three-dimensional structures of
120 cyclomodulins that were visualized with VMD (Visual Molecular Dynamics) software [14] are
121 presented and we provide representative examples of a cell cycle manipulation by bacterial
122 cyclomodulins. Special attention is paid to the deciphering of the consequences of host cell cycle
123 modifications.

124

125 **Highlights**

- 126 • The expression of many genes is cell cycle-regulated [15].
- 127 • Transcriptional and post-transcriptional mechanisms control cell cycle regulators [16].
- 128 • The cell cycle is also controlled by epigenetic mechanisms [6].

129

130 **3. Cycle inhibiting factor**

131 Enterohemorrhagic and enteropathogenic *E. coli* strains (EHEC and EPEC, respectively) are
132 major causes of infectious diarrhea in children worldwide. EHEC and EPEC use the Type 3 secretion
133 system (T3SS) encoded by the Locus of Enterocyte Effacement (LEE) to promote their establishment
134 within the host by delivering different virulence effectors to infected host cells (Table 1) [17]. Cycle
135 inhibiting factors (CIFs) are effectors produced by T3SS of EHEC and EPEC. CIF is not encoded by
136 the LEE but instead by a lambdoid prophage that has possibly been acquired through horizontal
137 transfer [17]. CIF causes cell arrest at both the G1/S and G2/M transitions (Fig. 1B) by accumulation

138 of the CDK inhibitors, p21^{Cip1} and p27^{Kip1} [18–20]. Such arrest is mediated by deamidation of the
139 ubiquitin-like protein NEDD8, which interacts with cullin-1 and promotes the inactivation of the SCF
140 (Skp1-Cullin1-F-box protein) complex, a Cullin-Ring ubiquitin Ligase (CRL) that promotes p21^{Cip1}
141 and p27^{Kip1} ubiquitinylation/degradation. Thus, this inhibition of CRL results in increased levels of
142 cell-cycle inhibitors such as p27^{Kip1} [21]. Cells exposed to CIF have also shown formation of stress
143 fibers and focal adhesion [22] beyond the cellular and nuclear enlargement that probably result from
144 inhibition of CRL-dependent RhoA degradation. In such cases, cells may restart the DNA synthesis
145 process without proceeding to division, thus increasing their DNA content [23].

146 Alignment between homologous sequences from different species revealed that CIF presents three
147 conserved residues in its catalytic motif: a cysteine C109, a glutamine Q185 and a histidine H165 [24].

148 This catalytic triad indicates that CIF is a member of a conserved family of cysteine proteases,
149 deamidases and acetyltransferases. Mutations of this motif have been shown to abolish a cell cycle
150 arrest by CIF. Moreover, the use of an irreversible inhibitor of cysteine proteases did not affect CIF
151 activity, suggesting that cysteine protease activity is not required for an induction of the phenotype
152 caused by CIF in eukaryotic cells [17].

153

154

155

Table 1. Cyclomodulin key features

	Alternative names	Toxin type	Species	Proteins	Ternary structure	Enzymatic activity	Cell cycle phase delay
Cycle Inhibiting Factor (CIF)	-	Cysteine protease	<i>E. coli</i> (EHEC, EPEC) <i>Y. pseudotuberculosis</i> <i>Pseudomonas sp.</i> <i>Enterobacter sp.</i> <i>Serratia sp.</i>	1 CIF protein	homodimer or monomer	cysteine protease deamidase acetyltransferase	G1/S G2/M
Mycolactone	-	Macrolide	<i>M. ulcerans</i>	-	12-member lactone ring	no	G1/S
γ-glutamyl transpeptidase (GGT)	-	Enzyme (EC 2.3.2.2)	<i>E. coli</i> <i>H. pylori</i>	1 protein with 2 chains cleaved by autocatalysis	dimer	EC 2.3.2.2	G1/S
Panton–Valentine leukocidin (PVL)	leucotoxin S component	β -pore-forming toxin Bi-component toxin	<i>S. aureus</i>	LukS-PV LukF-PV	octamer	no	G0/G1
Phenol soluble modulins (PSMs)	-	Peptides	<i>S.aureus</i>	PSM α 1 to PSM α 4 PSM β 1 and PSM β 2	monomer	no	G2/M
Cytotolethal Distending Toxin (CDT)	-	AB2 toxin AB intracellular acting toxin	<i>E. coli</i> <i>H. hepaticus</i> <i>S. enterica serovar Typhi</i> <i>many Gram - bacteria</i>	CdtB (enzymatic/A subunit) CdtA and CdtC (binding/B subunit)	trimer (1A & 2B)	A subunit : Dnase & phosphatase	G1/S G2/M
Shiga toxin (Stx)	Shiga toxin 2 Verotoxin 1	AB5 toxin	<i>S. dysenteriae serotype 1</i> <i>E. coli</i> (STEC)	stxA2 (A subunit) StxB2 (B subunit)	hexamer (1A & 5B)	A subunit : 3.2.2.22 (N-glycosidase)	S

Subtilase AB (SubAB)	-	AB5 toxin	<i>E. coli (STEC)</i>	SubA (A subunit) SubB (B subunit)	hexamer (1A & 5B)	A subunit	G1/S
Anthrax toxin	Edema toxin/ Lethal toxin	AB7 toxin Binary toxin Three-protein exotoxin	<i>B. anthracis</i>	Edema and/or Lethal factor (A subunit) Protective Antigen (B subunit)	2A & 7 or 8 B	Edema factor : adenylate cyclase (4.6.1.1) Lethal factor: zinc metalloprotease (3.4.24.83)	G1/S
Cholera toxin (Ctx)	Enterotoxin CT	AB5 toxin Heat-labile enterotoxin protein kinase A activator	<i>V. cholerae</i>	CTA (A subunit) CTB (B subunit)	hexamer (1A & 5B)	A subunit : 2.4.2.-	G1/S
Bordetella pertussis Adenylate Cyclase Toxin (ACT)	Pertussis Toxin	AB5 toxin	<i>B. pertussis</i>	S1 (A subunit) S2 to S5 (B subunit)	hexamer (1A & 5B)	A subunit : acetyltransferase	G1/S
Vacuolating toxin (VacA)	-	Pore-forming toxin Vacuolating cytotoxin	<i>H. pylori</i>	3 domains p33 p55 β-barrel	12 proteins	hypothetically	G1/S
Cytotoxic Necrotizing Factor 1 (CNF1)	-	Rho GTPases- activating toxin	<i>E. coli</i> <i>Vibrio sp.</i>	3 domains N-term (binding) C-ter (enzymatic) Central (translocation)	monomer	deaminase activity	G2/M

159

160 4. Mycolactone

161 The opportunistic pathogen *Mycobacterium ulcerans* is responsible for Buruli ulcer (BU), which is
162 a chronic disease that leads to skin necrosis in addition to a minimal inflammatory response [25]. The
163 exotoxin mycolactone A/B produced by *M. ulcerans*, was found to be a causative agent of BU (Table
164 1).

165 The mycolactone A/B is a macrolide that consists of two polyketide chains, one of which forms the
166 core lactone, a 12-member lactone ring, resulting in spontaneous cyclization [26].

167 Mycolactone-induced immunosuppression was found to be associated with the prevention of the
168 translocation (therefore, with the inhibition of the production) of proteins that pass through the
169 endoplasmic reticulum for a secretion [27]. Consequently, this effect impairs the immune functions
170 of various host cells such as monocytes, macrophages, T cells and dendritic cells that interact with
171 mycolactone during an infection [28,29].

172 It was found that the core lactone was sufficient for cytopathicity of mycolactone, while the fatty
173 acid side chain allows mycolactone to enter the host cells, enabling its interaction with intracellular
174 target molecules [30].

175 An exposé of cells to mycolactone *in vitro* leads to the cytoskeletal rearrangement and a
176 detachment of cells from the cell culture plate, which was associated with the direct binding of
177 mycolactone to a scaffolding protein, the Wiskott–Aldrich syndrome protein, resulting in the
178 alteration of actin dynamics [31]. Moreover, it was shown that mycolactone induces apoptosis as well
179 as an arrest in the G1 phase [32,33].

180

181 5. γ -glutamyltranspeptidase of *H. pylori*

182 Different bacteria such as *E. coli* and *H. pylori* produce a hydrolase γ -glutamyltranspeptidase,
183 (GGT), a bacterial virulence factor that catalyzes the transpeptidation and hydrolysis of the gamma-
184 glutamyl group of glutathione and related compounds (Table 1) [34].

185 *H. pylori* GGT is synthesized as a single 60 kDa precursor protein expressed by the gene HP1118 [35].
186 Following an autocleavage near the C-terminal portion inside the cell periplasm, GGT releases two
187 units, 40kDa and 20kDa, which form the active heterodimer [35]. A 20 kDa unit expresses the
188 enzymatic activity of GGT: the gamma-glutamyl binding site comprises the Tyr433 and the Arg 475
189 residues, while the C-terminus of the 20 kDa subunit are critical for its catalytic activity [36].

190 GGT as a virulence factor has gained increased attention during the past decade due to its harmful
191 effects on the host. GGT is mainly associated with colonization and gastric ulcer induction, as was
192 observed in some animal models [37,38]. GGT causes damage to gastric cells, including apoptosis
193 [35], the production of reactive oxygen species causing DNA damage [38,39], an induction of
194 inflammatory responses by an increase in the expression of cyclooxygenase-2 [40] and interleukin 8
195 [38], as well as cell cycle delay [41]. Exposure of human cancer cells AGS (gastric adenocarcinoma
196 cells) to GGT resulted in G1 phase arrest. The arrest was associated with an increased expression of
197 cyclin E, cyclin A, Cdk 4 and Cdk 6, and a decreased expression of the cyclin-dependent kinase (Cdk)
198 inhibitors p27^{Kip1} and p21^{Cip1}, suggesting that GGT hampers the G1-S phase transition [41].

199

200 6. Panton-Valentine leucocidin

201 *Staphylococcus aureus* (*S. aureus*) is an opportunistic pathogen responsible for a variety of diseases
202 ranging from skin and soft tissue infections to life-threatening systemic diseases in humans and
203 animals. *S. aureus* frequently promotes infections by producing powerful toxins. Pantone–Valentine
204 leukocidin (PVL) is one of the β -pore-forming toxins associated with the increased virulence of
205 certain *S. aureus* strains (Table 1). PVL is responsible for leukocyte destruction and necrotic
206 hemorrhagic pneumonia, a highly lethal infection that essentially affects healthy children and young
207 adults.

208 PVL is a bi-component toxin that acts due to the synergistic activity of two protein subunits, 33
209 and 34 kDa in size, denoted as S and F according to their slow or fast elution in cation-exchange
210 carboxy-methyl cellulose chromatography [42]. These proteins are encoded by two co-transcribed
211 genes, *lukS-PV* and *lukF-PV*, which reside on a prophage [43]. *LukS-PV* and *LukF-PV* subunits with
212 a beta-barrel structure acquire pore-forming conformation after binding to specific cellular receptors,
213 followed by hetero-oligomerization at the plasma membrane of the host defense cells. An insertion
214 of pores into the plasma membrane leads to ion influx and efflux followed by cell lysis [44].
215 It was observed that the *lukS-PV* subunit inhibits a proliferation of the human acute myeloid
216 leukemia cell line THP-1 [45]. An analysis of a cell cycle phase distribution of *LukS-PV*-treated THP-
217 1 cells identified cell cycle changes: *LukS-PV* reduced the number of S-phase cells while increasing
218 the number of G0/G1-phase cells. Moreover, *LukS-PV* significantly inhibited the expression of cyclin
219 D1 that regulates the cell cycle progression from phase G1 to phase S [10]. Collectively, these data
220 demonstrate a *LukS-PV*-induced G0/G1 arrest. In addition to cell cycle arrest, *LukS-PV* induces
221 apoptosis in THP-1 cells via the mitochondrial pathway, as was demonstrated by an increase in
222 caspase-8 expression, as well as an increase in caspase-9, -3 and Bax levels, and a decrease in Bcl-2
223 level [45].

224

225 7. Phenol soluble modulins

226 The phenol-soluble modulin (PSM) peptides were first identified in 1999 as a “pro-inflammatory
227 complex” isolated by hot phenol extraction from *S. epidermidis* [46]. PSMs have recently emerged as
228 a novel toxin family that contributes to increased virulence and the spread of highly aggressive *S.*
229 *aureus* isolates (Table 1) [47–49]. Formed by amphipathic peptides arranged in alpha helices, PSMs
230 are classified according to their size: short (20–25 amino acids) α -type peptides (PSM α 1–PSM α 4) and
231 δ -toxin, and long (44 amino acids) β -type peptides (PSM β 1 and PSM β 2) [50].

232 PSMs are encoded at three different locations in the genome. Four PSM α 1–PSM α 4 peptides are
233 encoded in the *psmA* operon; PSM β 1 and PSM β 2 are encoded in the *psm β* operon; and δ -toxin is
234 encoded within the coding sequence for RNAIII, the RNA effector molecule of the accessory gene
235 regulator (Agr) quorum-sensing system [51,52].

236 PSMs have a multiplicity of biological functions that are essential to staphylococcal pathogenesis
237 [53]. PSMs trigger inflammatory responses such as chemotaxis and a priming of human neutrophils,
238 and induce cytokine expression [49]. It was found that PSMs inhibit interleukin expression during
239 long-term infections, suggesting the involvement of PSMs in persistence [54]. PSMs lyse white and
240 red blood cells [55] and contribute to the formation of biofilms [56]. PSMs also present antimicrobial
241 and immunomodulatory properties [49,57,58].

242 Alekseeva *et al.* demonstrated that the original methicillin-resistant *S. aureus* strains commonly
243 known as MW2 induced both a decrease in the mitotic index and a cytopathic effect in host cells

244 [59]. Moreover, it was shown that MW2 induces a G2/M phase transition delay in host cells, which
245 was associated with the accumulation of the cyclin-dependent kinase Cdk1/cdc2 and
246 unphosphorylated histone H3. Recently, it was determined that PSMs were responsible for the G2/M
247 phase transition delay, suggesting that PSMs belong to a family of cyclomodulins. The PSM α -induced
248 G2/M phase delay was associated with increased bacterial efficiency and decreased production of
249 antibacterial peptides [60].

250

251 8. AB toxins

252 Some toxins, referred to as AB toxins, consist of distinct domains that interact with receptors on
253 the cell surface (B domain) and modify the action of intracellular host targets through their enzymatic
254 activities (A domain) [61,62]. Many bacterial cyclomodulins belong to the family of AB toxins.

255

256 9. Cytolethal distending toxin

257 Cytolethal distending toxin (CDT), produced by a variety of Gram-negative bacteria such as
258 *Escherichia coli*, *Helicobacter hepaticus*, *Salmonella enterica* serovar Typhi (*S. typhi*) and other bacteria
259 [63], belongs to the family of AB toxins (Table 1, Fig. 2). CDT blocks the cell cycle of the host cell and
260 induces DNA single and double strand breaks (DSB) [64].

261 CDT is the product of an operon encoding three proteins: CdtA, CdtB and CdtC. The A domain
262 of CDT is formed by a CdtB protein that exhibits the DNase activity responsible for DSB, and a
263 phosphatase activity that resembles phosphatidylinositol 3,4,5-triphosphatase [64–66]. CdtA and
264 CdtC proteins form the B domain, which allows it to bind to the susceptible cell, leading to toxin
265 internalization. CdtB is then relocated to the nucleus by a retrograde transport pathway via early and
266 late endosomes [67].

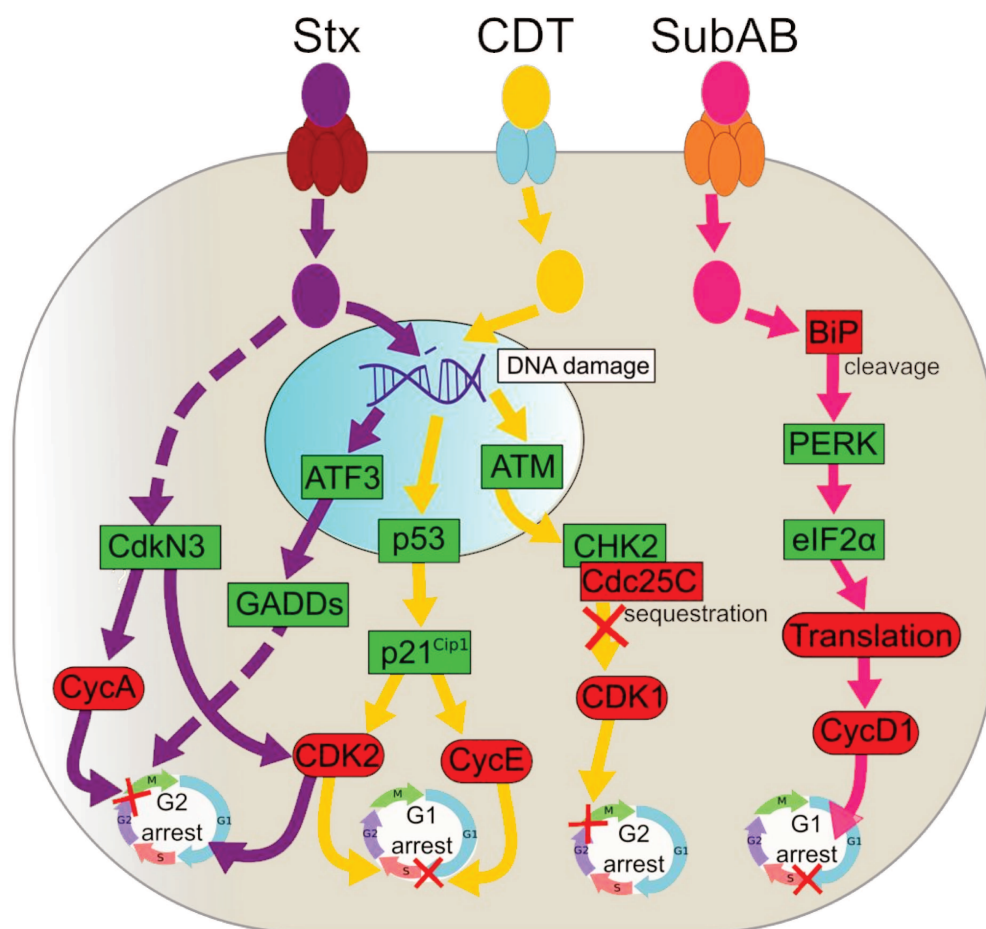
267 In contrast to other pathogens, in the *S. typhi* typhoid toxin, CdtB is associated with PltA (the
268 pertussis-like toxin A, homologous to the pertussis toxin ADP-ribosyltransferase subunit) and to PltB,
269 the pertussis-like toxin B, instead of being associated with CdtA and CdtC [67]. The atomic structure
270 of typhoid toxin shows that typhoid toxin is A2–B5 toxin. Two A subunits (StCdtB and PltA
271 proteins), covalently linked by a disulfide bond, are non-covalently associated with a pentameric B
272 subunit (PltB) [68]. The absence of CdtA does not prevent *S. typhi* CDT from delaying cell cycle
273 progression [69].

274 The CdtB subunit is highly conserved among various bacteria and has 25–40% identity with
275 phosphodiesterase enzymes, including DNase I [70,71]. The nuclease activity shown by this subunit
276 triggers a DNA damage response (DDR) [64]. The activation of the DDR is initiated by the ataxia
277 telangiectasia mutated (ATM) kinase that phosphorylates CHK2, a serine/threonine kinase that, in
278 turn, phosphorylates various cell cycle regulators such as phosphatases Cdc25A and Cdc25C (Fig. 2)
279 [67]. Phosphorylation of Cdc25C creates an interaction site for the 14-3-3 family of proteins that
280 sequester Cdc25C in the cytoplasm [72]. Thus, phosphatase Cdc25C is unable to activate the nuclear
281 hyper-phosphorylated (inactive) CDK1/cyclin B complex, leading to the delay in the G2/M phase of
282 the cell cycle [73]. Delay at the G1/S phase by CDT is also reported to result from an ATM/p53-
283 dependent accumulation of the CDK2-cyclin E inhibitor, p21^{Cip1}. Inhibition of CDK2-cyclin E blocks
284 the entry into the S phase of the cell cycle [74,75].

285

286 **Figure 2: Signaling pathways of Shiga toxin (Stx), Cytolethal Distending Toxin (CDT)**
 287 **and subtilase AB (SubAB).**

288 Activated and inactivated proteins are colored in green and red, respectively. Arrow
 289 colors match catalytic moieties of toxins. Dashed arrows are drawn when the precise
 290 mechanism is unknown. (i) Shiga toxin (Stx) binds to the cell membrane through the
 291 pentameric B subunit (dark red), followed by an internalization of the enzymatic A
 292 subunit (purple). Stx induces irreversible DNA damage that activates ATF3 and GADDs
 293 proteins, leading to cell cycle arrest in the G2 phase. Stx also induces CdkN3 that inhibits
 294 Cyclin A and CDK2 and results in cell cycle arrest in the G2 phase. (ii) Cytolethal
 295 Distending Toxin (CDT) binds to a cell membrane through CdtA and CdtC (blue),
 296 leading to an enzymatic CdtB (yellow) internalization. CDT causes DNA damage that
 297 leads to the activation of ATM, followed by Cdc25C sequestration by CHK2.
 298 Consequently, Cdc25C is not free to bind CDK1, leading to its inhibition and, ultimately,
 299 to cell cycle arrest in the G2 phase. DNA damage caused by CDT also activates p53 and
 300 p21^{Cip1}, causing CDK2 and CycE inactivation and cell cycle arrest in the G1 phase. (iii)
 301 Subtilase AB binds to the cell membrane with the pentameric B subunit (orange)
 302 followed by the entrance of the enzymatic A subunit (pink). SubAB cleaves the
 303 chaperone BiP that activates PERK and eIF2 α , leading to a translation inhibition. Finally,
 304 cyclin D1 is down-regulated and causes the arrest of the cell cycle in the G1 phase.



305

306

307 *In vitro* and *in vivo* evidence indicates that CDT is a beneficial virulence factor for bacterial survival.
308 Moreover, CDT enhances microbial pathogenicity. It was found that CDT participates in the increase
309 of bacterial gut colonization, stomach and intestinal inflammation, and deregulation of the immune
310 system in mice and rabbits [75,76]. Furthermore, the effect caused by CDT may lead to apoptosis or
311 necrosis of cells through the mitochondrial effectors Bax/Bcl-2, cytochrome C release and caspase
312 activation [77].

313 Another consequence of the genotoxic effects of CDT is related to the induction of cellular
314 senescence associated with persistently activated DNA damage signaling that may result in genomic
315 instability [67,78].

316

317 10. Shiga toxin

318 Shiga toxins (Stxs) are produced by *Shigella dysenteriae* serotype 1 bacteria and Shiga-like toxin-
319 producing *E. coli* (STEC), which are subdivided into types 1 and 2, depending on the type of toxin
320 (Table 1) [79]. Infections with *S. dysenteriae* and STEC are major public health concerns in developed
321 and developing countries since they may induce a bloody diarrhea (hemorrhagic colitis) or a
322 hemolytic-uremic syndrome (HUS) [80]. Stx belong to the family of AB₅ toxins that consist of the
323 ribosomal 32 kDa RNA-cleaving A subunit, which is non-covalently bound to five receptor-binding
324 7.7 kDa B subunits (Fig. 2) [81]. After internalization, the toxins are transported from an early
325 endosome, through the Golgi apparatus, to the endoplasmic reticulum (ER) [82]. During this
326 retrotranslocation, the A subunit dissociates from the B subunit due to a proteolysis and a disulfide
327 bond reduction. [81]. Once in the cytoplasm, the A subunit acquires a native conformation and
328 activates its enzymatic features, leading to the removal of an adenine residue from the ribosomal
329 RNA of eukaryotic cells and, subsequently, impairing a protein synthesis [81].

