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par

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Diversité moléculaire des effecteurs antimicrobiens chez l'huître creuse Crassostrea gigas: mise en évidence et rôle dans la réponse antimicrobienne.

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List of Abbreviations

ACN	Acetonitrile
AMPs	Antimicrobial Peptides
AU PAGE	Acid Urea PolyAcrylamide Gel Electrophoresis
BCA	Bicinchoninic Acid
bp	base pair
BPI	Bactericidal Permeability Increasing Protein
BSA	Bovine Serum Albumin
C55P	Undecaprenyl Phosphate
cDNA	Complementary DNA
CFU	Colony Forming Units
Cm	Chloramphenicol
Cq	quantification Cycle
C-ter	Carboxy-terminus
Def	Defensin
dNTP	Deoxyribonucleotide
EDTA	Ethylenediaminetetraacetic acid
EST	Expressed Sequence Tag
FIC	Fractional Inhibitory Concentration
gDNA	Genomic Deoxyribonucleic Acid
IPTG	Isopropyl β-D-1-thiogalactopyranoside
kDa	Kilodalton
LC MS/MS	Liquid Chromatography coupled to tandem Mass Spectrometry
LPS	Lipopolysaccharide
MALDI-TOF	Matrix Assisted Laser Desorption/Ionization Time-of-Flight
MIC	Minimal Inhibitory Concentration
N-ter	Amino-terminus
PCR	Polymerase Chain Reaction
pDNA	Plasmid Deoxyribonucleic Acid
Prp	Proline rich peptide
qPCR	quantitative Polymerase Chain Reaction
RACE	Rapid Amplification of cDNA Ends
RNA	Ribonucleic Acid
RP-HPLC	Reverse Phase High Performance Liquid Chromatography
RU	Resonance Units
SDS	Sodium Dodecyl Sulfate
SDS PAGE	SDS PolyAcrylamide Gel Electrophoresis
TEMED	*N,N,N',N'*-Tetramethylethylenediamine
TFA	Trifluoroacetic Acid
TPP+	[^{3}H]TetraPhenylPhosphonium bromide
UDP-GlcNAc	UDP-N-acetyl glucosamine
UDP-MurNAc-pp	UDP-N-acetylmuramyl-pentapeptide

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Résumé

Contexte d'étude et objectifs de la thèse

L'huître creuse, *Crassostrea gigas,* est un mollusque bivalve d'importance majeure pour l'aquaculture. Parmi les 15 espèces d'huîtres cultivées, *C. gigas* représente 98% de la production mondiale, avec 4.23 millions de tonnes produites en 2008. Avec une production annuelle de 3.9 millions de tonnes, la Chine est le plus grand producteur mondial. Viennent ensuite, La Corée du Sud, le Japon et la France, avec des productions entre 116000 et 314000 tonnes par an (FAO, 2009). L'ostréiculture est l'activité aquacole principale de la France où l'espèce *C. gigas* a été introduite, à partir de stocks en provenance du Canada et du Japon (Grizel and Heral, 1991) au début des années 1970, suite à la disparition de l'espèce alors cultivée, *C. angulata*, décimée par une maladie virale.

Depuis plusieurs décennies, les productions d'huître creuse connaissent des épisodes de mortalités anormales de juvéniles survenant au cours de périodes estivales. Ces phénomènes de mortalités récurrents sont rapportés dans différents pays au niveau mondial, incluant la France où les élevages sont affectés avec plus ou moins d'intensité selon les années. Les phénomènes de mortalités estivales ont fait notamment l'objet d'un programme de recherches pluridisciplinaires, dénommé défi MOREST (MORtalités ESTivales) coordonné par l'IFREMER entre 2000 et 2005 (Samain and McCombie, 2007). Ces études ont permis de montrer que les mortalités estivales reposaient sur un modèle général d'interactions multifactorielles impliquant l'animal (statut physiologique et/ou génétique), l'environnement (climatologie, hydrologie, zootechnie) et des facteurs biotiques incluant des agents infectieux (OsHV1 herpes virus et bactéries, *Vibrio splendidus* et *V. aestuarianus*). Depuis 2008, les mortalités estivales qui frappent l'ostréiculture constituent la crise la plus grave jamais observée depuis l'introduction de l'huître creuse en France. Ces mortalités sont caractérisées par (i) l'ampleur du phénomène, où tous les bassins ostréicoles français sont concernés avec des pertes de 60 à plus de 80% de leurs stocks de juvéniles, (ii) des mortalités qui affectent principalement les juvéniles jusqu'à 18 mois, toutes origines confondues, de captage naturel ou d'écloserie, et (iii) qui surviennent de façon quasi simultanée sur l'ensemble des façades maritimes françaises et dans des écosystèmes très variés. Enfin, le caractère infectieux de ces phénomènes est maintenant avéré, faisant intervenir de façon prédominante un variant de l'Herpès virus OsHV1 (IFREMER, 2009) et la bactérie *Vibrio splendidus* (Paillard *et al.*, 2004; Garnier *et al.*, 2008).