330 In addition to a protein synthesis inhibition, Stx induces the ribotoxic stress response and ER
331 stress that may result in apoptosis, autophagy or an activation of the innate immunity associated with
332 a cytokine/chemokine production that contribute to tissue damage in multiple organs [83,84].
333 Furthermore, it was shown that *E. coli* Stx-1, known as verotoxin, induces an arrest of epithelial cells
334 in the S phase of a cell cycle (Fig. 1B), which is probably a consequence of irreversible DNA damage
335 (Fig. 2) [85]. The delay in the S phase is associated with an up-regulation of stress response genes: the
336 GADD (Growth Arrest and DNA Damage) family genes and the ATF3 (Activating Transcription
337 Factor), as well as with the cell cycle-related phosphatase CdkN3 [85]. It was shown that ATF3 is able
338 to induce GADD34 and GADD153, which are inducer proteins of apoptosis [86], as well as an
339 expression of CdkN3, which is an inhibitor of CDKs [87]. However, the precise mechanisms of the
340 interaction between those proteins and the arrest of Stx-1-infected eukaryotic cells in the S phase of
341 the cell cycle has not yet been completely determined at this time.

342

343 11. Subtilase toxin

344 Subtilase toxin (SubAB) is an AB₅ type toxin that is produced by Shiga toxigenic strains of
345 *Escherichia coli* (STEC) (Table 1) [88]. Similar to other AB toxins, five 13 kDa B subunits of SubAB bind
346 to the receptors of host cells, while the 35 kDa A subunit possess an enzymatic activity that is essential
347 for cytotoxicity [89]. SubAB may contribute to the pathogenesis of a systemic hemolytic uremic
348 syndrome. However, the *in vivo* effects of SubAB have only been examined in mice and were shown
349 to cause microvascular injury, thrombosis and necrosis in various organs such as the brain, the liver

350 and the kidneys [90]. SubAB inhibits protein synthesis resulting from ER stress induced by cleavage
351 of the carboxy-terminal portion of chaperone BiP/Grp78, which is essential for a proper folding and
352 an assembly of nascent proteins (Fig. 2) [91]. SubAB-induced ER stress caused many other cellular
353 events such as activation of Akt-NF-B signaling, a down-regulation of gap junction expression and
354 an induction of apoptosis [92].

355 The cleavage of BiP/Grp78 was associated with a phosphorylation of a double-stranded RNA-
356 activated protein kinase-like ER kinase (PERK) and a eukaryotic initiation factor-2 α (eIF2 α) [93]. A
357 degradation of BiP/Grp78 leads to cyclin D1 down-regulation caused by both a SubAB-induced
358 translational inhibition and a continuous prolonged proteasomal degradation synthesis and results
359 in G1 phase arrest (Fig. 2) [93–95].

360

361 12. Anthrax toxin

362 *Bacillus anthracis* is a causative agent of anthrax, a disease with local or systemic clinical
363 manifestations. Cutaneous anthrax is characterized by edematous necrotic lesions that become black
364 eschars, while systemic anthrax has a multiplicity of symptoms, including of hypotension and shock,
365 followed by sudden death [96].

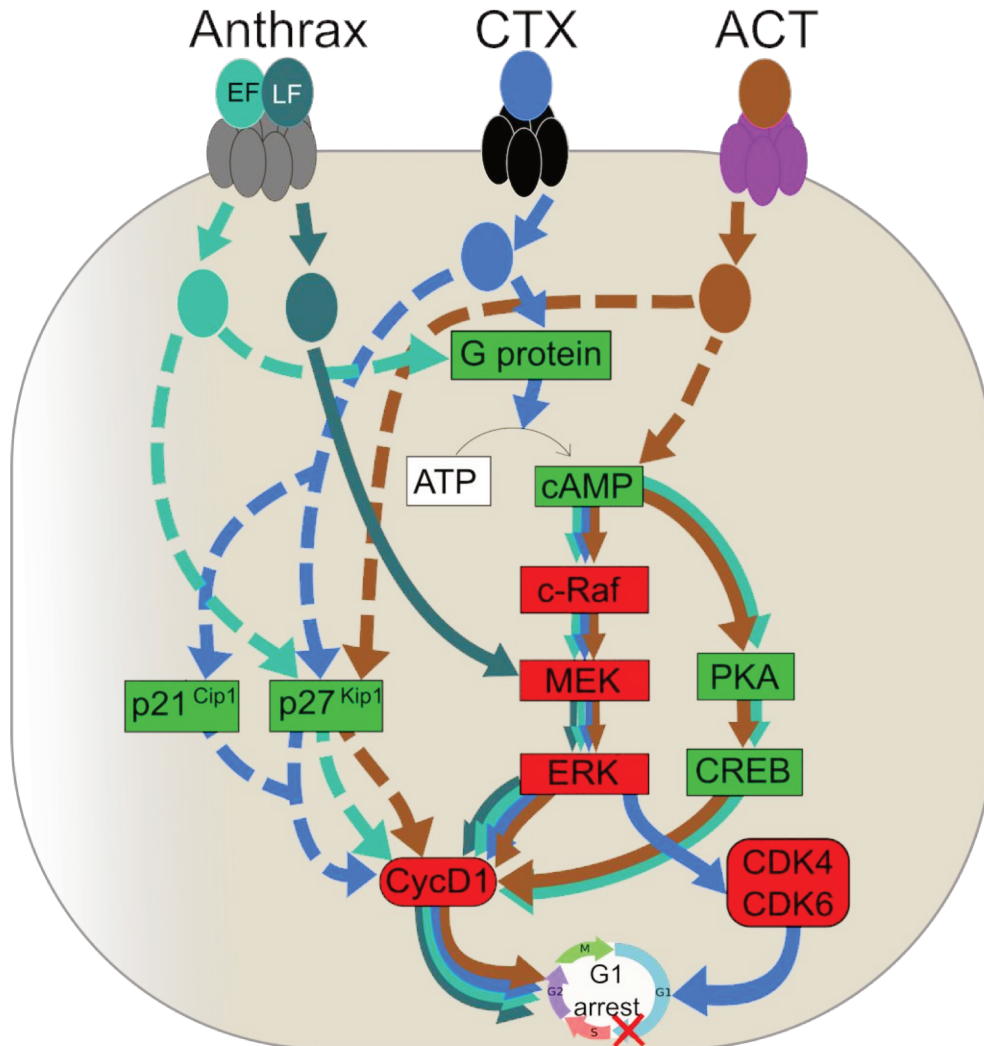
366 *B. anthracis* toxin is an AB toxin family and consists of the B subunits, the protective antigen (PA)
367 and two alternative A subunits: the lethal factor (LF), forming lethal toxin (LT), and the edema factor
368 (EF), forming edema toxin (ET) (Table 1) [97]. The 83kDa PA binds to protein receptors on the host
369 cells, such as tumor endothelial marker 8 (TEM8) and capillary morphogenesis gene product 2
370 (CMG2), followed by the entrance of the catalytic moieties of the toxins, ET or LT, into the host cell
371 cytosol. The EF subunit causes cutaneous edema and has been shown to be lethal to mice infected
372 intravenously [98]. ET is an adenylate cyclase toxin that upon a translocation into host cells and after
373 an association with a calcium-binding protein, calmodulin, catalyzes the production of an ubiquitous
374 second messenger cyclic AMP (cAMP), resulting in the impairment of multiple changes in cell
375 signaling pathways (Fig. 3) [99,100].

376

377 **Figure 3: Signaling pathways of adenylate cyclase AB toxins resulting in G1 phase** 378 **arrest of the eukaryotic cell cycle.**

379 Activated and inactivated proteins are colored in green and red, respectively. Arrow
380 colors match catalytic moieties of toxins. Dashed arrows are drawn when the precise
381 mechanism is unknown. (i) Anthrax toxin is formed by EF, LF and PA. Heptameric PA
382 (gray) binds to the membrane and leads to the entrance of EF and LF into the cell. EF
383 (green) induces cAMP production followed by inactivation of the c-Raf/MEK/ERK
384 cascade, leading to Cyclin D1 inactivation. A cAMP increase induces PKA and CREB,
385 leading to cyclin D1 inactivation. The level of p27^{Kip1} is increased by cAMP and leads to
386 cyclin D and, especially, Cyclin D1 inactivation. LF (dark green) directly inactivates
387 MEK, leading to ERK and Cyclin D1 inactivation. (ii) Cholera toxin (CTX) binds to the
388 cell membrane through the pentameric CTB subunit (black), leading to endocytosis of
389 the catalytic CTA subunit (blue). CTX activates the G protein and leads to cAMP
390 production followed by up-regulation of p27^{Kip1} and p21^{Cip1} and inactivation of the c-
391 Raf/MEK/ERK cascade, leading to Cyclin D1, CDK4 and CDK6 inactivation. (iii)
392 Adenylate cyclase toxin (ACT) bounds to a membrane through the pentameric subunit

393 (purple), and the catalytic subunit (brown) is translocated to the cytosol. In the same
 394 way as EF, ACT induces production of cAMP that leads to inactivation of the c-
 395 Raf/MEK/ERK cascade, activation of PKA and CREB, activation of p27^{Kip1} and, finally,
 396 inactivation of Cyclin D1. Inactivation of Cyclin D1, CDK4 and CDK6 leads to cell cycle
 397 arrest in the G1 phase.
 398



399
 400

401 The proliferation of many cells is controlled by the phosphorylation of extracellular signal-
 402 regulated kinase ERK due to the action of mitogen-activated protein kinase (MEK) that communicates
 403 a signal from cellular receptors to the nuclear DNA, thereby regulating the gene expression; this
 404 transduction pathway was influenced by cAMP [101]. ET-induced cAMP reduces the amount of the
 405 phosphorylated ERK and phosphorylates the Cyclic AMP Response-Element Binding (CREB) protein
 406 in part through cAMP-dependent Protein Kinase A (PKA) [102]. ET-induced cAMP accumulation
 407 leads to a decrease in the level of cyclin D1 that, together with CDKs, regulates the entry into the G1
 408 phase. The level of p27^{Kip1}, which inhibits the complex of cyclin D and CDKs, was augmented due to
 409 the cAMP increase. Consequently, an accumulation of cells in the G1/G0 phase and a decrease of cells
 410 in the S phase occurred in ET-treated cells [102].

411 LF, the other catalytic subunit of the anthrax toxin, is a zinc metalloprotease that cleaves and
 412 inactivates the N-terminal end of MEKs. It leads to the inhibition of ERK, followed by a down-

413 regulation of cyclin D1, cyclin D2 and checkpoint kinase 1 (Chk1), resulting in the arrest of cells in
414 the G1/G0 phase of the cell cycle [103].

415

416 13. Cholera toxin

417 The cholera toxin (CT or CTX) is the major virulence factor of *Vibrio cholera* and the main diarrhea-
418 causing enterotoxin (Table 1). CTX is a member of the AB₅ family of toxins that consist of a cell surface
419 receptor-binding homopentameric B subunit (CTB) that is linked to a catalytic A subunit (CTA),
420 which comprises the CTA1 and CTA2 domains (Fig. 3) [104]. A binding of the pentameric CTB
421 subunit to the GM1 ganglioside receptor on the intestinal cells (enterocytes) triggers CTX endocytosis
422 followed by the cleavage of CTA1, which then becomes an active enzyme. Once activated, CTA1
423 activates the G protein through an ADP-ribosylation reaction, thereby stimulating adenylate cyclase
424 to produce cAMP (Fig. 3). The high cAMP levels impede the electrolyte balance, causing a drastic
425 efflux of ions and water from enterocytes, leading to watery diarrhea [105].

426 The capacity of CTX to modulate the cell cycle progression of eukaryotic cells was reported by Li
427 et al. [106]. CTX induces an accumulation of cells in the G1 phase due to the down-regulation of cyclin
428 D1 and CDK2 proteins along with an up-regulation of the cell-cycle inhibitory proteins, p21^{Cip1} and
429 p27^{Kip1} (Fig. 3) [106]. More recently, the action of CTX was related to the cAMP-dependent
430 phosphorylation of a serine/threonine-specific protein kinase c-Raf by PKA. A kinase c-Raf is an
431 upstream regulator of the cascade involving MEK and ERK, thus regulating the gene expression.
432 When phosphorylated, c-Raf inhibits an expression of cyclin D1, Cdk4 and Cdk6 that leads to cell
433 cycle arrest [107].

434

435 14. *Bordetella pertussis* adenylate cyclase toxin

436 *Bordetella pertussis*, a Gram-negative bacterial pathogen, is responsible for respiratory infections
437 manifested by whooping cough, with possible lethal complications (Table 1).

438 Similar to *B. anthracis*, *B. pertussis* produces adenylate cyclase toxin (ACT), which belongs to the AB₅
439 toxin family (Fig. 3) [108].

440 ACT of *B. pertussis* contains an N-terminal domain of approximately 400 amino acid residues
441 and a repeat toxin hemolysin domain (RTX) of approximately 1300 amino acid residues [109]. These
442 species secrete ACT through the type I secretion system, which is formed by the proteins CyaB, D
443 and E. It has been shown that ACT of *B. pertussis* is activated through an acetylation of two lysine
444 residues that are located in the RTX domain by the CyaC acetyltransferase [109]. ACT is activated in
445 the cytosol by calmodulin, leading to the production of cAMP. Concentrations of ACT that are below
446 1 µg/ml lead cells to apoptosis, whereas ACT concentrations above 1 µg/ml induce an apoptosis-
447 independent cell death, as was shown using the J774 macrophage cell line. This fact is probably due
448 to the hemolytic and pore-forming synergism promoted by ACT, in addition to the decrease in the
449 intracellular ATP levels [102]. In the same way as the ACTs of *B. anthracis*, the ACTs of *B. pertussis*
450 have been shown to inhibit the proliferation of the J774 macrophages through cell cycle delay.

451 It is recognized that intracellular cAMP can elicit its biological effects via different mechanisms
452 (Fig. 3). An analysis of the effects of *B. pertussis* on the regulatory pathways controlling the cell cycle
453 reveals that ACT stimulates PKA, which activates CREB through its phosphorylation that, in turn,
454 decreases the Cyclin D1 level, followed by G1 phase arrest [102]. On the other hand, in the same way
455 as other toxins of the AC family, *B. pertussis* ACT-induced cAMP may activate the c-Raf/MEK/ERK

456 complex, followed by the inhibition of ERK phosphorylation, resulting in the decrease of the Cyclin
457 D1 level and, consequently, causing a block in cell cycle progression at the G1-S transition [102].
458 Furthermore, ACT may increase the cyclin-dependent kinase inhibitor, p27^{Kip1}. Such a regulatory
459 profile causes an accumulation of infected cells in the G1/G0 phase and a reduction of cells in the S
460 phase [102].

461

462 15. Vacuolating cytotoxin

463 *Helicobacter pylori* is a Gram-negative bacterium that colonizes the intestinal mucosa and causes
464 several diseases such as chronic gastritis, gastric cancer and ulcers. One of the major virulence factors
465 reported for *H. pylori* is vacuolating cytotoxin A (VacA), which is known for its ability to induce the
466 formation of large acid vesicles in the cytoplasm of gastric cells (Table 1) [110].

467 VacA is synthesized as a 140 kDa pre-protoxin, which undergoes proteolytic processing,
468 resulting in a mature secreted 88kDa monomer form of toxin [110]. Intracellular-acting exotoxin
469 VacA may be considered as a prototype of AB toxin due to the modulation of cellular homeostasis
470 by forming ion-conducting intracellular membrane channels, allowing the efflux of metabolic
471 substrates for the growth of bacteria [111]. VacA can also be embedded in the endosome via
472 endocytosis, resulting in large vacuole formation by water influx [112].

473 It was shown that VacA enters gastric epithelial cells, inserts itself into mitochondria, forms
474 membrane channels and modulates a mitochondrial membrane permeability [110,111]. This results
475 in cytochrome c release and an execution of apoptosis through an increase in the expression of cell
476 cycle/apoptosis regulators such as p53, p21^{Cip1} and Bax, beyond caspases 8 and 9, as was
477 demonstrated using gastric epithelial AGS cells [112,113].

478 An exposure of cells to a recombinant VacA was associated with the inhibition of cell growth
479 and resulted in morphological changes and DNA fragmentation; a cell cycle analysis revealed a
480 prolongation of the cell cycle progression in the G1 phase [114]. These findings indicate that VacA of
481 *H. pylori* induces apoptosis in gastric epithelial cells and suggests that VacA might mediate the
482 development of gastric diseases through a cell cycle arrest in the G1 phase [114]. Additionally, it was
483 found that VacA could efficiently block a proliferation of T cells by inducing an arrest during the
484 G1/S phase transition [115]. This influences the T cell receptor/interleukin-2 (IL-2) signaling pathway
485 through the Ca²⁺-calmodulin-dependent phosphatase calcineurin, resulting in down-regulation of
486 IL-2 transcription. Collectively, these data suggest that VacA may induce a local immune
487 suppression, explaining the peculiar chronicity of Hp infections [115].

488

489 16. Cytotoxic Necrotizing Factor

490 The Cytotoxic Necrotizing Factor 1 (CNF-1) is a toxin frequently produced by pathogenic *E. coli*.
491 This toxin is commonly associated with urinary tract and gastrointestinal infections as well as with
492 septicemia, neonatal meningitis and soft tissue infections (Table 1) [116]. *E. coli* CNF-1 is a 113.8 kDa
493 AB toxin comprising an N-terminal receptor-binding domain, a binder domain with two
494 hydrophobic helices involved in membrane translocation and a C-terminal catalytic domain [117].

495 The first description of CNF1 was associated with its ability to cause multinucleation in different
496 cell culture lines [118]. Investigations of molecular mechanisms of the action of CNF 1 demonstrate
497 that CNF1 activates proteins belonging to the family of Rho GTPases (e.g., Rho, Rac and Cdc42), by
498 promoting deamidation of glutamine residues through hydrolysis of GTP to GDP. Since Rho GTPases

499 modulate a large number of cellular functions, CNF-1 affects the cytoskeleton assembly, whereas Rho
500 GTPases regulate the polymerization of actin filaments [119] and CNF-1-activated Rho GTPases
501 induce macropinocytosis and phagocytosis [120].

502 Moreover, it was shown that CNF1 prevents the progression of cells from the G2 phase to the M
503 phase in uroepithelial cells [121]. It is recognized that Cyclin B1 expression varies during the cell cycle
504 progression with the highest level in the G2/M phase. It is also documented that the G2/M phase
505 transition is driven by an activation of a Cdk1/cyclin B1 complex [122]. Cyclin B1 is essentially found
506 in the cytoplasm region through the G2 phase until it translocates into the nucleus, which precedes
507 the nuclear envelope breakdown [121]. It was demonstrated that CNF-1 reduces cyclin B1 expression
508 and induces a sequestration of cyclin B1 in the cytoplasm that results in G2/M phase arrest [121,123].

509 As in the case of other cyclomodulins, CNF-1-induced G2/M arrest, which results in the damage
510 of the epithelium, might favor *E. coli* colonization and represent a long-term risk of carcinogenesis
511 due, to some extent, to the capacity of Rho GTPases to promote a great number of events such as the
512 motility of tumorigenic cells, metastasis, cell invasiveness and abrogation of cytokinesis [124].

513

514 **17. Alteration of the host cell cycle as a beneficial feature for bacterial fitness**

515 The toxins excreted by bacteria play a pivotal role in the enhancement of bacterial fitness during
516 an infection of eukaryotic hosts. The action of these toxins alters diverse cellular transduction
517 pathways, thus providing toxin-producing bacteria with a noteworthy fitness advantage against a
518 eukaryotic host as well as against competitor bacteria. For example, toxins with proteolytic functions
519 can degrade host matrix components and impede important host cell processes, in addition to
520 providing nutrients that can be used by the pathogen [125]. The ability of some toxins to promote
521 bacterial adhesion to the host cells and tissues is another example of how bacteria promote their
522 successful establishment within the host [126]. Moreover, bacterial toxins may favor adaptation to
523 the adverse host environment [127].

524 The beneficial role of a bacteria-induced host cell cycle alteration for a pathogen invasion was
525 observed for bacterial toxins as well. Thus, *E. coli*-produced toxin, CNF, which is shown to induce the
526 G2/M transition delay during infection, probably impairs the host epithelial layer turnover and,
527 therefore, favors bacterial colonization. [121]. Another *E. coli* toxin, CIF, which is produced by EPEC
528 and EHEC strains, induces the arrest of the G2 and the G1 phases of the cell cycle, which is correlated
529 with the accumulation of the CDK inhibitors, p21^{cip1} and p27^{kip1}. These CDK inhibitors are involved
530 in cell cycle exit, differentiation, carcinogenesis and survival. Consequently, CIF could prolong a
531 bacterial attachment and a local persistence by slowing down a crypt–villus cell renewal [128,129].

532 *S. aureus*, which commonly infects epithelial surfaces and leads to abscess formation, necrosis and a
533 compromise of the integrity of the host epithelial barrier, is also known to cause morphological
534 changes in epithelial cells such as cell enlargement, an increase in nucleus size and a decrease of a
535 host cell proliferation [59]. An *S. aureus*-induced decrease of the host cell proliferation was associated
536 with the G2/M transition delay, which was triggered by PSMs [59]. A PSMA-induced delay results in
537 an augmented staphylococcal internalization and in an enhanced intracellular proliferation, which
538 have been shown to be more effective in G2/M phase cells than in asynchronous cells. Moreover, a
539 PSMA-induced delay correlates with an alteration in defensin (antibacterial peptides) gene
540 expression: the expression was lower when the cells were in the G2/M phase. This suggests a

541 reduction of the antibacterial functions of epithelial cells, thus demonstrating an advantage of a
542 PSMA-induced cell cycle arrest for an infection of host cells with *S. aureus* [59].

543 The ACT cyclomodulins produced by *Bordetella* species and *B. anthracis* are capable of interfering
544 with a proliferation and a differentiation of T cells, reducing the production of TNF- α and increasing
545 the production of IL-10 in dendritic cells [130,131]. The continuous production of ACT during an
546 infection triggers mechanisms that compromise an inflammatory response by inhibiting the
547 regeneration of populations of defense cells [102,132]. Moreover, ACT was recently found to promote
548 an internalization of bacteria in non-phagocytic cells [133]. It is likely that an ACT-related increase of
549 a bacterial internalization is associated with an ACT-induced cell cycle alteration, similar to that of
550 staphylococcal PSMs.

551 A number of toxins with the ability to inhibit different stages of protein synthesis in eukaryotic
552 cells are commonly found in nature [134]. SubAB and Stx are some of them. While SubAB inhibits
553 the production of pro-inflammatory cytokines and chemokines during infection, Stx, which is also
554 involved in the inhibition of protein synthesis, induces an inflammatory response [135]. Both SubAB
555 and Stx have been reported to cause cell cycle delay as well as apoptosis [94]. Whereas a cell cycle
556 delay might be beneficial for an intracellular bacterial establishment due to impaired cellular activity,
557 the induction of apoptosis probably helps to disperse bacteria within the host as a result of the release
558 of bacteria from apoptotic cells, followed by their migration to other host cells [136,137].