L'importance économique de l'huître a motivé les efforts de recherche sur les processus moléculaires et cellulaires des mécanismes de défense de *C. gigas*. La réponse immunitaire chez les huîtres, comme chez tous les invertébrés, est dite innée ou non spécifique, par opposition à l'immunité dite acquise (à mémoire cellulaire) des vertébrés. Ce type de système immunitaire, dépourvu d'immunoglobulines, ne permet donc pas d'envisager chez les mollusques des traitements de masse telle que la vaccination pour limiter les infections. Les stratégies visant à améliorer la

résistance de ces animaux doivent alors passer par d'autres voies telles que la sélection génétique et des mesures prophylactiques d'élevage, nécessitant l'acquisition des connaissances sur les bases moléculaires de l'immunité. Ces connaissances sont de prime importance pour comprendre la susceptibilité des huîtres vis-à-vis de microorganismes agresseurs. Dans ce cadre, les projets de recherche de l'équipe « Réponse Immunitaire, Aquaculture, Environnement » (RIAE) concernent la compréhension à l'échelle moléculaire et cellulaire de la réponse immunitaire comme grande fonction physiologique des huîtres, en considérant les interactions avec les communautés biologiques qui constituent l'environnement des ces animaux en élevage. Ces travaux ont pour objectifs d'améliorer les connaissances sur les bases physiologiques et génétiques qui gouvernent la plus grande résistance des huîtres aux mortalités et une meilleure adaptation aux conditions d'élevage. Dans ce contexte, l'équipe de recherche d'Evelyne Bachère (RIAE) a identifié depuis plusieurs années par des approches de génomique et biochimique divers effecteurs de la réponse immunitaire de l'huître, tels que des peptides et protéines antimicrobiens (AMPs pour «AntiMicrobial Peptides»).

Dans ce contexte, le sujet de travail de ma thèse a concerné l'étude de la diversité des effecteurs de l'immunité de l'huître *C. gigas* avec une attention particulière aux peptides/protéines antimicrobiens. Les objectifs de cette thèse ont été (1) d'appréhender et caractériser la diversité de séquences observées chez les effecteurs antimicrobiens de l'huître *C. gigas* et (2) de déterminer le rôle de cette diversité dans les mécanismes de l'immunité innée chez cette espèce. Pour cela, j'ai étudié 3 familles des peptides et protéines antimicrobiens identifiées précédemment au laboratoire: des défensines (Gueguen *et al.*, 2006b; Gonzalez *et al.*, 2007a), un peptide riche en proline (Gueguen *et al.*, 2009), ainsi qu'une protéine appeée BPI pour«Bactericidal/Permeability-Increasing Protein» (Gonzalez *et al.*, 2007b). Les travaux exposés dans cette thèse ont été entrepris au sein de l'Unité Mixte de Recherche UMR 5119 Ecosystèmes Lagunaires IFREMER / CNRS / Université de Montpellier 2 / IRD.

Le premier chapitre de cette thèse est organisé en 4 parties et présente une revue bibliographique de données existantes sur le modèle d'étude de l'huître creuse *C. gigas*. Dans une première partie, l'aquaculture de *C. gigas* en France, les principaux agents pathogènes et les aspects généraux des mortalités estivales sont décrits. Dans une seconde partie sont présentés, les caractéristiques anatomiques et physiologiques de l'huître, et un état de l'art sur son système immunitaire incluant les effecteurs antimicrobiens. Dans une troisième partie, sont présentés le rôle des peptides antimicrobiens dans la réponse immunitaire, leur classification, et leurs différents mécanismes d'action. La quatrième partie concerne l'évolution des systèmes immunitaires, avec une attention particulière à la sélection naturelle, à la théorie de la coévolution et aux processus génétiques impliqués dans la diversification moléculaire. Pour finir cette partie, une comparaison

entre les réponses immunitaires acquise et innée est proposée, avec plusieurs exemples de diversification de récepteurs et d'effecteurs de l'immunité, tout en considérant le sens biologique de cette diversité.

Le deuxième chapitre présente les résultats obtenus dans le cadre du travail de cette thèse. Ceux-ci sont organisés en trois sections : (i) la mise en évidence de la grande diversité des peptides antimicrobiens chez *C. gigas* et les processus génétiques impliqués dans cette diversification (*cf. Section 1*), (ii) la détermination du mécanisme d'action de certains de ces peptides, les défensines (*cf. Section 2*) et (iii) l'approfondissement des connaissances sur l'implication des effecteurs antimicrobiens dans la réponse antimicrobienne de l'huître suite à une infection par une bactérie pathogène (*cf. Section 3*).

Le troisième chapitre synthétise l'ensemble de ces résultats qui font l'objet d'une discussion générale. Enfin des perspectives de recherche sont proposées sur l'implication de la diversité d'effecteurs antimicrobiens dans les réactions de défense de l'huître *C. gigas*.

Le quatrième chapitre présente les matériels et méthodes utilisés dans le cadre des ces travaux.

Chapitre 2 : Résultats

Section 1. Caractérisation de la diversité moléculaire des effecteurs antimicrobiens *Cg*-BPI, *Cg*-Defs et *Cg*-Prp de l'huître creuse *Crassostrea gigas*

Au cours des dernières années, une grande diversité moléculaire au sein des gènes de l'immunité des invertébrés a été mise en évidence (Schulenburg *et al.*, 2007; Du Pasquier, 2009). Ainsi, le paradigme indiquant que la diversité des gènes de l'immunité était exclusivement restreinte au système immunitaire des vertébrés est maintenant remis en cause. Des études sur l'évolution des effecteurs et récepteurs de l'immunité chez des invertébrés ont permis d'obtenir de nouvelles connaissances sur l'origine, la fonction et les mécanismes de diversification impliqués (Flajnik and Du Pasquier, 2004 ; Lee *et al.*, 2005; Litman *et al.*, 2005; Pujol *et al.*, 2008). L'étude de l'évolution des effecteurs antimicrobiens a été entreprise au travers de différents modèles (Patil *et al.*, 2004; Jiggins and Kim, 2005; Semple *et al.*, 2006). Dans plusieurs cas, il a été proposé que des hôtes, exposés à divers pathogènes, pourraient développer un répertoire plus large d'effecteurs antimicrobiens afin d'améliorer leur potentiel de défense (Pujol *et al.*, 2008). La diversification moléculaire des gènes de l'immunité repose sur l'hypothèse générale de co-évolution ou de «course aux armements». Cette hypothèse propose que les agents pathogènes évoluent continuellement pour «échapper» à la réponse immunitaire de l'hôte, et qu'en conséquence, la réponse immunitaire de l'hôte évolue aussi

en parallèle afin d'améliorer les mécanismes de défense contre ces pathogènes (Dawkins and Krebs, 1979).