559 Among the bacterial effectors that lead to cell cycle arrest through their ability to induce chromatin
560 injury, there is a CDT family of toxins produced by a number of unrelated Gram-negative species.
561 After DNA damage, CDT activates the DNA repair response partly through the ATM kinase that
562 modulates the activation of cell cycle checkpoints, leading to cell type-dependent cell cycle arrest [6].
563 The pathogen-induced cell cycle arrest may alter epithelial cell turnover and thus promote bacterial
564 colonization. Additionally, bacteria-induced long-lasting DNA damage may induce genetic
565 instability and lead to the development of cancer.

566 Epigenetic modifications that may develop as a result of bacteria-induced chromatin injury are
567 the focal point of an emerging topic in the field of host-pathogen interactions. There is only limited
568 knowledge at present about microbial factors that induce epigenetic modifications that make it
569 possible to impose a pathogen transcriptional signature onto host cells [6]. Consequently, the
570 following intriguing question is raised: do bacterial toxins that induce DNA damage followed by cell
571 cycle arrest promote epigenetic alterations in host cells at the same time?

572

573 18. Concluding remarks.

574 Bacterial cyclomodulins are a growing family of microbial virulence factors that not only alter host
575 cell cycle progression, but that also interfere with typical host cell activity, including cell
576 differentiation and development that slow down cell renewal, thus favoring host colonization as well
577 as the hijacking of host cell protective functions for their own benefit. Moreover, an alteration of the
578 eukaryotic cell cycle enhances an infective efficiency of bacterial pathogens. It should also be
579 emphasized that bacteria-induced long-lasting DNA damage may induce genetic instability and lead
580 to the development of cancer. On the basis of this review, it is apparent that cyclomodulin structures
581 and the mechanisms of bacteria-induced cell cycle alterations vary depending on the type of bacteria,
582 which is illustrated here by patterns of the three-dimensional structures of different cyclomodulins.
583 Most cyclomodulins consist of proteins, but the presence of nonproteinaceous effectors highlight the

584 capacity of bacteria to develop molecules of distinct compositions, thus enhancing their fitness during
585 infection. Deciphering the molecular mechanisms by which cyclomodulins hijack the main host cell
586 functions will ultimately provide us with clues for understanding the fundamental stages of the host-
587 pathogen interaction, as well as with the tools necessary for the development of therapeutic
588 approaches during bacterial infections.

589

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2 - Justification

L'altération du cycle cellulaire de l'hôte est une stratégie utilisée par des bactéries pathogènes pour augmenter leur succès lorsque l'hôte a subi une infection (Nougayrède et al., 2005; Oswald et al., 2005). Comme il a été mentionné antérieurement, *S. aureus* alter le cycle cellulaire d'eucaryotes à travers d'un mécanisme inconnu jusqu'à ce jour. Cette altération se produit par les effecteurs bactériens qui affectent la différenciation cellulaire, faire en sorte que les cellules-hôtes se maintiennent dans une phase déterminée du cycle cellulaire et modifier l'expression de gènes régulateurs (Haugwitz et al., 2006; Heimer et al., 2010; Sugai et al., 1992). En 2013, un travail a démontré que la leucocidine Panton-Valentine (PVL) provoque un retard dans la phase G1 chez les cellules eucaryotes (Bu et al., 2013).

Notre groupe de recherche a montré un effet cytopathique de souches de *S. aureus* provenant de mammites ovines gangréneuses et sub-cliniques et la souche MRSA *S. aureus* USA400 MW2, résistante à la méthicilline. Cet effet a été caractérisé par un grossissement nucléaire des cellules épithéliales (bovines et humaines) comme celles induit par des génotoxines, incluant des tailles cellulaires anormales et des noyaux augmentés (Taieb et al., 2006). L'effet cytopathique a été associé avec un ralentissement de la prolifération cellulaire et de la diminution des nombres de mitoses. Ensuite, notre groupe a montré que *S. aureus* induit un retard de la transition de phase G2/M par l'analyse des phases du cycle cellulaire en cytométrie de flux. Les niveaux de phosphorylation de Cdk1/cdc2, indispensable pour déclencher l'entrée en mitose, sont augmentés dans les cellules exposées à *S. aureus*, ce qui indique que la plupart des cellules ont été bloquées dans la phase G2. La phosphorylation de l'histone H3 commence en prophase, avec un niveau maximal en métaphase, suivie par une diminution en télophase. L'exposition à *S. aureus* diminue le niveau de l'histone H3 phosphorylée. Ces résultats confirment que la plupart des cellules infectées sont ralenties en phase G2 (Alekseeva et al., 2013). Les données présentées par notre groupe montrent que *S. aureus* produit des effecteurs responsables pour les altérations observées dans le cycle cellulaire de la cellule-hôte.

L'analyse de la prolifération de *S. aureus* internalisé dans les cellules épithéliales montre l'augmentation de la prolifération des bactéries en phase G2. Ceci

suggère que le retard de la transition de phase G2/M peut être utilisé par *S. aureus* pour sa propagation au sein de l'hôte.

Dans la ressemblance avec les cyclomodulines produites par d'autres bactéries *S. aureus* peut produire les effecteurs moléculaires, liés au retard du cycle cellulaire chez l'hôte, lesquelles peuvent influencer le *fitness* des bactéries. Par exemple, durant l'infection, la CDT, le cyclomodulin de *E. coli*, non seulement induit l'apoptose dans des cellules du système immunologique, mais se montre aussi bénéfique à la colonisation de l'intestin après infection (Fox et al., 2004; Ohara et al., 2004). Un autre exemple est celui de la toxine ACT, qui est produite par des bactéries comme *Bordetella* sp. et *Bacillus anthracis*. La ACT est capable d'inhiber la différenciation et la prolifération de cellules T, réduisant ainsi la production de TNF α et augmentant la production de IL-10 (Rossi Paccani and Baldari, 2011; Vojtova et al., 2006). L'identification des effecteurs médiateurs de l'interaction entre *S. aureus* et l'hôte permettra la caractérisation de nouveaux mécanismes de pathogénicité de cette bactérie. Par ailleurs, l'analyse des altérations dans la cellule eucaryote est d'une importance extrême pour la bonne compréhension de l'établissement des infections causées par *S. aureus*. Ces résultats pourront contribuer au développement de stratégies pour combattre des maladies causées par cette bactérie.

2 - Justificativa

A interferência das bactérias patogênicas no ciclo celular hospedeiro é uma estratégia utilizada para aumentar o sucesso durante a infecção do organismo hospedeiro (Nougayrède et al., 2005; Oswald et al., 2005). Como mencionado anteriormente, *S. aureus* é capaz de interferir no ciclo celular eucariótico através de um mecanismo ainda não desvendado. Essa interferência ocorre pela produção de efetores bacterianos que podem alterar a diferenciação celular, causar a permanência das células hospedeiras em uma determinada fase do ciclo celular e modificar a expressão de genes regulatórios (Haugwitz et al., 2006; Heimer et al., 2010; Sugai et al., 1992). Em 2013, um trabalho demonstrou que a PVL causa o atraso na fase G1 em células eucarióticas (Bu et al., 2013).

Nosso grupo de pesquisa demonstrou um efeito citopático de linhagens de *S. aureus* proveniente de mastite de ovinos subclínicos e da linhagem MW2 de MRSA. Esse efeito é caracterizado por aumento nuclear de células epiteliais (bovinas e humanas) como aqueles induzidos por genotoxinas, em adição as células também apresentam tamanhos anormais (Taieb et al., 2006). O efeito citopático é associado com uma diminuição da proliferação celular e da diminuição de células em mitose. Em acordo com essas definições, nosso grupo de pesquisa demonstrou que *S. aureus* ocasiona um atraso na progressão do ciclo celular. Esse atraso está relacionado ao aumento da CDK1 inativada, que é um dos moduladores da fase G2/M (Alekseeva et al., 2013). Além disso, houve uma diminuição no número de células em mitose e de Histona H3 fosforilada, um marcador para condensação cromossômica, após a infecção por *S. aureus*. (Alekseeva et al., 2013). Outra análise desse trabalho demonstrou que *S. aureus* internalizada dentro das células hospedeiras apresentam aumento da proliferação na fase G2 do ciclo celular, sugerindo que o retardo causado por essa bactéria pode ser benéfico para a progeração desse patógeno no hospedeiro. Assim, os dados apresentados pelos autores apontam para o fato de que *S. aureus* produz efetores responsáveis pelas alterações observadas no ciclo celular da célula hospedeira.

Os possíveis efetores moleculares de *S. aureus* relacionados ao atraso do ciclo celular no hospedeiro podem estar associados aos mecanismos de aumento do *fitness* bacteriano, como observado para ciclomodulinas produzidas por outras bactérias. Um exemplo envolve a toxina CDT, que é produzida por várias bactérias patogênicas, como

Escherichia coli e *Shigella dysenteriae*. Durante a infecção, a CDT induz apoptose em células do sistema imunológico, além de beneficiar a colonização do intestino após a infecção (Fox et al., 2004; Ohara et al., 2004). Outro exemplo é a toxina ACT, que é produzida por bactérias como *Bordetella* sp. e *Bacillus anthracis*. A ACT é capaz de inibir a diferenciação e proliferação de células T, reduzindo a produção de TNF α e aumentando a produção de IL-10 (Rossi Paccani and Baldari, 2011; Vojtova et al., 2006).

A identificação dos efetores mediadores da interação entre *S. aureus* e o hospedeiro possibilitará a caracterização de novos mecanismos de patogenicidade dessa bactéria. Além disso, a análise das alterações na célula eucariótica é de extrema importância para o entendimento do estabelecimento das infecções causadas por *S. aureus*. Esses resultados poderão contribuir para o desenvolvimento de estratégias para o combate das enfermidades causadas por *S. aureus*.

3 - Objectifs

3.1 - Objectif général

- Déterminer les mécanismes de retard de la transition de phase G2/M dans des cellules eucaryotes infectées par *S. aureus*.

3.1.1 - Objectifs spécifiques

- Obtenir des fractions de surnageant de *S. aureus* par la chromatographie d'exclusion et tester leur capacité à induire un retard de la transition de phase G2/M chez les cellules épithéliales humaines HeLa ;
 - Identifier la composition de la fraction responsable du retard par la spectrométrie de masse ;
 - Etudier la capacité des toxines PSM α 1 et PSM α 3 à altérer la progression du cycle cellulaire et à induire un retard de la transition de phase G2/M;
 - Evaluer la capacité cytolytique de PSM α 1 et PSM α 3 ;
 - Comparer la capacité de la souche sauvage de *S. aureus* et la souche mutante LAC Δ psm α déficient en PSM α 1-4 à altérer la progression normale du cycle cellulaire et à induire le retard de la transition de phase G2/M;
 - Examiner la capacité des PSM α 1 et PSM α 3 à altérer la production des défensines en fonction de la phase du cycle cellulaire de l'hôte;
 - Analyser la capacité des souches de *S. aureus* provenant de patients avec entérocolite à altérer la progression normale du cycle cellulaire et à induire un retard de transition de phase G2/M
 - Evaluer la production d'optineurine, d'optineurine phosphorylée sur la sérine-177 et de PLK1 pendant le retard du cycle cellulaire dans des cellules eucaryotes infectées par *S. aureus*.

3 - Objetivos

3.1 - Objetivo geral

- Determinar os mecanismos responsáveis pelo atraso do ciclo celular em células eucarióticas infectadas por *S. aureus*.

3.1.1 - Objetivos específicos

- Isolar as frações do sobrenadante de *S. aureus* por cromatografia de exclusão de tamanho e testá-las quanto à capacidade de alterar a progressão do ciclo celular na fase G2/M em células HeLa;
- Identificar a composição da fração ativa de *S. aureus* responsável pelo atraso no ciclo celular através da espectrometria de massa;
- Avaliar a capacidade das toxinas PSM α 1 e PSM α 3 de atrasar a progressão do ciclo celular na fase G2/M;
- Avaliar a capacidade citolítica das PSM α 1 e PSM α 3;
- Avaliar a capacidade da linhagem de *S. aureus* *LAC* Δ *psma* de alterar a progressão normal do ciclo celular na fase G2/M;
- Comparar a participação das PSMs no atraso do ciclo celular através da infecção de células HeLa com as linhagens selvagem e mutante para o operon *psma*, deficientes das PSM α 1-4;
- Avaliar a capacidade das PSM α 1 e PSM α 3 em diminuir a produção de defensinas em função da fase do ciclo celular hospedeiro;
- Observar a capacidade de linhagens isolada de pacientes, com enterocolite, em relação à alteração da progressão normal do ciclo celular na fase G2/M;
- Avaliar a produção de Optineurina, Optineurina fosforilada na serina 177 e PLK1 durante o atraso do ciclo celular em células eucarióticas infectadas com *S. aureus*.

4 - Resultados/Résultat

**Chapitre 1/Capitulo 1 – Phenol Soluble Modulins α induisent le retard dans la phase G2/M du cycle cellulaire des cellules HeLa/
Modulinas Soluveis em Fenol α induzem atraso na fase G2/M do ciclo celular de células de HeLa**

1 - Présentation

Ce chapitre de la Thèse est dédié à l'analyse d'un groupe de molécules produites par *S. aureus* MRSA qui induisent un retard de la transition de phase G2/M chez les cellules humaines HeLa infectées. Ces cyclomodulines appartiennent au groupe des PSMs qui présentent diverses fonctions liées à la colonisation et la survie de *S. aureus*. L'identification de ces modulines a été effectuée à partir du surnageant de culture de la souche USA400 MW2 de *S. aureus*, en utilisant la technique de chromatographie d'exclusion de taille. Les fractions chromatographiques sont analysées pour leur capacité à induire un retard de transition de phase G/2M. L'identification des modulines dans les fractions induisant le retard a été effectuée par la spectrométrie de masse. Les fonctions de PSM α 1 et de PSM α 3 dans le retard du cycle cellulaire ont été confirmées par des expériences avec des cellules HeLa exposées à ces cyclomodulines synthétisées ou infectées avec une souche de *S. aureus* déficiente en PSM α 1-4.

Les β -défensines appartiennent à un groupe de peptides antimicrobiens qui inhibent l'installation d'une variété de bactéries et de champignons. Dans ce travail, des essais de PCR quantitative ont montré que le retard de la transition de phase G2/M induit par le PSM α 1 ou le PSM α 3 est associé à l'altération de la réponse immunitaire, plus précisément à la diminution de l'expression de gènes codant des défensines. La production de ces peptides est plus faible dans la phase G2/M que dans la phase G1/S du cycle cellulaire. Ces résultats confirment que le PSM- α est un facteur clé de la virulence de *S. aureus*.

L'analyse des souches de *S. aureus* provenant de patients avec entérocolite a permis de détecter le PSM- α exclusivement dans le surnageant de souches qui altèrent le cycle cellulaire, ce qui confirme la pertinence biologique de nos résultats. Néanmoins, ces souches ont présenté différentes intensités dans le retard du cycle cellulaire de l'hôte. Ce fait peut s'expliquer par la régulation différentielle de l'expression de l'opéron *psma* ainsi que par la possibilité que d'autres toxines puissent être impliquées dans la régulation du cycle cellulaire.

1 - Apresentação

Esse capítulo da Tese apresenta um grupo de moléculas produzidas por *S. aureus* MRSA que mostrou ser capaz de atrasar a progressão normal do ciclo na fase G2/M em células humanas HeLa. Essas ciclodulinas pertencem ao grupo das PSMs, as quais apresentam diversas funções relacionadas à colonização e a sobrevivência de *S. aureus*. A identificação dessas modulinas foi feita a partir do sobrenadante da cultura da linhagem MW2 de *S. aureus*, utilizando a técnica de cromatografia de exclusão de tamanho. As frações da cromatografia foram analisadas para a sua capacidade de atrasar a transição da fase G2/M do ciclo celular. A identificação das modulinas nas frações do sobrenadante foi feita através de espectrometria de massa. As funções das PSM α 1 e PSM α 3 no atraso do ciclo celular foram confirmadas após experimentos com células HeLa sincronizadas e expostas a essas modulinas ou infectadas com a linhagem de *S. aureus* mutante para o operon *psmA*.

As β -defensinas pertencem a um grupo de peptídeos antimicrobianos que inibem o estabelecimento de uma variedade de bactérias e fungos. Neste trabalho, ensaios de PCR quantitativo demonstraram que as PSMs causam a diminuição da expressão de genes das β -defensinas nas células infectadas. Além disso, a produção destes peptídeos foi menor na fase G2/M do que na fase G1/S do ciclo celular. Assim, podemos inferir que o atraso na fase G2/M, causado pelas PSM α 1 e PSM α 3, pode ser um fator fundamental para que haja diminuição da produção de β -defensinas pelo hospedeiro.

Por último, quatro linhagens isoladas de humanos com enterocolite estafilocócica também foram testadas *in vitro* quanto à capacidade do seu sobrenadante de causar o atraso do ciclo celular nas células eucarióticas. Essas linhagens apresentaram diferentes intensidades na capacidade de atrasar o ciclo celular do hospedeiro. Esse fato pode ser explicado pela provável regulação diferencial da expressão do operon *psmA* e pela possibilidade de que outras toxinas possam estar envolvidas na regulação do ciclo celular.

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Phenol-soluble modulin α induces G2/M phase transition delay in eukaryotic HeLa cells

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ABSTRACT *Staphylococcus aureus* is a gram-positive bacterium responsible for a wide range of infections. Host cell cycle alteration is a sophisticated mechanism used by pathogens to hijack the defense functions of host cells. We previously demonstrated that *S. aureus* MW2 (USA400) bacteria induced a G2/M phase transition delay in HeLa cells. We demonstrate here that this activity is triggered by culture supernatant compounds. Using size exclusion chromatography of the MW2 supernatant, followed by mass spectroscopy analysis of corresponding peaks, we identified phenol-soluble modulin α (PSM α) peptides as the likely candidates for this effect. Indeed, synthetic PSM α 1 and PSM α 3 caused a G2/M phase transition delay. The implication of PSM α in cell cycle alteration was confirmed by comparison of *S. aureus* Los Angeles County clone (LAC) wild-type with the isogenic mutant LAC Δ psm α , which lacks the psm α operon encoding PSM α 1–4. PSM α -induced G2/M transition delay correlated with a decrease in the defensin genes expression suggesting a diminution of antibacterial functions of epithelial cells. By testing the supernatant of *S. aureus* human clinical isolates, we found that the degree of G2/M phase transition delay correlated with PSM α 1 production. We show that PSMs secreted by *S. aureus* alter the host cell cycle, revealing a newly identified mechanism for fostering an infection.—Deplanche, M., Filho, R. A. E.-A., Alekseeva, L., Ladier, E., Jardin, J., Henry, G., Azevedo, V., Miyoshi, A., Beraud, L., Laurent, F., Lina, G., Vandenesch, F., Steghens, J.-P., Le Loir, Y., Otto, M., Götz, F., Berkova, N. Phenol-soluble modulin α induces G2/M phase

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Key Words: *Staphylococcus aureus* • cell cycle alteration • defensins • toxin • innate immune response

STAPHYLOCOCCUS AUREUS, a highly versatile gram-positive bacterium, can cause a multitude of diseases ranging from mild superficial skin infections to life-threatening disseminated infections such as pneumonia, osteomyelitis, meningitis, endocarditis, and sepsis (1, 2). Intermittent *S. aureus* colonization occurs in 30–50% of healthy adults (3), 10% of whom harbor *S. aureus* in the gastrointestinal tract (4).

The occurrence of antibiotic-resistant strains and the absence of an effective vaccine complicate the treatment of staphylococcal infections. To colonize and propagate within the host, *S. aureus* expresses a wide range of virulence factors, such as surface proteins, that govern adhesion to and invasion of host cells, evasion of immune responses (5), and biofilm formation (5). Other types of virulence factors, such as toxins, induce host cell lysis or elicit inflammatory responses (6, 7).

The host epithelium is in perpetual contact with numerous microorganisms, resulting in a multiplicity of the host's defense mechanisms. The integrity of the epithelial barrier is dependent on a regeneration of epithelial cells (8). Pathogens use specialized strategies to disrupt key cell functions and invade the epithelium to establish persistent colonization. Some of those strategies rely on cell cycle alteration. This cycle comprises the G1 phase characterized by cell growth, the S phase characterized by DNA

Abbreviations: BHI, brain heart infusion; CFU, colony-forming unit; DTB, double thymidine block; FCS, fetal calf serum; GAPDH, glyceraldehyde-3-phosphate dehydrogenase; hBD, human β -defensin; LDH, lactate dehydrogenase; MI, mitotic index; MOI, multiplicity of infection; NCBI, National Center for Biotechnology Information; PI, propidium iodide; PPIA, peptidylprolyl isomerase A; PSM α , phenol-soluble modulin α ; SEC, size exclusion chromatography; WT, wild-type

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replication, the G2 phase in which cells are prepared for division, the M phase during which mitosis occurs, and the G0 phase during which cells can enter a quiescent state. Bacterial toxins may interfere with the host cell cycle machinery, e.g., cytolethal distending toxin of *Escherichia coli* or *Helicobacter* species, which induces the DNA-damage signaling pathways together with alteration of the host cell cycle (9, 10)

We recently found that *S. aureus* USA400 MW2 induces a G2/M phase transition delay in epithelial cells. The delay was associated with the accumulation of inactive cyclin-dependent kinase 1 and unphosphorylated histone [³H]. We also showed that bacteria preferred the G2 phase for intracellular replication (11). However, the nature of the bacterial factor that delays cell cycle phase transition was not identified.

We show here that the cell cycle is altered by *S. aureus* compounds that were secreted into the culture supernatant. It was determined that they belonged to the cytolytic phenol-soluble modulins (PSM α) peptides. PSM α -induced G2/M-transition delay was related to the decrease of the antibacterial functions of the epithelial cells. The expression level of antibacterial peptides such as human β -defensins (hBDs), the first line of defense against staphylococcal infection (12), was lower in G2/M-phase cells compared with cells in the G1 phase. We also tested clinical human *S. aureus* isolates and found that cell cycle delay activity was associated with PSM α 1 production.

MATERIALS AND METHODS

Reagents

N-formylated PSM α 1 and PSM α 3 peptides were provided by CekoLabs (Tübingen, Germany). Lactate dehydrogenase (LDH) was quantified using the Pierce LDH Cytotoxicity Assay (ThermoFisher, Rockford, IL, USA). All other reagents were provided by Sigma-Aldrich (Saint-Quentin Fallavier, France).

Eukaryotic cells

The human cervix cancer HeLa cells (American Type Culture Collection, Manassas, VA, USA) were cultured in complete DMEM (cDMEM) [GlutaMax; Life Technologies, Carlsbad, CA, USA; 10% fetal calf serum (FCS)] supplemented with 100 U/ml penicillin and 100 μ g/ml streptomycin sulfate at 37°C with 5% CO₂. Trypsin/EDTA (Gibco, Saint Aubin, France) was used to release adherent cells for subculturing.

Staphylococcus aureus strains and culture conditions

Staphylococcus aureus clinical isolates were obtained from patients diagnosed with staphylococcal enterocolitis. The methicillin-resistant *S. aureus* USA400 MW2 strain, *S. aureus* USA300 [Los Angeles County clone (LAC) wild-type (WT)], and its isogenic mutant LAC Δ psm α , which lacks the *psm* α operon encoding PSM α 1–4 (13), were obtained from the Laboratory of Human Bacterial Pathogenesis, National Institutes of Health (Bethesda, MD, USA).