Plusieurs effecteurs antimicrobiens ont été identifiés chez l'huître: des défensines, *Cg*-Defs (Gueguen *et al.*, 2006b ; Gonzalez *et al.*, 2007a), un peptide riche en proline, *Cg*-Prp (Gueguen *et al.*, 2009), ainsi qu'une protéine de type BPI, *Cg*-BPI (Gonzalez *et al.*, 2007b). Dans le cadre de la caractérisation de ces effecteurs, une diversité de séquences nucléotidiques et protéiques avait été partiellement mise en évidence. Dans la première partie de mon travail de thèse, nous avons donc caractérisé la diversité moléculaire des séquences de ces trois effecteurs antimicrobiens au niveau des gènes et des transcrits. Les séquences nucléotidiques ont été obtenues à partir d'une recherche *in silico* sur les ESTs disponibles chez *C. gigas* (http://public-contigbrowser.sigenae.org:9090/Crassostrea_gigas/index.html). Ce travail a été complété par un séquençage exhaustif des produits de PCR obtenus. Finalement, les séquences ont été analysées par des calculs de valeurs de polymorphisme, par la construction d'arbres phylogénétiques et par des tests de sélection.

Au vu des résultats obtenus sur la diversité des peptides antimicrobiens de *C. gigas*, nous avons ensuite étudié les mécanismes génétiques par lesquels la diversité pouvait être générée. Nous avons mis en évidence que ces peptides et protéine antimicrobiens ont été soumis à différentes "routes évolutives", aboutissant à une variabilité de certains effecteurs. Ainsi, la grande diversité de séquences identifiées pour les AMPs (*Cg*-Defs et *Cg*-Prp) est produite d'une part par la duplication de gènes couplée à des événements de recombinaison, d'homéoplasie phylogénétique et à des événements d'insertion/délétion («in-del»), et d'autre part, par des pressions de sélection directionnelle. A l'opposé, la protéine *Cg*-BPI est codée par une seule copie de gène et nous n'avons pas détecté de pressions de sélection directionnelles sur cet effecteur de l'immunité.

Ces résultats mettent en évidence des processus génétiques au cours desquels certains acides aminés sont soumis à une pression de sélection, aboutissant à la diversification des effecteurs antimicrobiens de l'huître. Les résultats de cette première partie ont été valorisés par une publication **(Molecular diversity of antimicrobial effectors in the oyster *Crassostrea gigas* (2010) BMC Evolutionary Biology, 10: 23).** À notre connaissance, il s'agit de la première analyse de la diversité moléculaire des AMPs d'huître. Cette étude a montré que les AMPs de *C. gigas* ont été soumis à différents «patrons de diversification» conduisant à une importante variabilité des séquences. En considérant les activités synergiques déjà décrites chez *C. gigas* entre les effecteurs *Cg*-Prp et *Cg*-Defm, (Gueguen *et al.*, 2009) ainsi que celles observées entre des variants d'un même AMP dans d'autres espèces (Lauth *et al.*, 2005; Mangoni *et al.*, 2008), nous nous sommes intéressés, dans la suite de nos travaux, aux aspects fonctionnels de cette diversité des effecteurs antimicrobiens de l'huître.

Section 2. Détermination du mécanisme d'action des défensines d'huître.

Il est resté longtemps admis que le mécanisme d'action des peptides antimicrobiens reposait seulement sur la perméabilisation des membranes des microorganismes. Cependant, l'hypothèse selon laquelle les peptides ne sont que des créateurs de pores ou des détergents membranaires uniformes et non spécifiques est révolue. L'existence de relations étroites entre la structure des peptides et les différents mécanismes d'action est maintenant reconnue depuis plusieurs années (Yeaman and Yount, 2003). En effet, alors que dans une étape précoce de leur mécanisme d'action, la vaste majorité des peptides antimicrobiens interagit avec les membranes anioniques bactériennes grâce à leur propriétés amphiphiles (équilibre entre leur charge cationique et leur hydrophobicité) (Shai, 2002; Bulet *et al.*, 2004), il existe différents mécanismes antimicrobiens ultérieurs. Les AMPs peuvent en effet tuer ou inhiber la croissance des microorganismes par déstabilisation membranaire mais aussi par l'altération de processus métaboliques telle que l'inhibition de la biosynthèse de la paroi cellulaire, de la synthèse d'acides nucléiques, de protéines ou d'activités enzymatiques (Brogden, 2005 89).

Dans la première partie de ce travail de thèse, nous avons montré que la diversité moléculaire des défensines, *Cg*-Defs, est produite par la duplication génique et par des pressions de sélection directionnelles. Les variants ont divergé en trois groupes distincts (*Cg*-Defh1, *Cg*-Defh2 et *Cg*-Defm), présentant pour chaque groupe une séquence de peptide mature extrêmement conservée. L'évolution et la divergence de plusieurs familles multigéniques d'AMPs ont été mises en évidence chez plusieurs vertébrés et invertébrés (Cohuet *et al.*, 2008; Hollox and Armour, 2008; Lazzaro, 2008; Nicolas and El Amri, 2008; Padhi and Verghese, 2008), mais ces études ne comportent pas d'approche expérimentale permettant de comprendre les conséquences biologiques de cette diversification. Cependant, l'apparition de nouveaux variants d'AMPs par duplication génique, ainsi que par des pressions de sélection a pu être rattachée à une diversification fonctionnelle de ces molécules (Lynch and Conery, 2000; Tennessen, 2005). Il a également été proposé que la production d'un grand nombre d'AMPs structurellement similaires au sein d'un même organisme, pourrait être une stratégie évolutive permettant d'élargir le spectre d'activités antimicrobiennes (Mangoni and Shai, 2009).

D'après ces hypothèses, la diversité de défensines d'huître nous a conduit à étudier une possible divergence fonctionnelle. Pour cela, trois variants représentatifs de la diversité des défensines d'huître (*Cg*-Defh1, *Cg*-Defh2 et *Cg*-Defm) ont été produits sous forme recombinante. Les activités antimicrobiennes et les mécanismes d'action des peptides produits ont été caractérisés. L'activité antimicrobienne des trois variants a été évaluée par la détermination de la concentration minimale inhibitrice (CMI). Les résultats ont montré que les défensines d'huître présentent de fortes

activités contre les bactéries à Gram-positif et une activité réduite contre les bactéries à Gram-négatif. De manière intéressante, nous avons montré qu'il existe des activités différentielles entre les variants, certains étant 8 à 40 fois plus actifs que d'autres. A l'issue de ces résultats, nous avons étudié le mécanisme d'action des défensines de *C. gigas* contre la bactérie à Gram-positif *Staphylococcus aureus*. Conformément à son spectre d'activité dirigé presque spécifiquement contre les bactéries à Gram-positif, nous avons mis en évidence que les défensines d'huître sont des inhibiteurs spécifiques de la biosynthèse du peptidoglycane, le composant principal de la paroi cellulaire des bactéries à Gram-positif, qui est «caché» dans l'espace périplasmique chez les bactéries à Gram-négatif. Ainsi, les trois défensines n'ont pas montré d'effet sur l'intégrité de la membrane de *S. aureus* mais ont inhibé la biosynthèse de la paroi cellulaire, comme mis en évidence par l'accumulation du dernier précurseur soluble du peptidoglycane UDP-N-acetylmuramyl-pentapeptide. De plus, des essais d'antagonisme de l'activité antimicrobienne, des essais de chromatographie en couche mince et de résonance plasmonique de surface ont démontré que les défensines piègaient le lipide II, inhibant ainsi la biosynthèse du peptidoglycane. À notre connaissance, cette étude représente la première analyse détaillée du mécanisme d'action de défensines antibactériennes produites par des invertébrés. De plus, les trois défensines ont montré des capacités d'accrochage différentes au lipide II, lesquelles semblent assez bien correspondre aux différentes activités antibactériennes observées. Ainsi, d'après nos données expérimentales et l'analyse de la diversité des défensines d'huître (article 1), nous avons pu proposer que la diversité des défensines est le résultat des forces sélectives qui ont (i) conservé les résidus impliqués dans le piégeage du lipide II, et (ii) diversifié des résidus impliqués dans l'interaction électrostatique avec les membranes bactériennes. Les résultats de cette deuxième partie de mon travail ont été valorisés au travers de la publication N°2 **(Insight into invertebrate defensin mechanism of action: oyster defensins inhibit peptidoglycan biosynthesis by binding to lipid II». Journal of Biological Chemistry (2010, sous presse).**

Section 3. Rôle des effecteurs antimicrobiens dans la réponse immunitaire de *Crassostrea gigas*

La caractérisation des processus moléculaires impliqués dans les réactions de défense de *C. gigas* représente l'objectif principal des travaux de recherche du laboratoire. Ainsi, au cours des dernières années, des avancées ont été réalisées avec la caractérisation de divers effecteurs antimicrobiens de l'huître, deux familles de peptides, *Cg*-Defs et *Cg*-Prp (Gueguen *et al.*, 2006b; Gonzalez *et al.*, 2007a; Gueguen *et al.*, 2009), et une protéine, *Cg*-BPI (Gonzalez *et al.*, 2007b). Ces travaux ont été poursuivis par la mise en évidence d'une grande diversité moléculaire de ces

effecteurs qui pourrait résulter de la coévolution ou de la «course aux armements» entre hôte et pathogènes (Schmitt *et al.*, 2010a).

Ces résultats nous ont conduits à aborder le rôle de ces effecteurs dans la réponse antimicrobienne et la protection de l'huître vis-à-vis d'infections bactériennes. Ces travaux ont été motivés par les phénomènes de mortalités estivales d'huître creuse qui affectent les élevages français. Au cours de ces épisodes de mortalité, des souches de la bactérie *Vibrio splendidus,* LGP31 et LGP32, ont pu être isolées chez les huîtres, et leur caractère infectieux a été démontré par la reproduction expérimentale de mortalités après leur injection dans le muscle adducteur d'huîtres (Gay *et al.*, 2004a; Gay *et al.*, 2004b). A ce jour, les mécanismes impliqués dans la pathogénicité et la virulence de *V. splendidus chez* l'huître restent encore très mal connus.

Dans cette partie, nous avons cherché à approfondir l'implication des différents effecteurs antimicrobiens de l'huître, *Cg*-Defs, *Cg*-Prp et *Cg*-BPI, dans la réponse à une infection par un *Vibrio* pathogène. Pour cela, une étude globale a été développée basée sur l'analyse de l'expression des transcrits des trois effecteurs dans différents tissus après une injection de *V. splendidus* LGP32, et la localisation de l'expression voire la co-localisation des effecteurs. De plus, afin d'évaluer si ces effecteurs pouvaient interagir dans la réponse antimicrobienne de l'huître, nous avons analysé *in vitro* les activités antimicrobiennes des différents effecteurs en synergie.