All *S. aureus* cultures were performed as follows: aliquots from overnight cultures on brain heart infusion (BHI) broth were diluted (1:50) in DMEM. The mutant (LAC Δ psm α) in which the *psm* α operon was exchanged for a spectinomycin-resistant cassette was grown in BHI containing 250 μ g/ml spectinomycin before inoculating into DMEM.

Strains were incubated at 37°C under anaerobic conditions until cultures reached an optical density of 0.6 at 600 nm, corresponding to 10⁸ colony-forming units (CFUs)/ml (14). The staphylococci were harvested by centrifugation, washed twice with PBS, and resuspended in the interaction medium (DMEM). Bacterial concentrations were estimated spectrophotometrically and confirmed by plate counts.

Preparation of bacterial supernatants

Bacteria were grown in DMEM and harvested at an OD_{600nm} of 0.6. The culture supernatants (pH adjusted to 7.4) were concentrated 10-fold in a SpeedVac (SpeedVac Concentrator SVC11 and Refrigerated Condensation Trap Savant, Thermo Fisher Scientific, Waltham, MA, USA), sterile filtered with a 0.22 μ m filter (Millipore, Billerica, MA, USA), and stored at –20°C. The total protein concentrations of the concentrated supernatants did not differ >10% between samples. Concentrated DMEM was used as a control.

Double thymidine block

The double thymidine block (DTB) was used to synchronize cells at the G1/S border (9, 11). Briefly, cells were grown in a 25 ml flask up to 30% confluence. After washing with PBS, the cells were cultivated in cDMEM containing 2 mM thymidine for 18 hours. Then, thymidine was removed by washing with PBS. The cells were cultivated in fresh cDMEM for 9 hours to release cells and then cultivated in cDMEM containing 2 mM thymidine for 17 hours.

Cell culture infection

HeLa cells (30% confluence at the beginning of DTB) were infected with *S. aureus* with a multiplicity of infections (MOIs, number of bacteria per cell at the onset of infection) of 100:1 at the periods indicated after DTB release (infection medium: DMEM). HeLa cell concentrations were determined using 1 of the 4 samples (11). The remaining samples were used for the analysis in triplicate. The low HeLa cell density at the beginning of the experiment was used to ensure cell proliferation during the entire experiment because cells cease proliferating when they reach confluence and enter a state of quiescence (15).

Bacterial concentrations were estimated spectrophotometrically and were confirmed by CFU determination. Unbound bacteria were removed 2 h after infection by washing the wells with PBS, followed by incubation in cDMEM with 3% FCS containing 20 μ g/ml of lysostaphin and 100 μ g/ml gentamicin for 2 hours, which eliminates the extracellular bacteria (16), followed by incubation in cDMEM containing 25 μ g/ml of gentamicin for the periods indicated. The incubation time was chosen in agreement with the recognized evaluation of the phases of the HeLa cell cycle and on the basis of our results (11).

Exposure of HeLa cells to the bacterial supernatants or to PSM α 1 and PSM α 3

Three hours after DTB release, cells were exposed to concentrated *S. aureus* supernatants. The concentrated DMEM was used as a control because bacteria were grown in DMEM for the preparation of bacterial supernatants. To analyze the role of PSM α , HeLa cells were exposed to *N*-formylated synthetic PSM α 1 or PSM α 3 ranging from 0.1 to 10 μ g/ml. Exposure of HeLa cells to the culture supernatants or synthetic PSM α was performed in DMEM without FCS (17).

Flow cytometry analysis

Detached cells were combined with adherent cells, which were collected by trypsin/EDTA treatment and fixed in 70% ethanol overnight. Cells were then stained with propidium iodide (PI) and analyzed with an Accuri C6 flow cytometer (Becton Dickinson, Le Pont de Claix, France) (11). Data were collected from 20,000 cells, and analysis was performed with CFlow software (Becton Dickinson).

Mitotic index evaluation

To estimate the degree of HeLa cell synchronization, mitotic indexes (MIs; the percentage of cells in mitosis from the total number of cells) were evaluated as previously described (11). Briefly, cells were grown on coverslips in 12-well plates (Nunc, NuclonTM Surface; Thermo Scientific, Langensfeld, Germany) followed by DTB synchronization. Plates were centrifuged to avoid loss of mitotic cells during washing, and the cells were then fixed with 4% paraformaldehyde/PBS for 1 hour. The coverslips were then mounted on slides with DAPI-containing ProLong antifade Vectashield medium (Vector Laboratory, Les Ulis, France). The MIs were determined from 400 cells per assay. The cells were counted with a fluorescence microscope using $\times 400$ magnification (Nikon, Tokyo, Japan). The cell cycle phases were determined at the same time by flow cytometry.

Size-exclusion chromatography

Samples concentrated 10-fold (either *S. aureus* culture supernatants or DMEM) were applied to size-exclusion chromatography (SEC) through a 200 μ l sample loop, using a Superdex 75 HR 10/30 column (GE Healthcare Bio-Sciences AB, Uppsala, Sweden). Elution with PBS buffer at a flow rate of 0.3 ml/min was performed at room temperature. The elution was monitored at 280 nm, and fractions were collected (150 μ l/tube) and tested for their capacity to modify the HeLa cell cycle. Samples that demonstrated cell cycle modulation activity were characterized by mass spectrometry.

Tandem mass spectrometry analysis of bioactive fractions

Mass spectrometry experiments were performed using a nanoRSLC Dionex U3000 system fitted to a Q Exactive mass spectrometer (Thermo Scientific, San Jose, CA, USA) equipped with a nanoelectrospray ion source as previously described (18). Briefly, a preliminary sample concentration step was performed on a μ -precolumn C18 pepMap100 (C18 column, 300 μ m internal diameter, 5 mm length, 5 μ m particle size, 100 \AA pore size; Dionex, Amsterdam, The Netherlands) followed by separation on a C18 reversed-phase column (PepMap100, 75 μ m inner diameter, 150 mm length, 3 μ m particle size, 100 \AA pore size; Dionex) using solvent A [2% (v/v) acetonitrile, 0.08% (v/v) formic acid, and 0.01% (v/v) trifluoroacetic acid in deionized water] and solvent B [95% (v/v) acetonitrile, 0.08% (v/v) formic acid and 0.01% (v/v) trifluoroacetic acid in deionized water]. Peptides were separated using a gradient of 4–20% solvent B for 15 min and 20–50% solvent B for 10 minutes at a flow rate of 0.3 μ l/min. Electrospray ionization of eluted peptides occurred at a voltage of 2 kV, and spectra were recorded in positive mode. A data-dependent acquisition mode was used to fragment the 10 most intense ions in the MS spectrum, and the instrument was externally calibrated according to the supplier's instructions.

Peptides were identified from MS/MS spectra using X! Tandem (TANDEM) pipeline software (Plateforme d'Analyse

Protéomique de Paris Sud-Ouest, Institut National de la Recherche Agronomique INRA, Jouy-en-Josas, France, <http://pappso.inra.fr>). To perform a database search with nonspecific cleavage rules, a small in-house database composed of 485 proteins from www.uniprot.org was used. Phosphorylation of serine and *N*-formylation of methionine was selected as variable modifications, and a minimum score corresponding to an *e*-value < 0.05 was considered a prerequisite for valid peptide identification.

Detection of PSM α in the culture supernatants

Culture supernatants of the human isolates were concentrated 10-fold, precipitated by cold methanol [dilution: 1/5 (v/v)], and left for 10 minutes on ice. After centrifugation, the supernatants were analyzed by reversed-phase HPLC mass spectrometry on an Agilent 1100 Series HPLC (Agilent Technologies, Santa Clara, CA, USA) using the C12 Uptisphere Strategy 100 \AA , 2.2 μ m (50 mm) column (Interchim, Montluçon Cedex, France).

A gradient from 42% to 90% of acetonitrile for 13 minutes in the presence of 0.1% formic acid was used. Conditions for PSM α detection were as follows.

The injection volume was 100 μ l with a flow rate of 0.220 ml/min at 45°C. Peptide masses were calculated from multiply charged ions obtained by the coupled electrospray ionization-equipped LC/MSD Trap SL mass spectrometer using Agilent LC/MSD 5.2 software. The PSM α peptide standard curve of the respective synthetic PSM α peptide was obtained for concentrations ranging from 0.1 to 40 μ g/ml. The quantity of PSM α in the sample was proportional to the surface of the specific peak corresponding to the PSM α on a chromatogram. This allowed the detection of PSM α peptides at concentrations as low as 0.1 μ g/ml.

Analysis of cytotoxic effects of PSM α

HeLa cells were grown to 80% confluence in 96-well culture plates. The cells then were exposed to PSM α for 24 hours in DMEM. PSM α concentration ranged from 0.1 to 10 μ g/ml. The cytotoxic activity of PSM α was estimated by release of LDH (Pierce LDH Cytotoxicity Assay Kit; Pierce, Rockford, IL, USA), a reliable biochemical indicator of cell injury (19). For controls, untreated cells were included for measurements of spontaneous LDH release and maximum LDH release, which was induced by lysis reagent. Background control was performed by measuring the LDH activity in DMEM medium at $A_{492 \text{ nm}}$.

Cytotoxicity levels were calculated as follows: LDH release of treated cells (%) = (LDH PSM-treated cells – LDH control untreated cells) / (maximum LDH release – LDH control untreated cells) $\times 100$.

Lysostaphin protection assay

A protection assay was performed as previously described (20) with the following modifications. HeLa cells were grown in 12-well plates overnight, confluent monolayers were infected with LAC WT strain or its isogenic mutant (LAC Δ psm α) at a MOI of 100:1 in DMEM medium. Infection of host cells was synchronized by centrifugation (500 g, 10 minutes). Cells were then incubated for 50 minutes. Afterward, cells were incubated for 1 hour in DMEM supplemented with 20 μ g/ml of lysostaphin and 100 μ g/ml of gentamicin. At the end of incubation (T2) and 6 h after infection (T6), cells were collected after trypsinization. Cell concentrations were determined by a Trypan blue exclusion assay from 1 sample. The remaining samples were used for the CFU analysis in triplicate: cells were lysed with 0.05% Triton X-100 in PBS, and CFUs were determined after overnight incubation on BHI agar.

Defensin expression analysis

Exposure of the synchronized HeLa cells to PSM α was performed as described above. Expression of hBD-1,-3, and -9 in PSM α -treated cells was evaluated by RT-qPCR. Total RNA was isolated with Qiazol Reagent (#79306; Qiagen, Germantown, MD, USA). The RNA concentration was measured by spectroscopy (NanoDrop, Wilmington, DE, USA), and cDNA was synthesized from 1 μ g of RNA, using a qScript cDNA Synthesis kit (Quanta Biosciences, Gaithersburg, MD, USA). Reactions devoid of reverse transcriptase and reactions containing H₂O instead of cDNA were used as a negative control.

Primers for hBD-1,-3, and -9 were designed according to the sequences available at the National Center for Biotechnology Information (NCBI; <http://www.ncbi.nlm.nih.gov/>). To avoid genomic DNA amplification, primer sequences were designed to cover ≥ 2 subsequent exons of hBD-1,-3, and -9 (NCBI accession #NM_005218.3, NM_018661, and NM_001037380, respectively; Supplemental Table S1). Glyceraldehyde-3-phosphate dehydrogenase (GAPDH, NCBI accession #NM2046.3) and peptidylprolyl isomerase A (PPIA; NCBI accession #NM_001300981) were used as housekeeping genes. Relative quantification relates the PCR signal of the target transcript in a treatment group to that of an untreated control. Each 13 μ l PCR mixture included 2 μ l of cDNA, 200 nM of primers, 12.5 μ l of IQ Sybr Green supermix (2 \times) (Bio-Rad, Marne la Coquette, France), and H₂O. Amplification was carried out on a CFX96 Real Time System (Bio-Rad) for 3 minutes at 95°C and 40 cycles of 2 steps consisting of 5 seconds at 95°C and 30 seconds at 60°C. The relative quantification of the mRNA levels of the target genes was determined using CFX Manager based on the Δ CT method (21). The amount of target was normalized to PPIA and GAPDH genes: Δ CT = CT (target gene) – CT (housekeeping gene), where CT represents the cycle number required to reach a defined threshold target abundance. The relative mRNA level was calculated as $x\Delta$ CT (x = primer efficiency).

Statistical analysis

At least 4 different assays were performed per experiment. The differences among the groups were assessed by ANOVA. $P < 0.05$ was considered significant. Tukey's honestly significant difference test was applied for comparison of means between groups. The values are expressed as mean \pm SD.

RESULTS

Evaluation of the degree of HeLa cells synchronization

The MI of the asynchronous cells was $2.8 \pm 0.5\%$ (Table 1). There was a variation in MIs during progression through the cell cycle (Table 1). The duration of the cell cycle phases was in agreement with the recognized evaluation of the phases of the HeLa cell cycle (10, 8, 3, and 1 hour, for G1, S, G2, and M, respectively). Three hours after DTB release, the MI of the majority of cells in the S phase was $0.9 \pm 0.5\%$. Eleven hours after DTB release, when the majority of cells were in G2/M phases ($>85\%$), the MI increased to $19.5 \pm 2.4\%$. The MI decreased to $1.9 \pm 1.1\%$ at 24 hours after DTB release with the majority cells in the G1 phase ($55 \pm 3.8\%$). Finally, we observed a faint increase of the MI (up to $4.7 \pm 1.1\%$) 33 hours after DTB release, when $25 \pm 2.3\%$ of the cells were in G2/M phases. Our findings were in the agreement with the observation of others (22). The degree of the synchronization was sufficient for the analysis of the impact of *S. aureus* on host cell cycle.

Staphylococcus aureus culture supernatants modulate host cell cycle similarly to *S. aureus* bacteria

We previously showed that cells of strain *S. aureus* MW2 induced a G2/M phase transition delay. To verify whether the cells secreted the active molecules, concentrated MW2 culture supernatant was compared with concentrated DMEM for its capacity to effect the cell cycle. Exposure time was similar to the period of bacterial infection (11). After 25 hours of exposure of synchronized HeLa cells to concentrated MW2 supernatants, the percentage of cells in the G2/M phase was higher ($57 \pm 4\%$) compared with untreated cells ($33 \pm 4\%$), whereas only $40 \pm 4\%$ of HeLa cells exposed to concentrated DMEM were in the G2/M phase. This proportion was stable because the number of cells exposed to concentrated MW2 supernatants in the G2/M phase was still higher ($54 \pm 3\%$) after 3 h compared with untreated cells ($25 \pm 6\%$), where $27 \pm 7\%$ of the cells were exposed to concentrated DMEM (Fig. 1). This result

TABLE 1. Evaluation of the degree of HeLa cells synchronization

Experimental conditions	G1%	S%	G2/M%	Mitotic index%
Asynchronous cells	69 ± 6.4	17 ± 4.5	14 ± 3.8	2.8 ± 0.5
Synchronous cells				
Time after DTB release (h)				
3	2.6 ± 1.3	95.4 ± 3.9	2.0 ± 1.6	0.9 ± 0.5
6	6 ± 1.8	76 ± 7.2	18 ± 3.9	2.0 ± 1.4
9	10 ± 3.8	30 ± 4.5	40 ± 3.9	13.3 ± 2.1
11	7 ± 3.7	8 ± 3.5	85.3 ± 7.1	19.5 ± 2.4
13	45 ± 4.7	15 ± 1.8	40 ± 4.8	9.4 ± 2.3
16	75 ± 6.2	5 ± 1.4	20 ± 2.3	2.4 ± 1.6
18	55 ± 4.3	25 ± 2.8	20 ± 2.3	2.2 ± 1.6
21	50 ± 5.1	35 ± 3.4	15 ± 1.8	2.0 ± 1.4
24	55 ± 3.8	30 ± 2.8	15 ± 1.6	1.9 ± 1.1
27	55 ± 4.6	35 ± 4.1	10 ± 1.6	1.7 ± 0.9
30	57 ± 5.3	28 ± 2.6	20 ± 2.5	3.3 ± 1.6
33	50 ± 4.4	25 ± 2.9	25 ± 2.3	4.7 ± 1.1
36	65 ± 5.2	20 ± 1.8	15 ± 1.5	1.8 ± 1.2

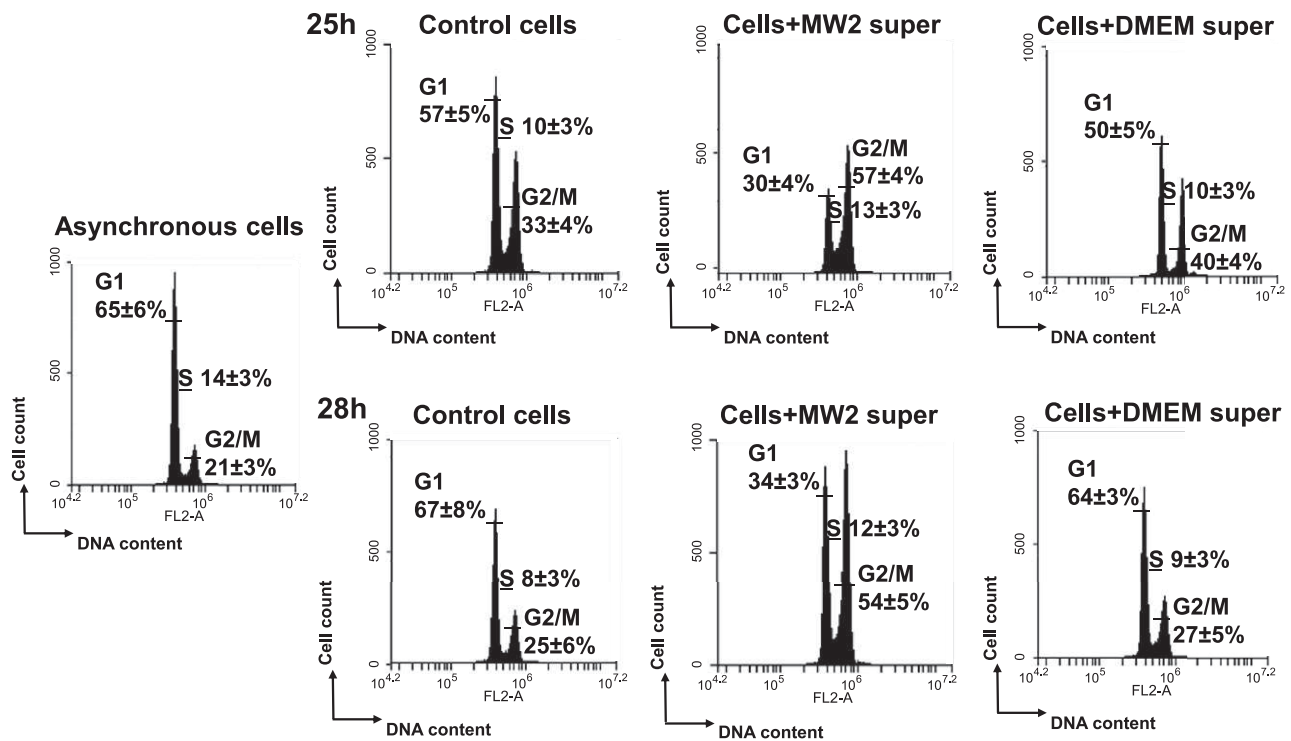


Figure 1. Culture supernatants of *S. aureus* MW2 strain induced G2/M transition delay. Synchronized HeLa cells were exposed either to MW2 supernatants or DMEM concentrated 10-fold. After 25 and 28 h of incubation, detached and adherent cells were fixed in 70% ethanol overnight, stained with PI, and analyzed by flow cytometry. Data were collected from 20,000 cells, and analysis was performed with CFlow software. The average percentage of cell cycle phase \pm SD is indicated. The values of the 1 representative assay of the 4 is shown. Exposure of the cells to MW2 supernatant induced a G2/M phase transition delay in a time-dependent manner. * $P < 0.05$.

suggests that the MW2 culture supernatant contains substances that interfere with the induction of G2/M transition.

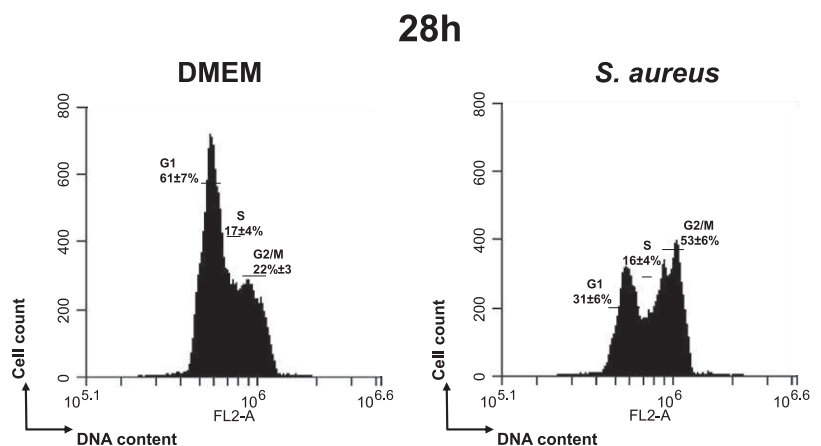
Detection of PSM α peptide derivatives in *S. aureus* supernatant chromatography fractions, which induce the G2/M phase transition delay

Concentrated MW2 culture supernatants and concentrated DMEM were fractionated using SEC. The fractions were pooled (2–4 fractions) and tested for their activities 28 hours post-treatment. The activities of *S. aureus* culture

supernatant fractions were compared with those of DMEM for their capacity to alter the HeLa cell cycle. The pool of culture supernatants containing fractions 23–26 induced the strongest G2/M delay ($53 \pm 8\%$ of cells), in contrast to the corresponding pool of DMEM fractions ($22 \pm 5\%$ of cells; $P < 0.05$). The majority of the cells exposed to that fraction pool of the MW2 supernatant were still in the G2/M phase, whereas the cells exposed to the corresponding pool of DMEM fractions had completed the G2/M phase and progressed to the G1 phase (Fig. 2).

We then analyzed fractions 23–26 by tandem mass spectrometry analysis. A number of molecules were

Figure 2. The pool of chromatography fractions 23–26 of MW2 supernatant induces G2/M transition delay. Synchronized HeLa cells were exposed to 100 μ l of size-exclusion chromatography fractions of concentrated MW2 supernatants or concentrated DMEM. After 25 hours, HeLa cells were analyzed by flow cytometry. The average percentage of HeLa cell cycle phase \pm SD is indicated on the histograms.



detected in the active fractions compared with the concentrated DMEM control, including several peptides derived from those of the PSM α peptide family. The majority of these peptides were derivatives of PSM α 1 or PSM α 3. However, PSM α 2 and PSM α 4 derivatives were also identified (Table 2). Based on these results, we considered PSM α 1 and PSM α 3 as the most likely candidates responsible for the G2/M transition delay.

PSM α 1 and PSM α 3 alter HeLa cell cycles to a different extent

Synthetic PSM α 1 and PSM α 3 at concentrations ranging from 0.1 to 10 μ g/ml were tested for their capacity to affect the cell cycle. Cytofluorometric analysis 25 hours after treatment revealed that PSM α 1 and PSM α 3 slowed down HeLa cell cycle progression. The untreated control cells completed the first cycle and progressed to the G2/M phase of the second cycle, whereas PSM α 1- and PSM α 3-treated (at concentrations of 0.1 and 1 μ g/ml) cells progressed to the G1 phase of the first cell cycle (Table 3). This resulted in a lower percentage of PSM-treated cells in the G2/M phase compared with the untreated control. Increasing the concentration of PSM α 1 to 10 μ g/ml resulted in an even stronger effect: the cell cycle progression was greatly slowed down and the majority of the treated cells were in the G2/M phase of the first cycle. The percentage of host cells in the G2/M phase was twice as high as untreated cells (53 ± 6.6 vs. $18 \pm 4.1\%$). PSM α 3 showed lower activity compared with PSM α 1 under the same conditions.