L'expression des transcrits de *Cg-Defhs, Cg-Defm, Cg-Prp* et *Cg-BPI* a été analysée par PCR en temps réel (qPCR) dans des hemocytes circulants et dans les branchies, le manteau et le muscle (site d'injection), prélevés 12h après l'injection de *V. splendidus*. Les résultats obtenus ont mis en évidence différents profils d'expression des transcrits pour chacun des antimicrobiens. Comme observé précédemment, *Cg-Defm* est exclusivement exprimé dans le manteau et son expression n'est pas modulée suite à une infection par *Vibrio* (Gueguen *et al.*, 2006b). Nous avons aussi confirmé que *Cg-BPI* a un niveau de transcrits constant dans des tissus comme la branchie et le manteau, et qu'il est l'unique effecteur régulé dans les hémocytes circulants (Gonzalez *et al.*, 2007b). De plus, nous avons montré que, l'injection de *V. splendidus* induit l'expression de *Cg-BPI* à l'inverse d'une injection d'eau de mer stérile. Les défensines hémocytaires *Cg-Defhs,* qui sont constitutivement exprimées dans les hémocytes d'huîtres non «stimulées», montrent une diminution de l'abondance des transcrits dans les hémocytes circulants suite à une injection de *V. splendidus*. Cette diminution des transcrits pourrait être liée à la migration des hémocytes exprimant *Cg-Defhs* vers les tissus, comme le suggère l'augmentation concomitante de l'abondance des transcrits de *Cg-Defhs* dans le muscle, site d'injection des bactéries. En revanche, l'abondance des transcrits de *Cg-BPI* n'augmente pas au niveau du site d'injection, ce qui suggère des propriétés chimiotactiques différentes entre les populations hémocytaires qui expriment respectivement ces deux effecteurs.

Dans le cadre de ces travaux, nous n'avons pas pu localiser les antimicrobiens par immunohistochimie sur des coupes histologiques d'huîtres entières, probablement à cause d'une trop faible concentration au niveau des tissus autres que les hémocytes. Par contre, sur des préparations d'hémocytes circulants, nous avons mis en évidence que *Cg*-BPI et *Cg*-Defhs sont colocalisés dans certains hémocytes, comment cela a déjà été décrit pour les AMPs *Cg*-Defhs et *Cg*-Prp (Gueguen *et al.*, 2009).

La colocalization de différents effecteurs antimicrobiens au niveau de populations hémocytaires, mais également leur co-localisation potentielle au sein des tissus d'huîtres, laissent penser que ces effecteurs puissent agir en synergie. Pour répondre à cette hypothèse, nous avons produit en système recombinant ou par synthèse chimique les variants les plus représentatifs de chaque famille d'antimicrobiens pour étudier leurs activités. Les spectres d'activité antimicrobienne de *Cg*-Defm, *Cg*-BPI et *Cg*-Prp ont été précédemment établis (Gueguen *et al.*, 2006b; Gonzalez *et al.*, 2007b). Dans le cadre de notre étude, nous avons montré que ces effecteurs antimicrobiens présentent des spectres d'activité différents qui peuvent être considérablement modifiés et élargis par des effets synergiques entre les différentes familles d'AMPs ou entre des variants d'une même famille.

Les huîtres sont en contact permanent avec un milieu riche en microorganismes qui peuvent constituer leur microflore endobionte. Sur la base des résultats obtenus, nous pensons que les niveaux constitutifs des transcrits d'AMPs présents dans l'huître peuvent contribuer au contrôle de la microflore endobionte. Nous pensons aussi que des concentrations plus élevées en AMPs de natures diverses pourraient être atteintes dans les tissus suite à une infection, grâce aux propriétés chimiotactiques des hémocytes et la colocalization des effecteurs antimicrobiens induite par ces mouvements hémocytaires. Ces mouvements pourraient ainsi contribuer à contrôler les infections au travers d'effets synergiques entre les effecteurs.

Données complémentaires: Identification de peptides antimicrobiens à partir de branchies de l'huître

Les épithéliums constituent une barrière physique et chimique aux infections. Cette défense innée est fondamentale notamment pour les organismes qui habitent dans des environnements aquatiques riches en microorganismes. Les épithéliums constituent une barrière mécanique, mais ils participent aussi à la réponse immunitaire et à la production de molécules actives (Ganz, 2002). Quand les pathogènes traversent ces barrières, ils sont confrontés à une réponse immédiate du système immunitaire au niveau épithélial, puis, les cellules immunitaires fonctionnent de manière coordonnée pour contrôler l'infection (Pruzzo *et al.*, 2005). Plusieurs effecteurs antimicrobiens ont

ainsi été isolés à partir des épithéliums d'organismes aquatiques (Park *et al.*, 1996; Birkemo *et al.*, 2003; Bergsson *et al.*, 2005).

Ces données, combinées à la mise en évidence de taux élevés de transcrits de défensines dans les branchies nous ont poussé à rechercher par des approches biochimiques la présence d'antimicrobiens dans cet organe. En utilisant une méthode mise au point pour enrichir les extraits de tissus en peptides cationiques, nous avons obtenu un mélange de peptides présentant une activité antimicrobienne vis-à-vis de bactéries à Gram-positifet à Gram-négatif. Par séquençage N-terminal et LC MS/MS, nous avons identifié dans nos extraits plusieurs fragments d'histones et de protéines ribosomales. Ces résultats préliminaires suggèrent qu'une grande variété d'AMPs est présente dans les branchies de l'huître incluant des antimicrobiens «non conventionnels» dérivés de l'histone. Des travaux complémentaires sont bien évidemment nécessaires pour établir le répertoire complet des effecteurs antimicrobiens présents dans les tissus de l'huître et leur rôle dans la protection contre les infections. Il sera notamment intéressant de déterminer si ces antimicrobiens dérivés d'histones et de protéines ribosomales sont également présents dans d'autres organes/tissus de l'huître et par quels mécanismes ils sont générés.

Discussion générale

Les objectifs de cette thèse étaient d'identifier et de caractériser la diversité moléculaire des effecteurs antimicrobiens de *C. gigas* et de comprendre l'importance de cette diversité dans l'immunité de l'huître. Plus précisément, j'ai étudié i) la diversité moléculaire de *Cg*-Défensines (*Cg*-Defs), du peptide riche en Proline *Cg*-Prp, et de la «Bactericidal/Permeability Increasing» protéine, *Cg*-BPI, ainsi que les mécanismes sous-jacents qui génèrent cette diversité; ii) les activités biologiques et le mécanisme d'action de trois variants de défensines d'huître contre la bactérie à Gram-positif *S. aureus* ; enfin j'ai développé une approche globale pour déterminer iii) le rôle de *Cg*-Defs, *Cg*-Prp et *Cg*-BPI dans la réponse immunitaire de l'huître à une infection par le pathogène d'huître *Vibrio splendidus* LGP32.

Diversifité de peptides antimicrobiens

La diversité et le polymorphisme de gènes de l'immunité ont été rattachés aux modèles théoriques de coévolution, comme l'hypothèse de la «Reine Rouge» (Van Valen, 1974) appliquée à l'interaction hôte/parasite (Combes, 2000). Cette hypothèse prédit une «course aux armements» entre l'hôte et le parasite, avec une évolution réciproque et un antagonisme entre les mécanismes de résistance de l'hôte et de l'infectiosité/virulence du parasite. Par conséquence, les molécules qui jouent un rôle essentiel dans l'interaction entre l'hôte et les pathogènes devraient aussi présenter

des hauts niveaux de diversité/polymorphisme pour assurer cette dynamique coévolutive. De ce fait, nous avons émis l'hypothèse que les effecteurs antimicrobiens de l'huître pourraient aussi présenter un haut niveau de diversité produite par la coévolution entre l'hôte et les pathogènes. En effet, plusieurs travaux mettent en évidence que toutes les espèces présentent un grand arsenal de familles d'antimicrobiens parfois très diverses (Vanhoye *et al.*, 2003; Conlon *et al.*, 2009; Nicolas and El Amri, 2009) qui pourraient jouer un rôle important dans la détermination de la pathogénicité d'un microorganisme dans cette espèce (Ganz, 2003). Ainsi, de gros efforts sont actuellement réalisés pour identifier l'arsenal exclusif d'antimicrobiens que chaque espèce peut développer, et pour comprendre les bases moléculaires de cette diversité (Clark and Wang, 1997; Lee *et al.*, 2005; Tennessen and Blouin, 2007; Cohuet *et al.*, 2008; Hollox and Armour, 2008).

Nos résultats ont montré une grande diversité moléculaire chez les AMPs de l'huître, *Cg*-Defs et *Cg*-Prp, laquelle est générée par la combinaison de différents mécanismes génétiques déjà décrits dans la diversification de plusieurs gènes de l'immunité (Holmes, 2004; Lazzaro, 2008). Comme il a été observé pour les pénaeidines chez la crevette, les classes d'AMPs d'huître sont composées par plusieurs variants générés par duplication génique et polymorphisme allélique, qui produisent différentes longueurs de séquences et du polymorphisme nucléotidique unique (O'Leary and Gross, 2006; Padhi *et al.*, 2007). Des analyses évolutives sur les pénaeidines ont indiqué que certains codons sont sous sélection diversifiante et la sélection sur ces sites pourrait donc être impliquée dans la fonction biologique des ces peptides (O'Leary and Gross, 2006). En conséquence, comme pour les pénaeidines, les AMPs de l'huître apparaissent être sous fortes pressions évolutives pour se diversifier et créer de nouveaux variants par différents mécanismes génétiques.

Contrairement aux résultats montrant l'évolution adaptative des AMPs chez la crevette et l'huître, chez la drosophile, certaines études ont suggéré que la sélection naturelle ne dirigerait pas l'évolution moléculaire des gènes codant les AMPs malgré la forte dynamique de duplication et de délétion génique qui existe sur ces gènes (Clark and Wang, 1997; Lazzaro and Clark, 2003; Schlenke and Begun, 2003; Sackton *et al.*, 2007). Cependant, l'état dynamique de duplication génique chez les AMPs de la drosophile suggère la génération possible de nouveaux gènes fonctionnels.

De façon intéressante, dans la classe des Insectes, un grand nombre de familles distinctes d'AMPs a été décrit, mais la plupart d'entre elles sont restreintes exclusivement à quelques espèces proches. Par ailleurs, chacune de ces espèces produit également un répertoire unique d'AMPs. Ainsi, il apparaît que chaque ordre ou famille d'insectes a développé un répertoire de molécules antimicrobiennes qui lui est particulier, ce qui pourrait être associé à une stratégie évolutive conférant une spécialisation de niche écologique. En conséquence, l'adaptation aux nouveaux pathogènes pourrait produire de hauts niveaux de diversité inter- et intraspécifique, comme cela a

aussi été proposé pour les AMPs d'amphibiens (Nicolas *et al.*, 2003; Lazzaro, 2008; Mangoni and Shai, 2009).

Ces données appuient notre hypothèse que l'existence de plusieurs variants chez les familles d'AMPs d'huître pourrait être indicative de processus de néo- ou sous-fonctionnalisation, du fait de (i) la forte exposition de l'hôte aux pathogènes, comme cela est observé chez les organismes aquatiques, et/ou de (ii) la spécialisation de niche écologique. Ces deux types de phénomènes sont souvent impliqués dans l'évolution de gènes de l'immunité et dans la «course aux armements» entre un hôte et ses pathogènes.