Nevertheless, with PSM α 3, the percentage of host cells in the G2/M phase was higher than that of untreated cells (31 ± 4.1 vs. $18 \pm 4.1\%$).

PSM α 1-induced G2/M transition delay is not related to the cytotoxic effect

To verify whether the observed G2/M phase transition delay was related to the cytotoxic effect of PSM treatment, the release of LDH was determined. LDH release from the

HeLa cells treated with PSM α 1 at concentrations of 0.1, 1, and 10 μ g/ml or PSM α 3 at concentrations of 0.1 and 1 μ g/ml did not reveal a significant increase of LDH release. We observed a small increase in LDH release by cells exposed to 10 μ g/ml of PSM α 3 (Supplemental Fig. S1).

Isogenic USA300 mutant lacking the PSM α operon does not induce G2/M phase transition delay

To further confirm the implication of PSM α in G2/M phase transition delay, the LAC WT strain and its isogenic mutant LAC Δ psm α were compared for their capacity to alter the host cell cycle. The exposure of synchronized HeLa cells to increasing numbers of LAC WT cells (MOI ranging from 10:1 to 50:1) resulted in an increase in the percentage of the G2/M phase, whereas there were no effects on cell cycle phases between the isogenic mutant LAC Δ psm α and the control cells (Table 4).

Internalized LAC WT replicated inside HeLa cells in contrast to its isogenic LAC Δ psm α mutant

We previously showed (11) that the G2 phase is a prerequisite for the intracellular proliferation of internalized *S. aureus*. There was no difference between the CFU number of LAC WT and LAC Δ psm α strains 2 hours after infection, indicating the equal number of internalized bacteria of both strains (Fig. 3). Given that PSM α peptides induce G2/M phase transition delay, we investigated whether they are responsible for the proliferation of internalized *S. aureus* inside of HeLa cells. Indeed, the CFUs of internalized LAC WT cells 6 hours after infection was 10-fold higher compared with LAC Δ psm α . The CFU number 6 hours after infection was 6-fold higher than the value 2 hours after infection for LAC WT ($P < 0.05$), which is caused by its replication over time. In contrast, there was no statistically significant difference between the CFU number 2 and 6 hours after infection with LAC Δ psm α , indicating that PSM α peptides not only induced cell cycle phase alteration, but also are responsible for bacterial intracellular proliferation.

TABLE 2. Detection of PSM α peptide derivatives in *S. aureus* chromatography fractions

Uniprot entry	Gene name	Description	Peptide sequence	X! Tandem e-value	SEC Fractions identification
PSMA1_STAAW	psmA1	Phenol-soluble modulin α 1	GIKVIKS	1,7E-3	23
PSMA1_STAAW	psmA1	Phenol-soluble modulin α 1	IIAGIHKV	1,2E-2	23, 24
PSMA1_STAAW	psmA1	Phenol-soluble modulin α 1	IHKVIKS	1,8E-2	23, 24
PSMA1_STAAW	psmA1	Phenol-soluble modulin α 1	LIEQFTGK	1,1E-2	23, 24, 25
PSMA1_STAAW	psmA1	Phenol-soluble modulin α 1	IIAGIHKVIKS	3,2E-5	23, 25
PSMA3_STAAW	psmA3	Phenol-soluble modulin α 3	FKDLLGKF	8,8E-4	23, 24, 25
PSMA3_STAAW	psmA3	Phenol-soluble modulin α 3	AKLKF	4,7E-2	24, 25, 26
PSMA3_STAAW	psmA3	Phenol-soluble modulin α 3	FFKDLLGK	3,6E-2	25
PSMA3_STAAW	psmA3	Phenol-soluble modulin α 3	FVAKLF	3,6E-2	25
PSMA3_STAAW	psmA3	Phenol-soluble modulin α 3	FVAKLKF	4,7E-3	26
PSMA2_STAAW	psmA2	Phenol-soluble modulin α 2	GIKFIK	4,6E-2	23
PSMA2_STAAW	psmA2	Phenol-soluble modulin α 2	IIAGIHKF	1,1E-3	23
PSMA2_STAAW	psmA2	Phenol-soluble modulin α 2	IHKFIKGL	5,1E-3	23
PSMA4_STAAW	psmA4	Phenol-soluble modulin α 4	IIDIFAK	4,2E-4	23, 24, 26
PSMA4_STAAW	psmA4	Phenol-soluble modulin α 4	IDIFAK	8,2E-3	24

TABLE 3. *PSMα1* and *PSMα3* induces the G2/M phase transition delay

Experimental conditions	G1%	S%	G2/M%
Control ^a	53 ± 7.9 ^a	29 ± 5.8 ^a	18 ± 4.1 ^a
Cells + 0.1 μg/ml <i>PSMα1</i>	85 ± 9.8	9 ± 5.2	6 ± 3.8*
Cells + 1 μg/ml <i>PSMα1</i>	81 ± 9.7	9 ± 4.9	10 ± 4.9*
Cells + 10 μg/ml <i>PSMα1</i>	30 ± 5.6	17 ± 5.6	53 ± 6.6*
Cells + 0.1 μg/ml <i>PSMα3</i>	81 ± 8.6	14 ± 4.8	5 ± 4.1*
Cells + 1 μg/ml <i>PSMα3</i>	79 ± 7.9	12 ± 4.2	9 ± 4.8*
Cells + 10 μg/ml <i>PSMα3</i>	51 ± 6.8	18 ± 4.8	31 ± 4.1*

^aCells complete the first cell cycle and progress within the second cell cycle. **P* < 0.05 vs. control.

Level of *PSMα*-induced expression of hBDs depends on the cell cycle phase

Quantification of hBD-1, -3, and -9 expression was analyzed at the time when the majority of the synchronized cells were either in the G1 or in the G2/M phases. The expression of hBD-1 was constant at all times of the exposure of the cells either to *PSMα1* or *PSMα3* (Figs. 4 and 5). The expression of hBD-3 and -9 was 3.2 ± 0.5- and 10 ± 0.7-fold higher, respectively, by the cells in G1 phase (87 ± 7%) compared with the cells in the G2/M phase (69 ± 6%; Fig. 4).

We observed a faint decrease (1.7 ± 0.5-fold) of hBD-3 expression during *PSMα3* treatment in the culture with 48 ± 6% of cells in the G2/M phase compared with those with the cells mainly in the G1 phase (70 ± 7%). There was no difference in hBD-9 expression, when the majority of the cells was either in the G1 or in the G2/M phase (Fig. 5).

Staphylococcus aureus clinical isolates are heterogeneous with respect to cell cycle delay activity

Thus far, we investigated the effect of *S. aureus* on cell cycles with clinical isolates that have been passaged extensively in laboratories. To understand whether the induction of cell cycle alteration is a common feature of *S. aureus* or is limited to certain strains, we tested the effect produced by freshly isolated clinical isolates.

The clinical isolate A980866 strongly delayed G2/M cell cycle transition, similar to MW2. Among HeLa cells that were exposed to concentrated A980866 supernatant for 20 hours, 86 ± 10.2% of cells were in the G2/M

TABLE 4. *LACΔpsmα* mutant does not induce the G2/M phase transition delay

Experimental conditions	G1%	S%	G2/M%
Control ^a	65 ± 10.2 ^a	19 ± 5.1 ^a	16 ± 4.3 ^a
Cells+ LAC WT MOI 10:1	49 ± 9.4	20 ± 4.9	31 ± 5.1*
Cells+ LAC WT MOI 25:1	39 ± 7.1	25 ± 5.4	36 ± 5.3*
Cells+ LAC WT MOI 50:1	36 ± 6.4	24 ± 4.3	40 ± 6.1*
Cells+ <i>LACΔpsm</i> MOI 10:1	66 ± 8.3	15 ± 3.9	19 ± 3.9
Cells+ <i>LACΔpsm</i> MOI 25:1	66 ± 7.9	14 ± 4.6	20 ± 4.3
Cells+ <i>LACΔpsm</i> MOI 50:1	62 ± 8.2	15 ± 3.7	23 ± 4.2

^aCells complete the first cell cycle and progress within the second cell cycle. **P* < 0.05 vs. control.

MOI 100:1

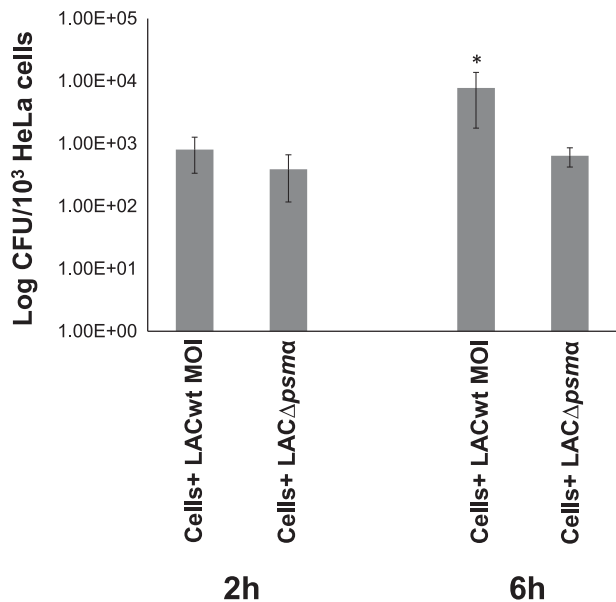


Figure 3. Replication of LAC WT strain internalized into HeLa cells. HeLa cells were infected with either LAC WT or its isogenic mutant *LACΔpsmα* at a multiplicity of infection of 100:1 for 1 hour in DMEM medium. HeLa cells were then incubated for 1 hour in DMEM supplemented with gentamicin. At T2 and T6 after infection, cells were lysed, and CFUs were determined. LAC WT strain replicates intracellularly because the CFU number of the LAC WT strain was increased 6 hours after infection. **P* < 0.05. The data are presented as the logarithm of CFU/10³ cells ± sd.

phase compared with 54 ± 8.3% in untreated cells and 10.4 ± 4.1% in cells exposed to concentrated DMEM (data not shown), whereas after 25 hours, 61 ± 8.1% of cells were in the G2/M phase compared with 29 ± 6.3% in untreated cells and 16 ± 5.4% in the culture exposed to concentrated DMEM (Table 5).

HeLa cell cycle phases were ~10, 8, 3, and 1 hour, for G1, S, G2, and M phases, respectively. Because synchronized HeLa cells were exposed to A980866 supernatant 3 hours after the second DTB release, synchronizing cells in the G1/S border, the results suggest that HeLa cells did not complete the cell cycle but were delayed during the G2/M phase transition (Table 5).

In HeLa cells exposed to concentrated supernatant of another clinical strain, strain DLY19990610, for 20 hours, 63 ± 6.4% of cells were in the G1 phase and only 17 ± 3.1% were in the G2/M phase (data not shown), whereas 25 hours after infection, 55 ± 7.8% of HeLa cells were in G2/M compared with 29 ± 6.3% in untreated cells vs. 16 ± 5.4% in the culture exposed to concentrated DMEM. This suggests that HeLa cells exposed to DLY19990610 supernatant for 25 hours achieved a complete cycle and progressed to the G2/M phase of the second cell cycle.

The exposure of cells to the A970 271 or LY 200 000 49 supernatants did not induce the difference in cell cycle phase distribution (Table 5).

Supernatants of 4 clinical isolates were analyzed by reversed-phase HPLC coupled to tandem mass

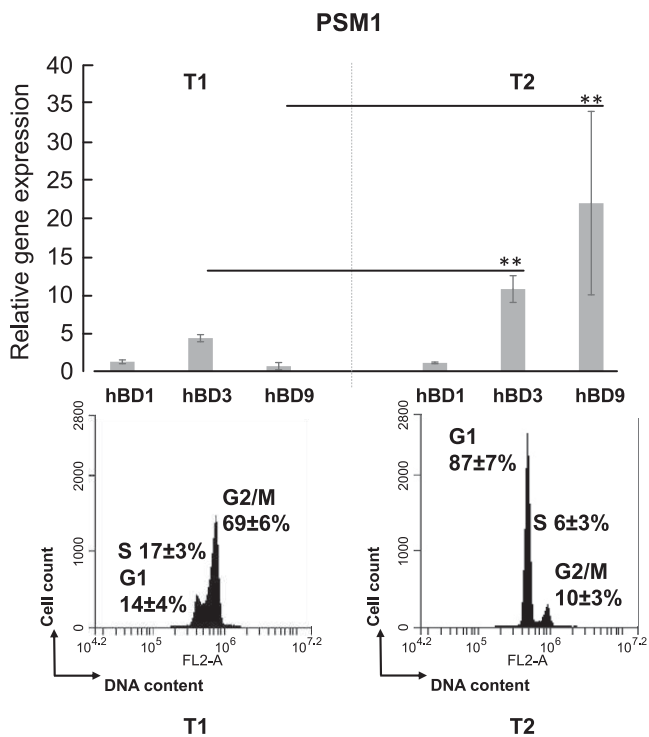


Figure 4. Analysis of mRNA levels of HBD-1, -3, and -9 in PSM α 1-treated cells. Synchronized HeLa cells were treated with 1 μ g/ml of PSM α 1 and analyzed when the cells were mainly either in the G1 or G2/M phases. Defensin mRNA level was measured by RT-qPCR. Expression of all genes was normalized to the expression of housekeeping genes GAPDH and PPIA. * $P < 0.01$.

spectroscopy. The results of analysis showed that a small amount of PSM α 1 was only detectable in A980866 supernatant that delay G2/M transition, whereas neither PSM α 1 nor PSM α 3 were detected in 3 other supernatants.

DISCUSSION

Pathogens have developed a large arsenal of strategies to subvert the host cell, such as the modulation of host cell apoptosis (23), the promotion of cell proliferation, and the inhibition of cell growth (24). Bacteria also have many virulence mechanisms that target the host cell cycle (9). Very few studies have described the capacity of *S. aureus* to modulate host cell cycle progression. It was shown that epidermal cell differentiation inhibitor affects keratinocyte differentiation (25) and that α -toxin increases the duration of S+G2/M phases (26).

We previously showed that *S. aureus* bacteria induce G2/M transition delay (11).

We demonstrated here that the capacity of *S. aureus* to alter the host cell cycle is caused by secreted products. SEC of *S. aureus* MW2 culture supernatant followed by mass spectrometry analysis of the active fractions revealed that the majority of the sequences of identified compounds in the biologically active fractions matched the sequence of peptides derived from PSM α 1 and PSM α 3. Consequently, we examined PSM α peptides as potential candidates to alter the host cell cycle.

PSMs have recently emerged as a novel toxin family that contributes to increased virulence and the spread and severity of *S. aureus* infection (27). PSMs stimulate inflammatory responses, are involved in biofilm development, and cause lysis of red and white blood cells (13). PSMs are divided into 2 groups, depending on their size: the short (20–25 amino acids) α -type peptides (PSM α 1–PSM α 4 and δ -toxin) and the long (44 amino acids) β -type peptides (PSM β 1 and PSM β 2). The PSM α peptide genes are grouped in 1 operon and apparently arose from gene duplication events (27). The production of PSMs is controlled by the accessory gene regulator (*agr*), a quorum sensing system that tightly controls virulence expression in *S. aureus* (6).

N-formylated synthetic PSM α 1 and PSM α 3 were tested for their capacity to alter the host cell cycle. With respect to cell cycle delay, PSM α 1 was more active than PSM α 3. This result was unexpected because PSM α 3 has been reported to exert the strongest lytic activity against eukaryotic cells (6, 28) by far.

The decreased effect on G2/M transition delay of the internalized USA300 Δ *psm* α mutant lacking the *psm* α operon further confirmed the involvement of PSM α on host cell cycle alteration. Our results suggest that PSM α act as a bacterial cyclomodulin, which would be a new property of PSMs. Cyclomodulins are a growing family of toxins that hijack the eukaryotic cell cycle (29). The role of cyclomodulins in bacterial pathogenicity is not well established thus far. However, the high prevalence of mucosa-

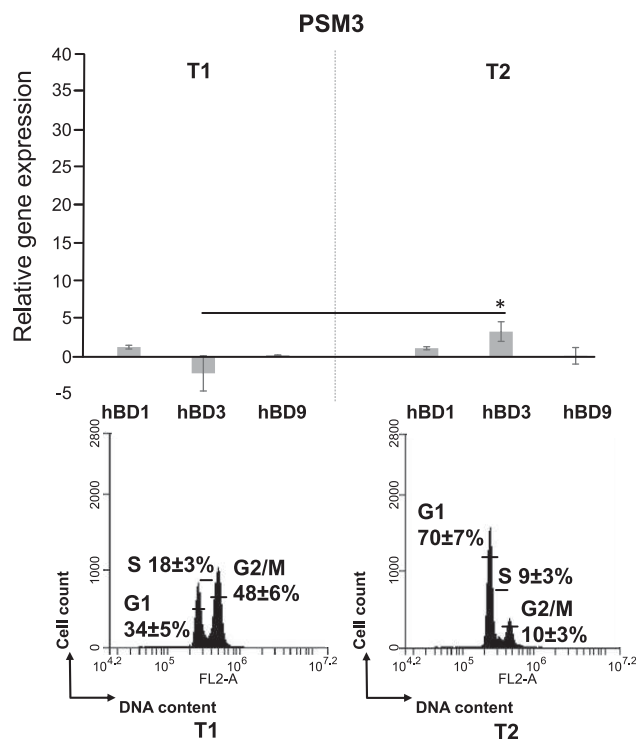


Figure 5. Analysis of mRNA levels of HBD-1, -3, and -9 in synchronized PSM α 3-treated cells. Synchronized HeLa cells were treated with 1 μ g/ml of PSM α 3 and analyzed when the majority of the cells were either in the G1 or G2/M phases. Defensin mRNA level was measured by RT-qPCR. Gene expression was normalized to the expression of housekeeping genes GAPDH and PPIA. ** $P < 0.05$.

TABLE 5. Cell cycle distribution of HeLa cells exposed to *S. aureus* clinical isolates

Experimental conditions	G1%	S%	G2/M%
Control ^a	52 ± 7.5 ^a	19 ± 3.8 ^a	29 ± 6.3 ^a
DMEM	68 ± 8.1	16 ± 3.6	16 ± 5.4
Cells + A980866 strain	32 ± 6.2	7 ± 3.5	61 ± 8.1*
Cells + DLY19990610 strain ^a	29 ± 5.2 ^a	16 ± 4.5 ^a	55 ± 7.8 ^{a,*}
Cells + A970271 strain	75 ± 7.4	12 ± 4.3	13 ± 4.6
Cells + LY20000049 strain	63 ± 6.6	19 ± 4.0	18 ± 3.9

^aCells complete the first cell cycle and progress within the second cell cycle. **P* < 0.05 vs. DMEM.

associated *E. coli* that produce cyclomodulin and genotoxin in colon cancer has been described (30).

To verify the capacity to alter the host cell cycle by freshly isolated *S. aureus* strains and to determine whether G2/M transition delay is a common feature of *S. aureus* strains, we tested 4 human clinical isolates. A delayed G2/M phase transition was shown in only 1 clinical isolate and was correlated with the detection of PSM α 1 in its supernatant. This result corroborated our findings with LAC WT and LAC Δ psm α mutant strains.

It is worth mentioning that synthetic PSM α 1 induced G2/M transition delay in HeLa cells at a concentration of 0.1 μ g/ml, but a stronger effect was observed at a concentration of 10 μ g/ml, suggesting a dose-dependent effect. PSM α 1 concentration in the supernatant of the *S. aureus* clinical isolate was <0.1 μ g/ml, although it induced a clear G2/M delay. At this level, we cannot exclude the presence of other molecules in bacterial supernatants that influence the host cell cycle, as has been shown with the multiplicity of *E. coli* cyclomodulins (29).

Some pathogens arrest the cells in a cell cycle phase, which is advantageous for their own life cycle. For example, it has been reported that the respiratory syncytial virus induces G1/G0 phase arrest of the airway cells and that this phase was favorable for virus production (31). Analysis of the proliferation of LAC WT and its isogenic mutant LAC Δ psm α showed higher proliferation rates of internalized LAC WT. These results are in accordance with findings that demonstrated intracellular replication of USA300 LAC WT within human embryonic kidney 293 cells, in contrast to its LAC Δ psm α mutant (20). On the one hand, PSM α causes a delay of the host cell cycle, which appears to be crucial for intracellular proliferation, and, on the other, PSM α plays a key role in intracellular replication. Consequently, the role of PSM α as a virulence factor is strengthened.

Upregulation of hBD-3 (32, 33) and downregulation of hBD-9 (32) were found in the *S. aureus*-infected skin (33) and in the ocular surface of the patients with bacterial keratitis (32). The hBD-3 expression was increased during the first day of the infection and then was constant within the following 2 days, as was observed in the human *S. aureus*-infected epidermal keratinocytes (12).

We show here that PSM α 1 strongly induces hBD-3 and -9 expression in the G1 phase cells compared to the G2/M phase cells. In contrast, only faint difference in hBD-3 expression were observed between cells in the G1 phase and

the G2/M phase in PSM α 3-treated cells. This discrepancy between PSM α 1 and PSM α 3 treatments may be explained by the lower capacity of PSM α 3 to induce the G2/M transition delay.

Given that hBDs expression was lower, when the cells were in the G2/M phase, it is likely that PSM α induces G2/M phase delay deteriorates antibacterial state of the epithelial surface and impairs the innate immune response during *S. aureus* infection.

In addition to the antimicrobial effect of hBD-3 within the innate immune system, it was shown that hBD3 is chemotactic for a broad spectrum of leukocytes, such as immature dendritic cells and T cells, monocytes, macrophages, and neutrophils, which are indispensable at the site of the infection (34). Thus, our data suggest that PSM α -induced host cell cycle alteration, which results in decreased hBD-3 expression, may impair an adaptive immune response during *S. aureus* infection.

In conclusion, our study shows that particularly PSM α 1 interferes in various ways with host cell cycle and host cell response. PSM α 1 induces G2/M phase transition delay, which prolongs the period of low hBD3 and -9 expression. Moreover, PSM α 1 promotes intracellular proliferation of *S. aureus* in HeLa cells. This is a novel mechanistic strategy of how *S. aureus* is able to subvert cell cycle progression accompanied by decreased immune response. **[F]**

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Phenol-soluble modulin α induces G2/M phase transition delay in eukaryotic HeLa cells

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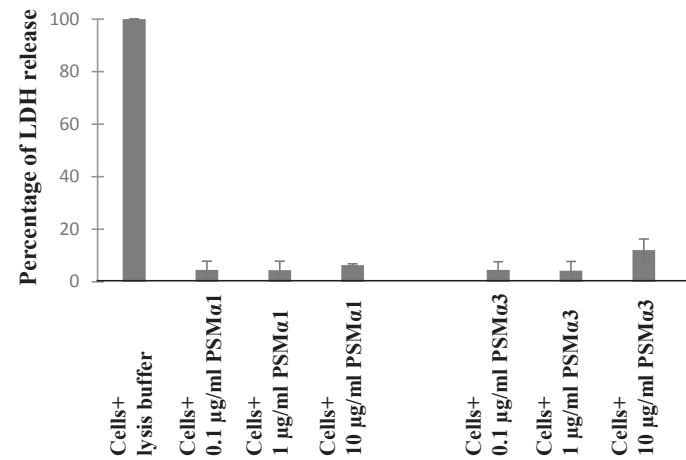
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Supplementary Figure S1. Estimation of LDH release by PSM-treated HeLa cells. HeLa cells were exposed to either PSMα1 or PSMα3 ranging from 0.1 to 10 μg/ml. After 24h of incubation, LDH release was measured using a Cytotoxicity Detection Kit. Results are shown as the difference of the mean value of LDH release of treated cells and untreated cells divided by the difference of the mean value of the maximum release by lysed cells and untreated cells. The error bar presents ±SD from three independent experiments performed in triplicate.