Importance biologique de la diversité des effecteurs antimicrobiens de l'huître

La diversité des AMPs de l'huître, comme conséquence directe des pressions coévolutives entre hôte et pathogènes, ont motivé l'étude (i) des activités antimicrobiennes et du mécanisme d'action de trois variants des défensines (*Cg*-Defh1, *Cg*-Defh2 et *Cg*-Defm) et (ii) de l'interaction entre les différentes familles d'effecteurs antimicrobiens. Les résultats obtenus montrent que la diversité de ces effecteurs se traduit par les différences des spectres d'activités antimicrobiennes et par l'existence d'effets synergiques entre les familles et entre les variants des AMPs.

Dans le cadre de notre étude, nous avons confirmé que les *Cg*-Defs sont principalement actives contre les bactéries à Gram-positif, que *Cg*-Prp est peu actif, et que *Cg*-BPI est actif contre les bactéries à Gram-négatif. De manière intéressante, notre travail a mis en évidence que les différents variants de chaque famille d'AMPs possèdent des activités antimicrobiennes différentes. De telles variations au niveau des spectres d'activité antimicrobienne de variants d'AMPs ont été décrites chez d'autres invertébrés. Chez la moule *Mytilus galloprovincialis*, les quatre variants de la famille des mytilines (mytilin B, C, D, et G1) montrent différentes activités antimicrobiennes (Mitta *et al.*, 1999a). Alors que les mytilines B, C et D sont actives contre les bactéries à Gram-positif et à Gram-négatif, la mytiline G1 montre une activité seulement contre les bactéries à Gram-positif. De plus, les mytilines B et C possèdent des activités différentielles contre le champignon *Fusarium oxysporum* et les bactéries à Gram-négatif. Chez les crevettes, les spectres d'activité des penaeidines de *Litopenaeus setiferus, Litset* Pen4-1 et *Litset* Pen3-4, ont été comparés au spectre de *Litvan* Pen3-1 de *L. vannamei*. La classe 3 des pénaeidines a un spectre d'activité plus large et elle est plus efficace contre certaines souches bactériennes par rapport à la classe 4 (Destoumieux *et al.*, 1999; Cuthbertson *et al.*, 2005). La classe 4 apparaît être plus sélective en ce qui concerne certaines souches bactériennes (Cuthbertson *et al.*, 2008). En conséquence, ces résultats appuient la théorie selon laquelle différents variants dans une famille d'AMPs pourraient résulter de leur divergence fonctionnelle permettant d'élargir le spectre d'activité antimicrobienne.

Les différences d'activité antimicrobienne entre les défensines d'huître ainsi que la présence d'une sélection diversifiante sur certains codons suggèrent que les pressions de sélection sur les AMPs seraient un phénomène adaptatif conduisant à augmenter la divergence fonctionnelle et ainsi à améliorer l'efficacité antimicrobienne. En effet, les défensines d'huître les plus actifs, *Cg*-Defh2 et *Cg*-Defm, présentent dans leur séquence un acide aminé chargé sous sélection diversifiante à une position spécifique (Lys16 et Arg16, respectivement), au lieu de Gly16 dans *Cg*-Defh1, qui est le variant le moins actif (Schmitt *et al.*, 2010a; Schmitt *et al.*, 2010b). Ces résidus sont très exposés sur la surface des molécules et seraient impliqués aux niveaux des interactions électrostatiques, mécanisme initial par lequel les AMPs arrivent à tuer les microorganismes (Yeaman and Yount, 2003; Brogden, 2005). Nous pouvons supposer que la substitution de cet acide aminé améliorerait l'interaction initiale avec les membranes bactériennes. Ceci a été proposé pour la plectasine, une défensine de champignon, chez laquelle la présence d'une lysine additionnelle en surface améliorerait l'activité antibactérienne, probablement grâce à une meilleure interaction avec la membrane bactérienne (Schneider *et al.*, 2010). Il a aussi été démontré que les substitutions d'acides aminés sur des sites soumis à la sélection diversifiante améliorent l'activité antimicrobienne des β-défensines de mammifères contre des pathogènes (Antcheva *et al.*, 2004; Higgs *et al.*, 2007). Ces résultats suggèrent fortement que **les variations d'activité antibactérienne entre les *Cg*-Defs dépendent en partie de la distribution de charges conférée par de la sélection diversifiante.**

Le piègeage du Lipide II par les défensines, un mécanisme ancien conservé au cours de l'évolution ?

Il a été longtemps reconnu que le mode d'action principal des AMPs était la formation non spécifique de pores et la déstabilisation de la membrane bactérienne. Nous avons démontré ici que les défensines de l'huître *C. gigas* ont un mode d'action différent. En effet, ils inhibent la biosynthèse du peptidoglycane en piégeant le lipide II, un précurseur de la paroi cellulaire impliqué dans la biosynthèse du peptidoglycane. Ce piégeage des précurseurs du peptidoglycane est commun à d'autres antimicrobiens, incluant des glycopeptides, lipopeptides, lipodepsipeptides, lantibiotiques, et autres AMPs (Schneider and Sahl, 2010). Récemment, un tel mécanisme d'action a aussi été identifié pour une défensine de champignon, la plectasine (Schneider *et al.*, 2010) et pour deux défensines de mammifères, la α-défensine HNP-1 (Leeuw, 2010), et la β-défensine HBD-3 (Sass *et al.*, 2010).