Supplementary Figure 1

Supplementary Table 1S. Primer sequences for RT-PCR

Primer sequences were designed to cover at least two subsequent exons in order to avoid genomic DNA amplification. GAPDH and PPIA were used as housekeeping genes. The relative quantification of the mRNA levels of the target genes was determined using CFX Manager based on the deltaCT –method.

Primers	Sequences
hBD1f	5'-CAGGTGGTAACTTTCTCACAGG-3'
hBD1r	5'-AATAGAGACATTGCCCTCCACT-3'
hBD3f	5'- TCCATAGGGAGCTCTGCCTTACCATT- 3'
hBD3r	5'- GACTGGATGAAAAGGTGTGCTTGGTC- 3'
hBD9f	5'-ATCCCCAGTAAGAGGTGGTTTG-3'
hBD9r	5'-GCAGGCACCAATTTGATCTTC-3'
GAPDHf	5'-CAAGGGCATCCTGGGCTAC-3'
GAPD Hr	5'-GGTGGTCCAGGGGTCTTACT-3'
PPIAf	5'-GACCCAACACAAATGGTTCC-3'
PPIAr	5'-TCGAGTTGTCCACAGTCAGC-3'

Chapitre 2/Capítulo 2 – L'indentification de la voie de signalisation eucaryotique, modifiée par les PSM α , qu'induit le retard dans la phase G2/M du cycle cellulaire des cellules HeLa/ A identificação da via de sinalização eucariota, modificada pelas PSM α , que leva ao atraso do ciclo celular na fase G2/M de células HeLa

1 - Présentation

À partir des découvertes présentées au premier chapitre, ayant trait à la participation des PSMs de *S. aureus* dans le retard du cycle cellulaire, il a été possible d'orienter les travaux de cette Thèse pour identifier la voie de signalisation eucaryote qui est affectée par ces cyclomodulines. La modulation de la phase G2/M dans les cellules eucaryotes est réalisée par l'action de diverses protéines, dont des kinases, des protéases et des protéines structurales. Parmi les protéines qui régulent les processus de transition de la phase G2/ M et la fin de la phase M, on souligne les protéines suivantes : CDK1, PLK1, CDC25, Optineurine, MYPT1/PP1 β , Myt1, Weel/Mik1 et actine. Notre groupe de recherche a démontré que les cellules HeLa infectées par *S. aureus* présentent un retard du cycle cellulaire dans la phase G2/M. Par ailleurs, durant l'infection, on observe une augmentation de CDK1 phosphorylée en tyrosine 15, l'état dans lequel la kinase est inactive. La CDK1 participe à un réseau d'interactions avec plusieurs protéines du cycle cellulaire, comprenant l'optineurine et la PLK1. Les activités de ces deux dernières protéines pourraient donc être différentiellement modulées en présence des PSMs de *S. aureus*.

Ce chapitre présente les résultats préliminaires d'expériences d'infection de cellules HeLa avec des *S. aureus* productrices de PSMs, indiquant une possible régulation de la production d'optineurine, d'optineurine phosphorylée sur la sérine-177 et de PLK1 par ces cyclomodulines. Comme on s'y attendait, les souches de *S. aureus* ont diminué la production d'optineurine dans les cellules HeLa infectées. La diminution des niveaux d'optineurine pourrait maintenir la PLK1 active durant une période plus longue que celle nécessaire pour clore la phase M, provoquant le retard observé dans la progression du cycle cellulaire. Cependant, il n'y a pas d'altération statistiquement différente entre les niveaux d'expression de PLK1 chez cellules HeLa lors de l'infection à *S. aureus* vs le contrôle non infecté. Puisque l'Optineurine participe à l'inactivation de PLK1, en éliminant le groupe phosphate du résidu de thréonine 210 dans cette protéine, il existe une possibilité d'évaluer les niveaux de PLK1 déphosphorylée par rapport à la PLK1 phosphorylée et active.

1 - Apresentação

A partir das descobertas apresentadas no Capítulo 1, relacionadas à participação das PSMs de *S. aureus* no atraso do ciclo celular, foi possível direcionar os trabalhos da Tese para a identificação da via de sinalização eucariótica que é afetada por estas ciclomodulinas. A modulação da fase G2/M em células eucarióticas é realizada pela ação de diversas proteínas, incluindo quinases, proteases e proteínas estruturais. Dentre as proteínas que regulam os processos de transição da fase G2 para M e o encerramento da fase M, se destacam a CDK1, PLK1, CDC25, Optineurina, MYPT1/PP1 β , Myt1, Weel/Mik1 e actina. Nosso grupo de pesquisa demonstrou que células HeLa infectadas com *S. aureus* apresentam o ciclo celular atrasado na fase G2/M. Além disso, durante a infecção, foi observado um aumento da CDK1 fosforilada na tirosina 15, estado esse em que a quinase encontra-se inativa. A CDK1 participa de uma rede de interações com várias proteínas do ciclo celular, incluindo a Optineurina e a PLK1. Estas duas últimas proteínas, portanto, poderiam ter suas atividades diferencialmente moduladas na presença das PSMs de *S. aureus*.

Esse capítulo apresenta os resultados preliminares dos experimentos de infecção de células HeLa com *S. aureus* produtora de PSMs, indicando uma possível regulação da produção de Optineurina, Optineurina fosforilada na serina 177 e PLK1 por estas ciclomodulinas. Como esperado, as linhagens de *S. aureus* diminuíram a produção de Optineurina nas células HeLa infectadas. A diminuição dos níveis de Optineurina poderia manter a PLK1 ativada durante um período maior do que o necessário para encerrar a fase M, causando o atraso observado na progressão do ciclo celular. No entanto, não foi observada alteração nos níveis de expressão de PLK1 durante a infecção por *S. aureus*. Uma vez que a Optineurina participa da inativação de PLK1, removendo o grupo fosfato do resíduo de treonina 210 nessa proteína, existe a perspectiva de avaliarmos os níveis de PLK1 desfosforilada em relação à PLK1 fosforilada e ativada.

Optineurin mediates the G2/M arrest in HeLa cells infected by PSM α -producing *S. aureus*

Introduction

Staphylococcus aureus is a versatile Gram-positive pathogenic bacterium that poses serious threats to humans such as skin infections, bacteremia, endocarditis, sepsis and abscess formation (Gordon and Lowy, 2008). Humans are a natural reservoir for *S. aureus* and the nasal anterior cavity is the main locus of colonization by this bacterium. Asymptomatic colonization is far more common than infection; however, infection frequently occurs in an opportunistic manner in hospitalized patients (Rasigade and Vandenesch, 2014). About 20% of the world population presents persistent colonization, whereas 30% presents intermittent colonization (Brown et al., 2014). The infectious process is triggered by the disruption of the epithelial barrier, allowing bacteria to spread from the primary colonization site to the gastrointestinal tract, perineum, soft tissues and bloodstream (Brown et al., 2014; Thomer et al., 2016).

The pathogenic process of *S. aureus* infection is mediated by a large variety of virulence factors. Among them are: (i) alpha-toxins, which form pores in the membrane of host cells; (ii) adhesins, consisting of bacterial surface proteins that recognize adhesive matrix molecules (MSCRAMMs) and promote host cell adhesion and internalization; (iii) *S. aureus* enterotoxins (SE), such as SEB, that interact with the host cell receptors and lead to gastrointestinal inflammation and death (Argudín et al., 2010; Foster et al., 2014; Otto, 2014a). Furthermore, together with the alpha-toxins and adhesins, a class of toxins comprising the Phenol-Soluble Modulins (PSM) are capable of regulating the host immune response (Deplanche et al., 2016; Otto, 2014a). PSM also favors a *S. aureus* infection by promoting biofilm formation, antimicrobial activity against bacteria of other species and host cells lysis (Otto, 2014a).

Our research group has recently demonstrated that the methicillin resistant *Staphylococcus aureus* (MRSA) modulates the host cell cycle. Cell cycle arrest caused by bacterial toxins usually leads to reduced regeneration of epithelial and immune cells, as well as to the spread of bacteria in the host and evasion of the immune system (Aronoff et al., 2006; Falzano et al., 2006; Nougayrède et al., 2005; Oswald et al., 2005). MRSA strains alter cyclin-dependent kinase 1 (CDK1) to its inactive form, blocking G2/M cell cycle transition (Alekseeva et al., 2013). Subsequently, our group

was able to correlate the cell cycle arrest caused by *S. aureus* with the level of the PSM α toxins produced by this bacterium. (Deplanche et al., 2015). Bacterial cyclomodulins often interact with cyclin-dependent kinases (CDKs), which are major cell cycle regulators in eukaryotic cells. CDKs are constantly produced in the cells and activated only in the presence of cyclins, whose expression levels change at specific checkpoints of the cell cycle progression (Lim and Kaldis, 2013; Vermeulen et al., 2003). Cytolethal distending toxin (CDT), which is produced by some *Escherichia coli* strains, is a well-known cyclomodulin that induce the G2/M phase arrest, similar to PSM α . CDT inhibits the activation pathway of the Cell Division Cycle 25 (CDC25) protein, which is the activator of CDK1 (Taieb et al., 2015; Malumbres, 2014; Vermeulen et al., 2003).

However, the mechanism of PSM-induced the G2/M transition delay is not well understood. Regulation of the transition of G2 to M phase occurs through CDK1 activation by its binding to cyclinB. The CDK1/cyclinB complex is regulated by phosphorylation and dephosphorylation of three main amino acid residues of the CDK1 protein structure. Activation of the CDK1/cyclinB complex occurs through phosphorylation of threonine 161 by CDK-activating kinase (CAK), and dephosphorylation of threonine 14 and tyrosine 15 by CDC25 phosphatase in the late G2 phase. CDC25 is associated with 14-3-3 repressor proteins during interphase, which prevents premature entry of cells into M phase (Schafer, 1998). Dissociation of proteins belonging to the 14-3-3 family is mediated by phosphorylation of CDC25 in its N-terminal portion by Polo-Like Kinase 1 (PLK1) in its nuclear export signal portion; more precisely, in serine 198 (Ser198) (Porter and Donoghue, 2003). Furthermore, PLK1 phosphorylation leads the CDK1/cyclinB complex to the nucleus and phosphorylates one ubiquitous protein called Optineurin (Toyoshima-Morimoto et al., 2001). Optineurin mediates the connection of the CDK1/cyclinB and myosin phosphatase complexes (MYPT1/PP1 β), enabling the resulting larger complex to dephosphorylate the threonine 210 residue of PLK1, thereby deactivating this kinase and leading to the end of M phase.

Methodology

Bacterial strains and growth conditions

The MRSA MW2 strain was provided by the Laboratory of Human Bacterial Pathogenesis, NIH, USA. These strains were cultured in Brain-Heart Infusion (BHI) broth as described by Deplanche, et al. (2015). Bacteria cultured overnight were diluted in Dulbecco's modified Eagle medium (DMEM) (GlutaMax, Life Technologies) in the ratio of 1:50 and allowed to grow under anaerobic conditions at 37°C until they reached optical density (OD) of 0.6 at 600 nm (corresponding to 10⁸ Colony Forming Units (CFUs) per milliliter) (Deplanche et al., 2015). Cells were then harvested, washed and suspended in fresh DMEM for subsequent assays. Bacterial CFUs were estimated using serial dilutions in sterile 0.9% saline solution and plating on BHI agar petri dishes.

Maintenance of eukaryotic cells and infection conditions

Infection of eukaryotic cells was performed according to Nougayrède (2006) and Deplanche et al. (2015). Briefly, human cervical cancer cells (HeLa) were cultured in DMEM containing 10% fetal calf serum (cDMEM) at 37°C and 5% CO₂. Trypsin/Ethylenediamine Tetraacetic Acid (EDTA) 0.25% (Invitrogen) was used to detach the HeLa cells from culture flasks for subculturing. For cell cycle assays, HeLa cells were synchronized at the G1/S phase using the Double-Thymidine Block (DTB) method. Cells were recovered from a T50 flask and plated in a T25 flask with a 30% confluence and kept overnight at 37°C and 5% CO₂. Then, cells were washed in Phosphate Buffered Saline (PBS) solution and incubated for 18 hours with Thymidine. Thymidine blockage was interrupted by incubating the cells in fresh cDMEM for 9 hours. Subsequently, cells were reincubated in cDMEM containing 2 mM thymidine for 17 hours. DTB-treated HeLa cells were infected with *S. aureus* at Multiplicity of Infection (MOI) 100 and 200, for 26 h and 28 h, respectively. The BD Accuri™ C6 Flow Cytometer was used to confirm cell cycle arrest (Alekseeva et al., 2013; Deplanche et al., 2015).

Western blot analysis

Western blot analysis was performed according to Alekseeva et al. (2013) with modifications. Total proteins from synchronized and infected HeLa cells were obtained using 100 μ L of Laemmli loading buffer (Laemmli, 1970). The proteins were resolved in 4-15 % Mini-protean[®] TGX[™] Precast Protein Gels (Biorad, France) and subsequently transferred to a PVDF membrane (Alekseeva *et al*, 2013). The PVDF membrane was blocked for 1 h at room temperature in 10% of the skimmed milk. The membrane was exposed overnight to the primary antibodies anti-Optineurin (Eurogentec), anti-PLK1 (Santa Cruz Biotechnology, Inc) and anti- β -actin (Cell Signaling Technology[®]) in concentrations according to manufacturer's instructions. It was then washed with Tris(hydroxymethyl)aminomethane-Buffered Saline solution (15 mM Tris-HCl, 4 mM Tris base, 150 mM NaCl) containing 0.025% Tween-20 (TBS-T). This membrane was next incubated with secondary either anti-mouse or anti-rabbit horseradish peroxidase-conjugated antibodies (Cell Signaling Technology[®]) at room temperature for 2 h, according to manufacturer's instructions. The Amersham[™] ECL[™] Prime Western Blotting Detection Reagent (GE Healthcare, France) was used to detect chemiluminescence, whose intensity was evaluated using the G:BOX image system (Syngene, model Chemi XT4, United Kingdom). The membrane was then re-probed with anti- β -actin antibody (Cell Signaling Technology[®]) as a loading control.

Immunofluorescence assays

HeLa cells were seeded at 2×10^5 cells per well on 18-mm-diameter cover slips (Marienfeld, Germany) in 24 well plates (Nunc, Nuclon TM Surface) in triplicate and grown for 16 h at 37°C. The low cell density (30% confluence) at the beginning of the experiment was used to ensure cell proliferation during the entire experiment since cells cease to proliferate at high cell density and enter a viable state of quiescence, HeLa cells were synchronized as described above. After washing the cover slips with 5% BSA/PBS HeLa cells were infected with *S. aureus* and left for 26h. Subsequently, the coverslips containing the HeLa cells were removed from the wells, washed, fixed and permeabilized with 4 % paraformaldehyde in PBS for 20 min. The blockage procedure was performed using goat serum diluted in PBS at a ratio 1:10. The blocking buffer was

then removed and the coverslips were exposed to anti-optineurin antibody for 16 h at 2-8 °C, according to the manufacturer's instructions. The coverslips were then washed and exposed for 1 hour at room temperature to anti-optineurin and anti-optineurin phosphorylated at serine 177 antibodies conjugated with fluorescein isothiocyanate (FITC), and exposed to anti-fade mounting medium with 4',6-diamidino-2-phenylindole (DAPI) (Vectashield Mounting medium with DAPI, Vector Laboratories-France) and transferred to slides. Immunofluorescence was visualized using an immunofluorescence microscope (Nikon Eclipse FN1, United States) at x40 and x400 magnifications. To estimate the percentage of cells presenting optineurin phosphorylated at serine 177, more than 300 mitotic cells were analysed in each of the following groups: non-infected control HeLa cells, HeLa cells infected with *S. aureus* at MOI 100 and HeLa cells infected with *S. aureus* at MOI 200.

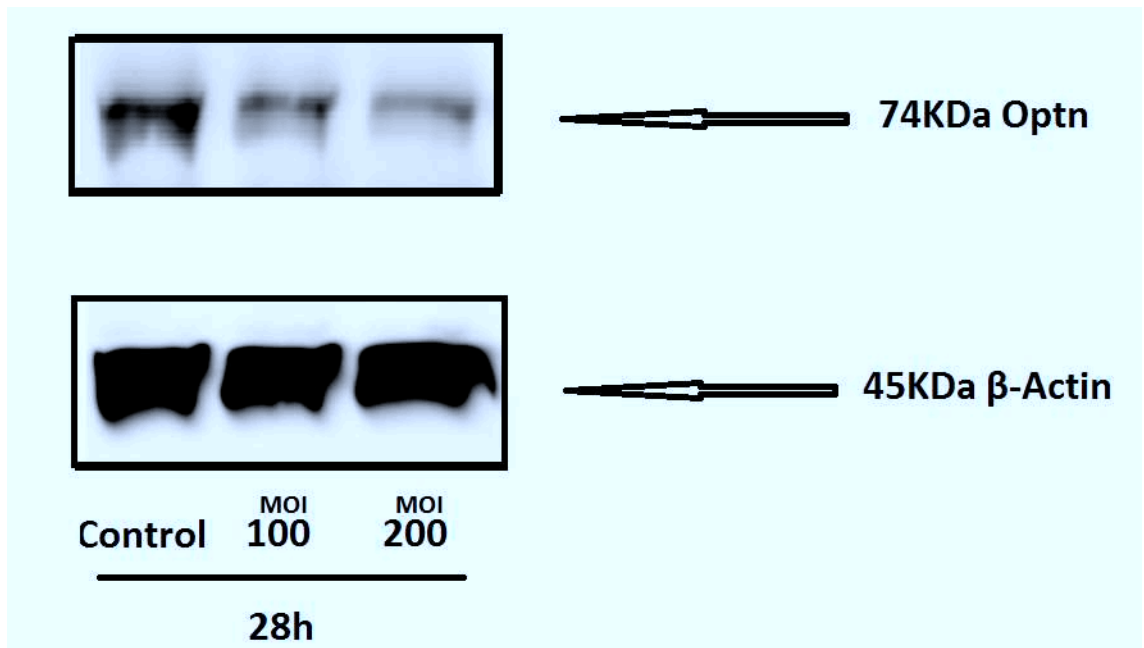
Results

Estimation of the relative amount of Optineurin and PLK1 during *S. aureus* infection

Optineurin is a multifunctional protein that is responsible for regulating the G2/M cell cycle transition in eukaryotic cells (Kachaner et al., 2012a). Optineurin is activated through the phosphorylation of serine 177 by PLK1 and is released from Ras-related protein 8 (Rab8) that are associated with the Golgi complex. The activated optineurin interacts with a protein complex that deactivates PLK1, allowing the completion of mitosis (Kachaner et al., 2012b). PLK1 participates in the cell cycle by regulating processes such as the formation of the mitotic spindle and separation of sister chromatids. The downregulation of this kinase might induce a delay in mitosis and cause cell abnormalities, including the formation of multinucleated cells, giant cells and fragmented nuclei (Mundt et al., 1997).

To determine whether optineurin is involved in the eukaryotic signaling pathway that leads to the cell cycle arrest during *S. aureus* infection, we evaluated the relative amount of optineurin in the total protein extract of infected HeLa at MOI 100 and 200 using Western blot analyses with anti-optineurin antibody. Compared with non-infected synchronized HeLa cells, a slight and not statistically significant reduction of optineurin level was observed at MOI 100:1 (Figure. 6). The increase of MOI to 200:1 resulted in the significant ($P \leq 0.05$) decrease of the optineurin level in infected synchronized HeLa cells, compared with non-infected synchronized cells. (Figure. 6). The result of Western blot analysis of the PLK1 expression levels indicated a decrease in the relative amount of PLK1 in infected cells at MOI 200:1, compared with non-infected synchronized control cells. However, this difference did not reach statistically significant level (Figure 7).

a)



b)

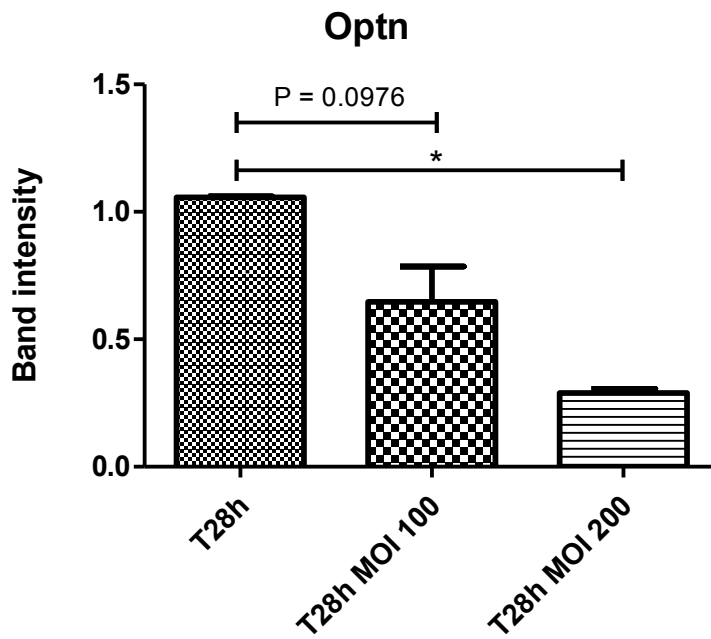
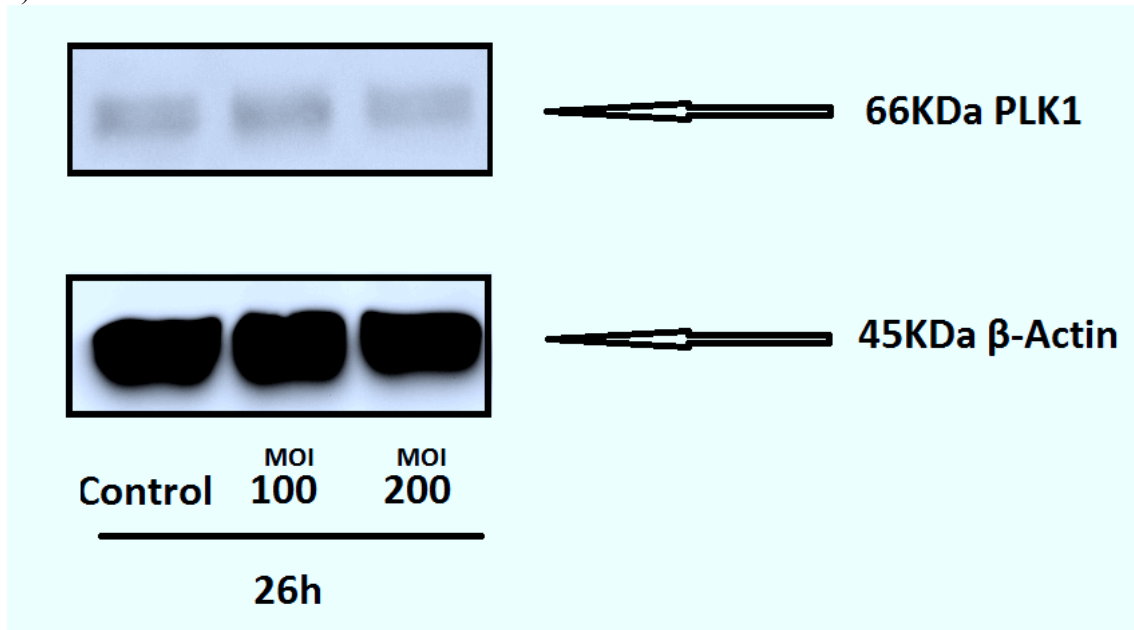


Figure 6 - Western blot to detect Optineurin. a) Blots are representative of two separate experiments. b) There is no statistical difference between the control and MOI 100 tests. There is statistical difference between the control and MOI 200 tests. Data are presented as mean \pm SD from two densitometry scans. Student T Test (*); P-value \leq 0.05.

a)



b)

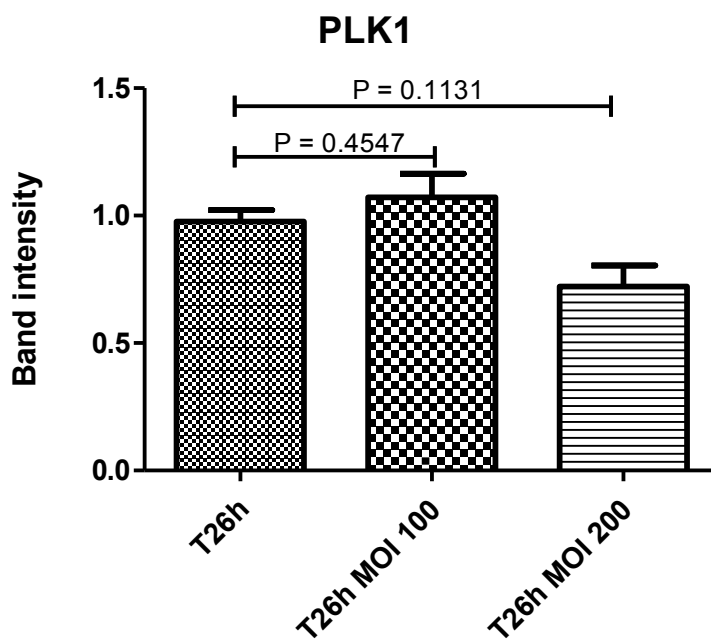


Figure 7 - Western blot to detect PLK1. a) Blots are representative of two separate experiments. b) There is no statistical difference between the control and test groups. Data are presented as mean \pm SD from two densitometry scans. Student T Test (*); P-value \leq 0.05.

Immunofluorescence assays using infected HeLa cells

It has been demonstrated that HeLa cells infected with *S. aureus* MW2 present a decrease in Optineurin production (Figure 6?). To confirm the involvement of this protein in the G2/M transition delay caused by *S. aureus*, we performed a qualitative immunofluorescence test that indicated a decrease in Optineurin levels in cells infected at MOI 200, compared with uninfected cells (Figure 8).

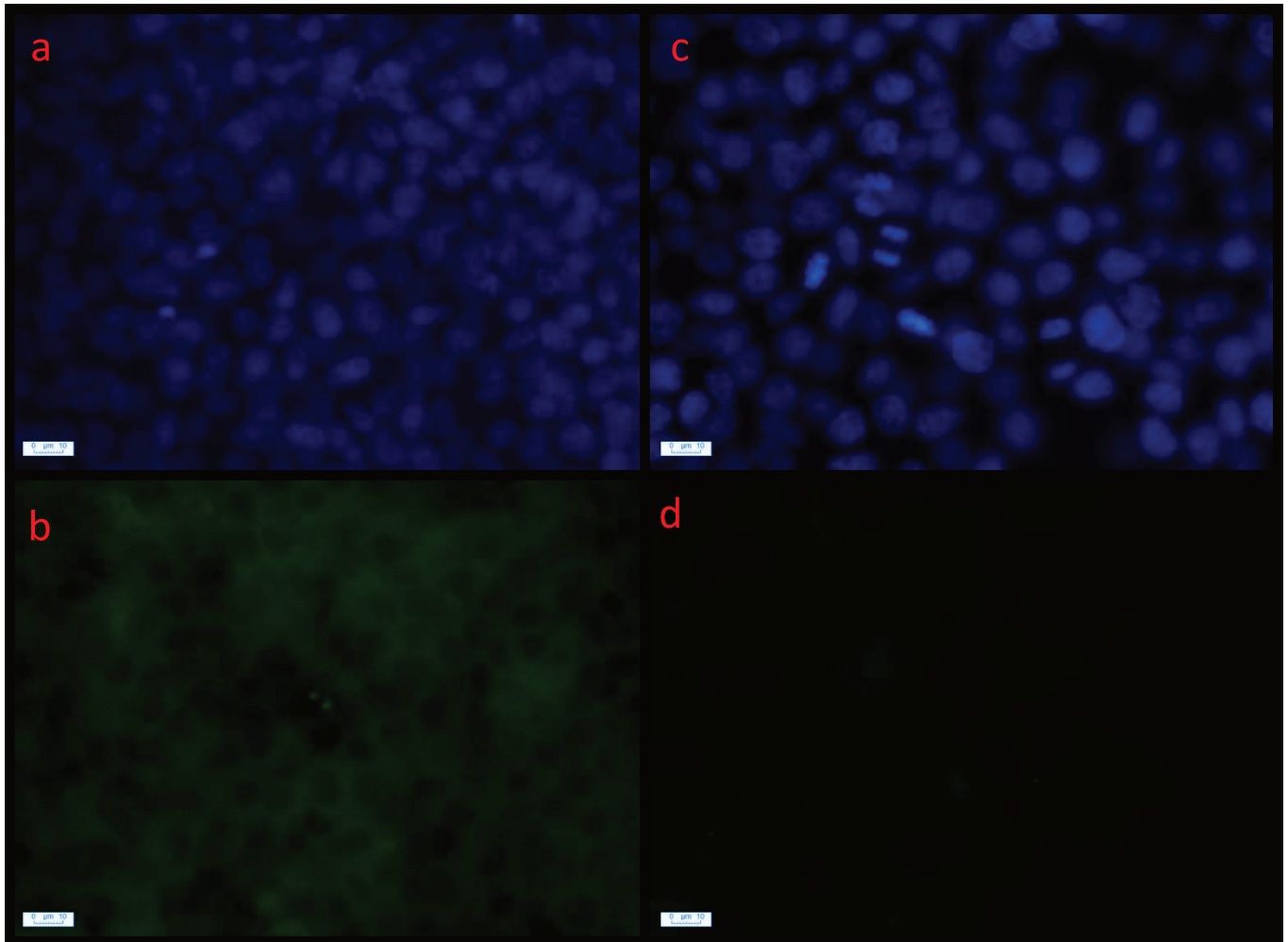


Figura 8 - Immunofluorescence assay for Optineurin.. **a)** Synchronized and uninfected HeLa cells, labeled with DAPI (blue). **b)** Synchronized and uninfected HeLa cells, labeled with anti-Optineurin (green). **c)** Synchronized HeLa cells, infected with MW2 strain (MOI 200) for 26h, labeled with DAPI (blue). **d)** Synchronized HeLa cells, infected with MW2 strain (MOI 200) for 26h, labeled with anti-Optineurin (green).

Mitotic HeLa cells presenting Optineurin phosphorylated at serine 177

Activated Optineurin, which is phosphorylated at serine 177, modulates the G2/M cell cycle transition by deactivating PLK1. To verify if *S. aureus* induces the activation of optineurin, immunofluorescence assays were conducted using an anti-p177ser-optineurin antibody in mitotic HeLa cells. After 26h of infection of synchronized HeLa cells, *S. aureus* reduced the percentage of mitotic cells presenting phosphorylated optineurin, This decrease was stronger at MOI 200 compared with MOI 100 (Figure 9).

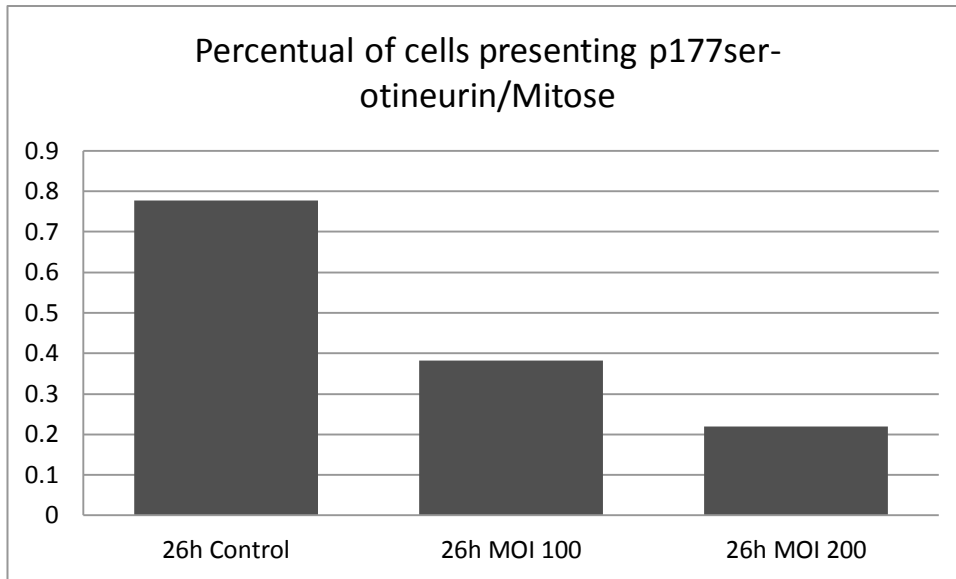


Figura 9 - Index of mitotic HeLa cells presenting Optineurin phosphorylated at serine 177 after 26h of infection by *S. aureus*. Detection of p177ser-Optineurin decreased upon infection in a MOI-dependent way. Data are representative of a single experiment.

Discussion

The eukaryotic cell cycle progression is controlled mainly by the CDK family of proteins, serine-threonine kinases and cyclins activators. Other proteins also participate, to a lesser extent, in cell cycle regulation (Vermeulen et al., 2003). During the G2/M transition, the action of PLK1 and CDK1 are directly involved in the regulation of events that govern these phases (Glover et al., 1998; Malumbres, 2014). Recently, our group of research demonstrated that HeLa cells infected with *S. aureus* present G2/M transition delay associated with increased inactive phosphorylated tyrosine 15 of CDK1 (Alekseeva et al., 2015). CDK1 participates in a wide network of interactions with various regulatory cell cycle proteins, including Optineurin and PLK1 (Glover et al., 1998; Kachaner et al., 2012b). Optineurin is a ubiquitous protein which performs different functions in eukaryotic cells. These functions include participation in Golgi organization, trafficking, vesicles secretion, innate immune response and mitotic regulation (Kachaner et al., 2012a).

Optineurin and PLK1, detected in synchronized HeLa cells infected with *S. aureus* strain MW2, were quantified using the Western blotting technique (Figures 6 and 7). Decreased optineurin level was observed to be bacteria concentration-dependent, suggesting that PSM α produced by *S. aureus* MW2 interferes with the cell cycle by modulating an optineurin expression (Figure 6 and 8). PLK1 phosphorylates optineurin at the serine 177 and allows translocation of optineurin from the Golgi apparatus to the cell nucleus during mitosis. Optineurin, together with CDK1 and the MYPT1-PP1 β phosphatase complex, participate in PLK1 deactivation by dephosphorylation of threonine 210 in the T-Loop of the kinase domain.

The decrease of the relative amount of phosphorylated optineurin has been associated with the cell cycle arrest at the G2/M phase (Figure 9) (Kachaner et al., 2012b). Cells overexpressing PLK1 could enter mitosis and establish an apparently normal bipolar spindle. However, in contrast, progression through mitosis is transiently delayed and cytokinesis appears to be disturbed, leading to a significant increase in large cells with multiple, often fragmented nuclei (Mundt et al., 1997). Therefore, the reduction of both optineurin and phosphorylated optineurin levels in infected cells could lead to the G2/M arrest by maintaining the activity of PLK1. In addition, HeLa cells infected with *S. aureus* MW2 do not present a significant reduction of PLK1 (Figure 7).

However, the further experiments have to be performed in order to confirm this conclusion.

The ability of bacteria to induce protein production is associated with host antimicrobial response (Lemaitre and Girardin, 2013). The shiga-toxigenic *E. coli* interacts directly with the BiP/Grp78 chaperone of eukaryotic cells, cleaving it and decreasing the protein production by disturbing the protein fold (Nours et al., 2013). In addition, the bacterial toxins which induce a cell membrane damage are associated with the decrease of a protein production of the host cell by accessing the Target of Rapamycin and Gcn2 kinase pathways (Kloft et al., 2010). Therefore, the ability of PSM α to disrupt host membranes, by its α -helix structure that is associated with pore membrane toxins, could explain the decrease of optineurin level (Cheung et al., 2014; Mueller et al., 2009).

Conclusion

As a partial conclusion, our study demonstrated that PSM α -producing *S. aureus* strain alter pathways that participate in host cell cycle regulation. *S. aureus* MW2 induces G2/M phase transition delay by decreasing optineurin production/activation. Moreover, HeLa cells infected with *S. aureus* MW2 did not present a statistically significant decrease in the production of PLK1, one of the most important G2/M phase regulators.

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5 - Discussion générale

Différentes bactéries pathogènes ont développé des stratégies pour faciliter leur établissement dans les cellules-hôtes et provoquer des pathogénies. Parmi ces stratégies nous pouvons noter la production de molécules effectrices capables de: (i) interagir avec des structures du cytosquelette de la cellule-hôte, conduisant à des altérations dans la membrane cellulaire qui facilitent l'internalisation bactérienne, (ii) modifier les modèles de signalisation en inhibant la présentation d'antigènes par des cellules du système immunitaire, et (iii) moduler le cycle cellulaire de l'hôte, ce qui peut conduire à une diminution de la quantité de lymphocytes (Bhavsar et al., 2007; Oswald et al., 2005).

Les cellules épithéliales sont la première ligne de défense contre de nombreux micro-organismes, comprenant *S. aureus* et *E. coli*. La barrière épithéliale est responsable de la production de nombreux composés notamment antibactériens et anti-inflammatoires qui pourraient induire des réponses immunitaires (Mitterhuemer et al., 2010). En outre, la réduction du niveau de certaines cytokines, telles que l'IL-32, par *S. aureus*, peut être associée à l'induction d'infections chroniques dans le tissu épithélial (Deplanche et al., 2016). L'IL-32 est associée à l'induction de cytokine IL-6, un modulateur inflammatoire puissant qui permet le développement des infections chroniques (Kang et al., 2012). Ainsi, cette étude a utilisé comme modèle expérimental des cellules épithéliales humaines (lignée HeLa).

La production de molécules effectrices bactériennes peut être essentielle pour réduire la prolifération de cellules du système immunitaire, augmenter la croissance bactérienne et détruire la cellule-hôte (Nougayrède et al., 2005). Récemment, notre groupe a démontré que *S. aureus* MRSA peut induire un retard de la transition de la phase G2/M du cycle cellulaire de cellules épithéliales HeLa (Alekseeva et al., 2013). D'autres bactéries, comme *Helicobacter pylori*, *Campylobacter jejuni* et *Actinobacillus actinomycetemcomitans*, se sont aussi montrées capables de sécréter des toxines modifiant la progression normale du cycle cellulaire (Ohguchi et al., 1998; Whitehouse et al., 1998).

Dans ce travail de Thèse, l'exposition des cellules HeLa au surnageant de culture de la souche MW2 de *S. aureus* a révélé que le retard dans la phase G2/M du cycle cellulaire est causé par un effecteur sécrété par la bactérie (Chapitre 1, Figure 1). Les effecteurs du surnageant ont été séparés par la technique de chromatographie

d'exclusion de taille et les fractions obtenues ont été utilisées pour la réalisation de tests *in vitro* avec des cellules HeLa. La fraction du surnageant responsable du retard de cycle cellulaire de l'hôte a ensuite été analysée par la spectrométrie de masse, ce qui a révélé la présence des toxines PSM α 1 et PSM α 3 (Chapitre 1, Figure 2, Tableaux 2, 3 et 4). Les PSMs sont un groupe de peptides produits par certaines espèces du genre *Staphylococcus*, comme *S. epidermidis* et *S. aureus* (Otto, 2014a). Ces peptides présentent une toxicité pendant les processus infectieux et sont aussi des modulines capables de provoquer le retard du cycle cellulaire dans la phase G2/M (Bibalan et al., 2014). Par ailleurs, les PSMs contribuent à la dissémination et à la virulence de *S. aureus*, menant à la formation de biofilms et à la modulation de la réponse immunitaire de l'hôte (Cheung et al., 2014; Deplanche et al., 2016).

Ainsi, les surnageants de cultures de quatre souches de *S. aureus*, isolées chez des patients souffrant d'entérocolite staphylococcique, ont été testés quant à leur capacité à provoquer le retard du cycle cellulaire dans des cellules HeLa (Chapitre 1, Tableau 5). Les résultats démontrent que ces souches sont hétérogènes concernant la capacité à altérer le cycle cellulaire eucaryote dans la phase G2/M. Cette hétérogénéité peut s'expliquer par les différences existant dans la régulation de l'expression de l'opéron *psma*, qui est promue par le système Agr (Peschel and Otto, 2013). Les séquences des gènes qui codent pour les composants du système Agr peuvent subir des variations entre les souches de *S. aureus*, provoquant différents niveaux d'expression de ce système et, par conséquence, de l'opéron *psma*. De plus, il est possible que d'autres modulines produites par *S. aureus* participent à la régulation du cycle cellulaire, comme pour des bactéries pathogènes telle que *E. coli* (Nougayrède et al., 2005).

La capacité à altérer la progression du cycle cellulaire des cellules-hôtes, attribuée aux PSM α , classe ces peptides dans la catégorie des cyclimodulines. Les modulines qui provoquent des altérations dans l'ADN conduisant à l'apoptose, à la nécrose, ainsi qu'à d'autres processus liés à la mort cellulaire, peuvent être classées dans la catégorie des génotoxines (Roos and Kaina, 2006). Il n'a cependant pas été possible d'attribuer de mort cellulaire au retard de la phase G2/M causé par les PSM α 1 et PSM α 3, comme cela a été observé dans le test de viabilité cellulaire LDH (Chapitre 1, Figure supplémentaire S1). De la même façon, le facteur inhibiteur du cycle (Cif), produit par des souches de *E. coli*, mène au retard du cycle cellulaire sans endommager l'ADN (Taieb et al., 2006).

La présence de l'opéron *psma* a été capable d'augmenter la survie de *S. aureus* internalisée dans des cellules HeLa (Chapitre 1, Figure 3). La souche de *S. aureus* mutante pour l'opéron *psma* a été récupérée en moindre quantité à partir de cellules HeLa après 6h d'infection, en comparaison à la souche sauvage parentale (Chapitre 1, Figure 3). Le meilleur taux de survie de la souche sauvage peut être attribué à la capacité de PSM α 1 à retarder le cycle des cellules-hôtes dans la phase G2/M, qui est préférentielle pour la multiplication de *S. aureus* (Alekseeva et al., 2013). De plus, ce résultat peut s'expliquer par la capacité de la PSM α 3 à altérer les membranes cellulaires à travers (i) la formation de pores, (ii) le changement de la courbe ou (iii) la solubilisation des membranes des cellules-hôtes, permettant aux bactéries d'échapper au phagolysosome dans le milieu cytoplasmique (Grosz et al., 2014; Vandenesch et al., 2012).

Ce travail de thèse a aussi montré que les PSM α 1 et les PSM α 3 réduisent l'expression des β -défensines hBD3 et hBD9 associées à la phase G2/M du cycle cellulaire des cellules-hôtes (Chapitre 1, Figures 4 et 5). La défensine hBD3 induit la production de cytokines et de chimiokines, comme IL-6, IL-10 et la protéine inflammatoire de macrophages-3 α . Ces molécules stimulent la migration et la prolifération de cellules dendritiques, cellules T, monocytes, macrophages et neutrophiles (Winter and Wenghoefer, 2012). Par ailleurs, la réduction de la production de hBD3 et hBD9 dans les cellules exposées à PSM α 1 peut être reliée à la plus grande capacité de cette moduline à retarder le cycle cellulaire de l'hôte, quand on compare à PSM α 3. En plus de la modulation des défensines, il a récemment été démontré que la souche sauvage de *S. aureus*, quand on la compare à ses mutants isogéniques pour l'opéron *psma* e/ou *psm β* , présente une diminution de l'expression de cytokines pro-inflammatoires, comme IL-32, IL-6 et IL-8 (Deplanche et al., 2016).

Bien que la progression du cycle cellulaire dans les cellules eucaryotes soit principalement contrôlée par les CDKs, d'autres protéines jouent des rôles fondamentaux dans la régulation du cycle cellulaire et sont impliquées dans la progression de la phase G2/M (Glover et al., 1998; Malumbres, 2014; Vermeulen et al., 2003). Notre groupe de recherche a montré que les cellules HeLa infectées avec *S. aureus* présentent un retard du cycle cellulaire dans la phase G2/M, ainsi qu'une augmentation des niveaux de CDK1 phosphorylée sur la tyrosine 15 – état dans lequel cette kinase est inactive (Alekseeva et al., 2013). La CDK1 participe à un réseau d'interactions avec diverses protéines du cycle cellulaire, y compris l'Optineurine et la

PLK1 (Kachaner et al., 2012b). Les résultats préliminaires du Chapitre 2 ont montré qu'il y a une réduction de la production d'optineurine et d'optineurine phosphorylée sur la sérine-177, sans aucune altération de la production de PLK1 (Figures 6, 7, 8 et 9). Alekseeva et al. (2013) ont montré que les cellules HeLa infectées avec *S. aureus* présentent un retard dans le cycle cellulaire concomitant avec une augmentation de CDK1 inactive. Aussi, il est probable que le retard observé dépende de l'interaction entre CDK1 et optineurine. Cette dernière est impliquée dans la même voie de signalisation qui régule la transition de la phase G2/M et est phosphorylée sur la sérine177 par la PLK1 (Kachaner et al., 2012b).

Conjointement à la CDK1 et au complexe MYPT1-PP1 β phosphatase, l'optineurine participe au processus d'inactivation de PLK1, à travers l'élimination du groupe phosphate présent dans la thréonine 210 du domaine kinase de cette protéine (Kachaner et al., 2012b). Ainsi, la réduction d'Optineurine dans les cellules HeLa exposées à *S. aureus* productrice de PSMs pourrait provoquer un effet similaire à celui de la surexpression de PLK1, laquelle se maintiendrait active plus longtemps que nécessaire pour une division cellulaire normale. Corroborant cette hypothèse, les cellules HeLa infectées avec la souche MW2 de *S. aureus* ont présenté une augmentation de la taille cellulaire globale, ainsi qu'une augmentation de la taille des noyaux, comme cela a été observé pour des cellules dont la production de PLK1 est altérée (Alekseeva et al., 2013; Mundt et al., 1997).

La production de PSM α par *S. aureus* pourrait diminuer l'expression d'optineurine dans la cellule-hôte de diverses manières. Comme exemple, nous pouvons prendre les souches shiga-toxines de *E. coli* qui peuvent altérer la synthèse protéique en empêchant les modifications de structure correctes de protéines dans le réticulum endoplasmique (Morinaga et al., 2008). Par ailleurs, des études avec des toxines formant des pores ont montré que la réduction de la traduction de protéines hôtes peut être liée aux dommages que ces toxines provoquent dans la membrane cellulaire. Ces toxines peuvent interférer dans les voies de *Target of Rapamycin* (TOR) et Gcn2, lesquelles régulent l'initiation et la durée de la traduction génique. (Kloft et al., 2010). Les PSM α ont un effet cytolytique sur les cellules hôtes, attribué à sa structure en α -hélice amphipathique, laquelle permet la formation de pores dans les membranes cellulaires (Cheung et al., 2014; Mueller et al., 2009; Vandenesch et al., 2012). Aussi, la voie de transduction de signaux impliquée dans la réduction des niveaux d'optineurine

par les PSM α peut être liée aux dommages que ces moduliens causent dans les membranes de la cellule eucaryote.

5 - Discussão Geral

Diferentes bactérias patogênicas têm desenvolvido estratégias para facilitar o estabelecimento nas células hospedeiras e causar patogenias. Dentre essas estratégias podemos destacar a produção de moléculas efetoras capazes de: (i) interagir com estruturas do citoesqueleto da célula do hospedeiro, levando a alterações na membrana celular que facilitam a internalização bacteriana, (ii) modificar padrões de sinalização no hospedeiro, inibindo a apresentação de antígenos por células do sistema imunológico, e (iii) modular o ciclo celular hospedeiro, o que pode levar à diminuição da quantidade de linfócitos (Bhavsar et al., 2007; Oswald et al., 2005).

Células epiteliais formam a primeira linha de defesa contra muitos microorganismos, incluindo *S. aureus* e *E. coli*. A barreira epitelial é responsável por produzir muitos compostos antibacterianos e inflamatórios, induzindo as respostas imunológicas (Mitterhuemer et al., 2010). Além disso, a redução de certas citocinas, como a IL-32, por *S. aureus*, pode estar associada à indução de infecções crônicas no tecido epitelial (Deplanche et al., 2016). A IL-32 está associada à indução da citocina IL-6, um forte modulador inflamatório que propicia o desenvolvimento de infecções crônicas (Kang et al., 2012). Desta forma, o presente trabalho utiliza como modelo experimental as células epiteliais humanas da linhagem HeLa. de ordem inata e adaptativa necessárias à eliminação de patógenos

A produção de moléculas efetoras bacterianas pode ser essencial para diminuir a proliferação de células do sistema imune, aumentar o crescimento bacteriano e destruir a célula hospedeira (Nougayrède et al., 2005). Recentemente, o nosso grupo de pesquisa demonstrou que *S. aureus* MRSA pode induzir o atraso na fase G2/M do ciclo celular de linhagens tumorigênicas epiteliais (Alekseeva et al., 2013). Outras bactérias, como *Helicobacter pylori*, *Campylobacter jejuni* e *Actinobacillus actinomycetemcomitans*, também são capazes de secretar toxinas que modificam a progressão normal do ciclo celular (Ohguchi et al., 1998; Whitehouse et al., 1998).

Neste trabalho de Tese, a exposição de células HeLa ao sobrenadante do cultivo da linhagem MW2 de *S. aureus* revelou que o atraso na fase G2/M do ciclo celular é causado por algum efector secretado pela bactéria (Capítulo 1, Figura 1). Os efetores do sobrenadante foram separados por cromatografia e as frações obtidas utilizadas para a realização de testes *in vitro* com células HeLa. A fração do sobrenadante responsável

pelo atraso no ciclo celular hospedeiro foi então avaliada por espectrometria de massa, a qual revelou a presença das toxinas PSM α 1 e PSM α 3 (Capítulo 1, Figura 2, Tabelas 2, 3 e 4). As PSMs são um grupo de peptídeos produzidos por algumas espécies do gênero *Staphylococcus*, como *S. epidermidis* e *S. aureus* (Otto, 2014a). Esses peptídeos apresentam toxicidade durante os processos infecciosos e atuam como modulinas capazes de causar o atraso do ciclo celular na fase G2/M (Bibalan et al., 2014). Além disso, as PSMs contribuem para a disseminação e a virulência de *S. aureus*, levando à formação de biofilmes e modulação da resposta imune do hospedeiro (Cheung et al., 2014; Deplanche et al., 2016).

Da mesma forma, os sobrenadantes dos cultivos de quatro linhagens de *S. aureus* isoladas de pacientes com enterocolite estafilocócica, não MRSA, foram testados quanto à capacidade de causar atraso no ciclo celular em células HeLa (Capítulo 1, Tabela 5). Os resultados demonstraram que essas linhagens são heterogêneas para a capacidade de alterar o ciclo celular eucariótico na fase G2/M. Essa heterogeneidade pode ser explicada pelas diferenças na regulação da expressão do operon *psma*, que é promovida pelo sistema Agr (Peschel and Otto, 2013). As sequências dos genes que codificam para os componentes do sistema Agr podem sofrer variações entre as linhagens de *S. aureus*, ocasionando diferentes níveis de expressão desse sistema e, conseqüentemente, do operon *psma*. Além disso, é possível que outras modulinas produzidas por *S. aureus* participem da regulação do ciclo celular, como observado em bactérias patogênicas como *E. coli* (Nougayrède et al., 2005).

A capacidade de alterar a progressão do ciclo celular das células hospedeiras, atribuída às PSM α , classifica esses peptídeos como ciclomodulinas. Modulinas que causam alterações no DNA, levando à apoptose, necrose e outros processos relacionados à morte celular, podem ser classificadas como genotoxinas (Roos and Kaina, 2006). No entanto, não foi possível atribuir morte celular ao atraso na fase G2/M causado pelas PSM α 1 e PSM α 3, como observado no teste de viabilidade celular LDH (Capítulo 1, Figura suplementar S1). Em conformidade com esses resultados, o fator inibidor do ciclo (Cif), produzido por linhagens de *E. coli*, leva ao atraso no ciclo celular sem causar dano ao DNA (Taieb et al., 2006).

A presença do operon *psma* foi capaz de aumentar a sobrevivência de *S. aureus* internalizada em células HeLa (Capítulo 1, Figura 3). A linhagem de *S. aureus* mutante para o operon *psma* foi recuperada em menor quantidade a partir de células HeLa após 6h de infecção, em comparação com a linhagem selvagem parental (Capítulo 1, Figura

3). A maior taxa de sobrevivência da linhagem selvagem pode ser atribuída à capacidade de PSM α 1 atrasar o ciclo das células hospedeiras na fase G2/M, que é preferencial para a multiplicação de *S. aureus* (Alekseeva et al., 2013). Além disso, esse resultado pode ser explicado pela capacidade da PSM α 3 em alterar as membranas celulares através da (i) formação de poros, (ii) mudança da curvatura ou (iii) solubilização das membranas das células hospedeiras, possibilitando o escape das bactérias do fagolisossomo para o meio citoplasmático (Grosz et al., 2014; Vandenesch et al., 2012).

Este trabalho de tese também mostrou que as PSM α 1 e PSM α 3 diminuem a expressão das β -defensinas hBD3 e hBD9 associadas à fase G2/M do ciclo celular das células hospedeiras (Capítulo 1, Figuras 4 e 5). A defensina hBD3 induz a produção de citocinas e quimiocinas, como IL-6, IL-10 e a proteína inflamatória de macrófagos-3 α . Estas moléculas sinalizadoras estimulam a migração e a proliferação de células dendríticas, células T, monócitos, macrófagos e neutrófilos (Winter and Wenghoefer, 2012). Além disso, a diminuição da produção de hBD3 e hBD9 nas células expostas a PSM α 1 pode estar relacionada à maior capacidade dessa modulina de atrasar o ciclo celular hospedeiro, quando comparada com a PSM α 3. Além da modulação das defensinas, foi demonstrado recentemente que a linhagem selvagem de *S. aureus*, quando comparada com seus mutantes isogênicos para o operon *psma* e/ou *psm β* , apresenta uma diminuição da expressão de citocinas pró-inflamatórias, como IL-32, IL-6 e IL-8 (Deplanche et al., 2016).

Embora a progressão do ciclo celular nas células eucarióticas seja controlada principalmente pelas CDKs, outras proteínas possuem papéis fundamentais na regulação do ciclo celular e estão envolvidas na progressão da fase G2/M (Glover et al., 1998; Malumbres, 2014; Vermeulen et al., 2003). Nosso grupo de pesquisa mostrou que células HeLa infectadas com *S. aureus* apresentam o ciclo celular atrasado na fase G2/M, com aumento nos níveis de CDK1 fosforilada na tirosina 15 - estado em que essa quinase encontra-se inativa (Alekseeva et al., 2013). A CDK1 participa de uma rede de interações com várias proteínas do ciclo celular, incluindo a Optineurina e a PLK1 (Kachaner et al., 2012b). Os resultados preliminares do Capítulo 2 demonstram que houve diminuição na produção de Optineurina e Optineurina fosforilada na serina 177, sem alteração da produção de PLK1 (Figuras 6, 7, 8 e 9). Alekseeva et al. (2013) demonstraram que as células HeLa infectadas com *S. aureus* apresentam atraso no ciclo concomitante com o aumento de CDK1 inativada. Deste modo, é provável que o padrão

de atraso observado seja dependente da interação entre CDK1 e Optineurina, a qual se encontra na mesma via de sinalização que regula a transição da fase G2/M e é fosforilada na serina 177 pela PLK1 (Kachaner et al., 2012b).

Juntamente com a CDK1 e o complexo MYPT1-PP1 β fosfatase, a Optineurina participa do processo de inativação de PLK1, através da remoção do grupo fosfato presente na treonina 210 do domínio quinase desta proteína (Kachaner et al., 2012b). Portanto, a redução de Optineurina e Optineurina fosforilada na serina 177 em células HeLa expostas a *S. aureus* produtora de PSMs poderia ocasionar um efeito similar ao da superexpressão de PLK1, a qual ficaria ativa por mais tempo que o necessário para uma divisão celular normal. Corroborando com essa hipótese, células HeLa infectadas com a linhagem MW2 de *S. aureus* apresentaram-se aumentadas e com núcleos de tamanho aumentado, como observado em células com alteração da produção de PLK1 (Alekseeva et al., 2013; Mundt et al., 1997).

A produção de PSM α por *S. aureus* poderia diminuir a expressão de Optineurina na célula hospedeira de diversas formas. Como exemplo, as linhagens shiga-toxigênicas de *E. coli* são capazes de alterar a síntese protéica ao impedirem o correto dobramento de proteínas no retículo endoplasmático (Morinaga et al., 2008). Além disso, estudos com toxinas formadoras de poros mostraram que a redução da tradução de proteínas hospedeiras pode estar relacionada aos danos que estas toxinas causam na membrana celular. Tais toxinas têm a capacidade de interferir nas vias de *Target of Rapamycin* (TOR) e Gcn2, as quais regulam a iniciação e a duração da tradução gênica. (Kloft et al., 2010). As PSM α possuem um efeito citolítico sobre as células hospedeiras que é atribuído a sua estrutura de α -hélice anfipática, a qual permite a formação de poros em membranas celulares (Cheung et al., 2014; Mueller et al., 2009; Vandenesch et al., 2012). Assim, a via de transdução de sinais envolvida na redução dos níveis de Optineurina pelas PSM α pode estar relacionada ao dano que estas modulinas causam nas membranas da célula eucariótica.

6 - Conclusion

Les toxines PSM α 1 et PSM α 3 produites et secrétées par *S. aureus* ont induit le retard dans la phase G2/M du cycle cellulaire des cellules HeLa infectées. Ces toxines augmentent la multiplication intracellulaire de *S. aureus* et diminuent la production de β -défensines associées à la défense de l'hôte. Par ailleurs, l'analyse des souches de *S. aureus* provenant de patients avec entérocolite a permis de détecter le PSM- α exclusivement dans le surnageant de souches qui altèrent le cycle cellulaire, ce qui confirme la pertinence biologique de nos résultats..

Les résultats obtenus indiquent que le retard de la transition de phase G2/M, qui est induit par les PSM de *S. aureus*, est médié par l'optineurine, protéine de cellules eucaryotes, régulatrice de la transition de phase G2/M. Cependant, le mécanisme par lequel ces PSM α conduisent à une réduction des niveaux d'optineurine demeure inconnu. Nos résultats préliminaires avec des cellules Hela démontrent la réduction de l'expression d'une autre protéine, la PLK1 lors de l'infection à *S. aureus*. Néanmoins, la différence de l'expression de PLK1, qui joue un rôle important dans la transition G2/M et est modulée par l'optineurine, dans cellules infectées vs le contrôle n'était pas statistiquement significative. La réalisation de nouvelles expériences est indispensable pour la confirmation cette observation.

6 - Conclusão

As toxinas PSM α 1 e PSM α 3 produzidas e secretadas por *S. aureus* induziram o atraso na fase G2/M do ciclo celular de células HeLa infectadas. Estas toxinas promovem o aumento da multiplicação intracelular de *S. aureus* e causam a diminuição da produção de β -defensinas associadas à resposta imune do hospedeiro. Além disso, os sobrenadantes dos cultivos de diferentes linhagens de *S. aureus* isoladas de humanos causaram o atraso no ciclo celular de maneira heterogênea, em que algumas linhagens atrasaram mais intensamente o ciclo do que outras.

Os resultados obtidos indicam que o atraso no ciclo celular causado por *S. aureus* produtora de PSM α é mediado pela Optineurina, proteína eucariótica reguladora da fase G2/M. Entretanto, o mecanismo pelo qual as PSM α levam à redução dos níveis de Optineurina permanece desconhecido. Outra proteína eucariótica, a PLK1, que tem papel importante na transição G2/M e é modulada pela Optineurina, não teve sua expressão significativamente alterada em células HeLa infectadas por *S. aureus* produtora de PSM α . No entanto, os resultados preliminares apontam para uma pequena redução da produção da PLK1 durante a infecção, o que deverá ser confirmado pela realização de novos experimentos.

7 - Perspectives

La découverte des peptides PSM α 1 et PSM α 3 de *S. aureus* comme modulateurs de la phase G2/M du cycle cellulaire (Chapitre 1) nous a conduit à chercher à comprendre les mécanismes par lesquels ces toxines affectent les voies de signalisation du cycle cellulaire de l'hôte. Les expériences réalisées dans la seconde partie de cette thèse (Chapitre 2) ont démontré que la modulation du cycle cellulaire promue par les PSM α est médiée par l'optineurine, protéine impliquée dans la voie de régulation de la phase G2/M, et dont l'expression a été réduite dans les cellules synchronisées et infectées par *S. aureus* porteur de l'opéron *psma*. Aussi, notre intention est d'entreprendre des expériences qui nous permettront d'identifier d'autres protéines du cycle cellulaire eucaryote qui sont probablement modulées par les PSM α .

La suite de ce travail impliquera l'évaluation de la production ainsi que l'activation des protéines qui déclenchent la voie de modulation de la phase G2/M dans les cellules eucaryotes exposées aux PSM α ou à des bactéries productrices de ces modulines. Parmi les régulateurs qui seront évalués, il faut souligner : (i) la PLK1 phosphorylée sur la thréonine 210, qui est l'état activé de la PLK1 et qui agit dans l'organisation du fuseau mitotique, la séparation des chromosomes homologues et la cytokinèse ; (ii) l'optineurine phosphorylée sur la sérine 177, qui participe à la régulation de l'activité de la PLK1 activée ; (iii) la CDC25, qui participe à l'activation du complexe CDK1/cycline B, fondamental pour l'activation de gènes impliqués dans la mitose. La production de ces protéines pourra être évaluée en utilisant des techniques de PCR quantitative et de Western blot avec les anticorps primaires adéquats.

Notre groupe de recherche a montré que les certaines souches cliniques de *S. aureus* induisent la phosphorylation de histone H2AX, l'un des marqueurs les plus importants et plus anciens de dommages de l'ADN chez les eucaryotes. Notre intention est aussi d'évaluer la capacité des PSM α à endommager l'ADN des cellules eucaryotes et à induire le système de réparation. Les dommages infligés au matériel génétique induisent en effet le système de réparation de l'ADN et altèrent le processus normal de la division cellulaire eucaryote. La CDT produite par certaines bactéries pathogènes comme *E. coli*, *Shigella dysenteriae* et *Salmonella enterica* serovar *Typhi*, est un exemple de cyclomoduline qui endommage l'ADN de la cellule-hôte. La CDT présente une activité de DNase et provoque le retard du cycle cellulaire par la modulation de

l'*Ataxia Telangiectasia Mutated* (ATM), protéine qui agit dans une voie de signalisation impliquée dans le système de réparation de l'ADN.

L'implication des PSM α dans l'activation du système de réparation de l'ADN sera ainsi évaluée à travers des marqueurs comme l'histone H2AX phosphorylée sur la sérine 139, les protéines ATM et ATM-Related (ATR), et les *Checkpoint Kinases* CHK1 et CHK2, responsables de l'inactivation de la protéine CDC25. Nous voulons aussi évaluer les niveaux de fragmentation de l'ADN eucaryote par le biais de l'Essai Comète ainsi que par la visualisation, par microscope optique, des chromosomes durant la mitose afin d'identifier de possibles aberrations chromosomiques induites par les PSM α .

7 - Perspectivas

A descoberta dos peptídeos PSM α 1 e PSM α 3 de *S. aureus* como moduladores da fase G2/M do ciclo celular (Capítulo 1) nos levou a buscar compreender os mecanismos pelos quais estas toxinas interagem com as vias de sinalização do ciclo celular hospedeiro. Os experimentos realizados na segunda parte desta tese (Capítulo 2) demonstraram que a modulação do ciclo celular promovida pelas PSM α é mediada pela Optineurina, proteína que está inserida na via regulatória da fase G2/M e cuja expressão foi reduzida em células HeLa sincronizadas e infectadas por *S. aureus* portador do operon *psma*. Portanto, pretendemos executar experimentos que nos permitirão identificar outras proteínas do ciclo celular eucariótico que são possivelmente moduladas pelas PSM α .

A sequência deste trabalho envolverá a avaliação da produção e ativação das proteínas que desencadeiam a via de modulação da fase G2/M em células eucarióticas expostas às PSM α ou a bactérias produtoras destas modulinas. Dentre os reguladores a serem avaliados, destacamos: (i) a PLK1 fosforilada na treonina 210, que é o estado ativado da PLK1 e que atua na organização do fuso mitótico, separação dos cromossomos homólogos e citocinese; (ii) a Optineurina fosforilada na serina 177, que participa da regulação da atividade da PLK1 ativada; (iii) a CDC25, que participa da ativação do complexo CDK1/ciclina B, fundamental para a ativação de genes envolvidos na mitose. A produção destas proteínas poderá ser avaliada por meio das técnicas de PCR quantitativa e Western blot com os anticorpos primários adequados.

Ainda, temos como perspectiva avaliar a capacidade das PSM α de causar danos ao DNA da célula eucariota e induzir o sistema de reparo desta molécula. A ocorrência de danos ao material genético induz o sistema de reparo do DNA e altera o processo normal de divisão celular eucariótica. A CDT produzida por algumas bactérias patogênicas, como *E. coli*, *Shigella dysenteriae* e *Salmonella enterica* serovar *Typhi*, é um exemplo de ciclomodulina que danifica o DNA da célula hospedeira. A CDT apresenta atividade de DNase e causa o atraso no ciclo celular pela modulação da *Ataxia Telangiectasia Mutated* (ATM), proteína que atua em uma via de sinalização envolvida no sistema de reparo do DNA.

Dessa forma, o envolvimento das PSM α na ativação do sistema de reparo do DNA será avaliado através de marcadores como a histona H2AX fosforilada na serina

139, as proteínas ATM e ATM-Related (ATR), e as *Checkpoint Kinases* CHK1 e CHK2, responsáveis pela inativação da proteína CDC25. Pretendemos também avaliar os níveis de fragmentação do DNA eucarioto por meio do Ensaio Cometa e pela visualização em microscópio óptico dos cromossomos durante a mitose, identificando possíveis aberrações cromossômicas induzidas pelas PSM α .

8 - Bibliografia/Bibliographie

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RÉSUMÉ

ABSTRACT

Mécanisme de la dérégulation du cycle cellulaire de l'hôte par *Staphylococcus aureus*

Staphylococcus aureus est une bactérie Gram positive qui colonise la peau des animaux et des humains sains. Dans certaines conditions, telles que la perturbation du microbiote, *S. aureus* peut induire différentes maladies en déjouant les fonctions de défenses de la cellule hôte. Récemment, notre équipe a montré que les *S. aureus* méthicilline-résistant (MRSA) souche MW2 (USA400) étaient capables d'induire un retard de la transition de phase G2/M des cellules HeLa. Dans ce travail, nous avons démontré que cette action est initiée par des composants du surnagent de culture de *S. aureus*. Différentes fractions de surnagents de culture de MW2 ont été obtenues par la chromatographie d'exclusion et analysées par la spectrométrie de masse. Ces techniques nous ont permis d'identifier les peptides phenol-soluble modulins alpha (PSM α) comme responsables du retard du cycle cellulaire des cellules hôtes. Confirmant l'implication de ces modulines, la souche LAC Δ psm α déficiente en PSM α 1 - 4, n'a pas affecté la progression normale du cycle cellulaire de cellules épithéliales HeLa. De plus, le traitement de ces cellules avec des PSM α 1 et PSM α 3 synthétiques a induit un retard de la transition de phase G2/M qui a été associé à la diminution de l'expression de gènes codant des défensines β . Enfin, nous avons démontré que la souche MW2 diminue le niveau d'optineurine et d'optineurine phosphorylée sur la sérine-177, une protéine hôte qui est impliquée dans la transition de phase G2/M. Ce travail représente une étape importante de la compréhension du mécanisme d'interférence de *S. aureus* avec le cycle cellulaire de la cellule hôte, révélant un nouveau rôle de PSM α , molécule produite par cette bactérie.

Mots clés : *Staphylococcus aureus*, retard de la transition de phase G2/M, phenol-soluble modulins alpha, optineurine, cellules HeLa

Mechanisms of regulation of the host cell cycle by Staphylococcus aureus

Staphylococcus aureus is a Gram-positive bacterium that colonizes the skin of healthy animals and humans. In certain conditions, including the disruption of the commensal microbiota, *S. aureus* can cause different diseases by deviating the host defense functions. Recently, our group has shown that the methicillin-resistant *S. aureus* (MRSA) MW2 (USA400) strain causes delay in the transition of the G2/M phase of HeLa cells. In the present work, we demonstrated that this action is initiated by components of the supernatant of the *S. aureus* culture. Different supernatant fractions were obtained by size exclusion chromatography and were analyzed by mass spectrometry, which allowed to identify phenol-soluble modulins alpha (PSM α) as responsible for the host cell cycle delay. Confirming the involvement of these modulins in the delay, the MRSA LAC Δ psm α strain, which is deficient in PSM α 1-4, did not affect the normal progression of the cycle in HeLa cells. In addition, the treatment of these cells with synthetic PSM α 1 and PSM α 3 caused delay in the transition of the G2/M phase associated with the decreased production of host β -defensins. Lastly, we demonstrated that the MW2 strain, which produce PSM α , decreases the level of optineurin and optineurin phosphorylated at serine 177, a host protein that is involved in the G2/M phase transition. The work conducted in this thesis represents an important achievement in the understanding of how *S. aureus* interferes with the host cell cycle, revealing a new role for PSM α produced by this bacterium.

Keywords: *Staphylococcus aureus*, G2/M phase transition delay, phenol-soluble modulins alpha, optineurin, HeLa cells



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