Les *Cg*-Defs sont très similaires, en terme de séquences et de structure tridimensionnelle, à la plectasine [GenBank: CAI83768], la défensine de la tique [GenBank BAB41028], de la moule [GenBank AAD45118], de la libellule [GenBank AAB24032], de l'araignée [GenBank AAW01792] et du

scorpion [GenBank AAB27538]. Il est intéressant de noter que les défensines les plus proches de celles de l'huître et de la moule proviennent des espèces représentatives de taxa «d'anciens» arthropodes (Bulet *et al.*, 1992; Volkoff *et al.*, 2003; Froy and Gurevitz, 2004) et de champignons saprophytes (Mygind *et al.*, 2005). Bien que l'évolution convergente puisse en partie expliquer les similitudes observées entre ces défensines, une origine génétique commune pour les défensines de champignons, de mollusques et «d'anciens» arthropodes semble plus probable. Cette théorie est appuyée par la conservation entre ces AMPs du motif Cs$\alpha\beta$ et de quatre acides aminés en position N-terminale, qui chez la plectasine sont impliqués dans l'interaction du peptide avec le lipide II (Schneider *et al.*, 2010). De plus, nous avons mis en évidence que chez *Cg*-Defs, ces mêmes sites sont sous sélection négative et par conséquence, sous forte contrainte fonctionnelle (Schmitt *et al.*, 2010a). Cependant, les trois variants de défensines d'huître montrent une liaison différentielle avec le lipide II, phénomène qui est corrélé avec la variation de son activité antimicrobienne. En conséquence, nous ne pouvons pas exclure la possibilité que d'autres acides aminés puissent aussi être impliqués dans la liaison au lipide II.

Les défensines d'invertébrés sont phylogénétiquement distantes des défensines de mammifères (Crovella *et al.*, 2005; Semple *et al.*, 2006). Toutefois, des représentants des deux familles de défensines de mammifères (α et β) , dont l'activité de perméabilisation membranaire a précédemment été documentée (Selsted and Ouellette, 2005; Hadjicharalambous *et al.*, 2008; Morgera *et al.*, 2008) ont récemment montré leur aptitude à lier le lipide II (Leeuw, 2010; Sass *et al.*, 2010). C'est aussi le cas des bactériocines, peptides antibactériens sécrétés par les procaryotes, qui a l'inverse des défensines ne sont pas le produit d'un gène mais celui d'une synthèse mutienzymatique. Ainsi, la nisine (dont la structure est totalement distincte de celle des défensines) est un exemple très bien renseigné d'une **activité antibactérienne résultant à la fois de perméabilisation membranaire et de piégeage du lipide II** (Breukink and de Kruijff, 1999). Ainsi, nous proposons, **qu'au travers de l'évolution convergente, des peptides antimicrobiens des différentes espèces du règne vivant ont façonné leurs structures de sorte à réaliser ces deux mécanismes extrêmement complémentaires pour l'activité antimicrobienne.** On peut en effet facilement concevoir que l'accès au lipide II, qui est impossible en l'absence de perméabilisation membranaire chez les bactéries à Gram négatif, se trouve grandement facilité pour les peptides à activité perméabilisante. De la même manière, chez les bactéries à Gram positif, la perméabilisation de la membrane permettrait l'accès au lipide II intracytoplasmique. La perméabilisation serait alors une étape qui permet (Gram negatif) ou facilite (Gram positif) l'accès à la cible cellulaire.

Activités synergiques entre les effecteurs antimicrobiens de l'huître

Un autre point important qui appuie l'hypothèse de la diversité fonctionnelle des effecteurs antimicrobiens de l'huître est l'existence d'activités synergiques entre les variants permettant d'élargir le spectre d'activité des effecteurs. Les activités synergiques entre les AMPs de l'huître ont été dans un premier temps mises en évidence entre *Cg*-Defm et un variant de *Cg*-Prp (Gueguen *et al.*, 2009). Dans le cadre de cette étude, nous avons montré qu'une forte synergie peut également s'exercer entre les variants d'une même famille d'antimicrobiens et entre familles, comme pour *Cg*-Defhs et *Cg*-BPI. De plus, nous avons observé une variation de l'efficacité de l'activité synergique selon la combinaison des variants, ce qui appuie d'autant plus la théorie de la diversité fonctionnelle des variants. **En conséquence, la production d'un grand nombre d'AMPs structurellement similaires est probablement une stratégie évolutive permettant d'améliorer le spectre d'activité antimicrobienne en utilisant la combinaison de variants.**

Les activités biologiques des AMPs ont été pendant longtemps évaluées au niveau individuel pour une séquence donnée. Maintenant, l'existence d'activités synergiques entre les AMPs est totalement reconnue, et il a été démontré que les AMPs peuvent réagir simultanément contre des microorganismes du fait de leur colocalisation (Yeaman and Yount, 2003). Chez les amphibiens, plusieurs familles d'AMPs montrent des activités synergiques. Les variants de dermaseptines S ont des activités synergiques entre elles, ce qui permet d'améliorer l'activité antibactérienne d'un facteur 100 (Mor *et al.*, 1994). Comme cela a été observé pour les *Cg*-Defs ou *Cg*-Prp quand ils sont combinés avec *Cg*-BPI, les temporines 1Ta et 1Tb chez les amphibiens sont très peu actives contre les bactéries à Gram-négatif, mais de fortes activités synergiques sont obtenues quand chacun des peptides est combiné avec la temporine1Tl (Mangoni and Shai, 2009).

Les différences observées au niveau des spectres d'activité antimicrobienne et les activités synergiques entre les antimicrobiens de l'huître, nous permettent de proposer un modèle qui pourrait expliquer en partie, les mécanismes sous-jacents de leur interaction. Les effecteurs antimicrobiens de l'huître présentent différents mécanismes d'action. *Cg*-BPI agit par déstabilisation de la membrane des bactéries à Gram-négatif tandis que les *Cg*-Defs sont des inhibiteurs de la biosynthèse du peptidoglycane peu ou pas actifs sur les membranes bactériennes. Concernant *Cg*-Prp et bien que son mécanisme d'action ne soit pas encore caractérisé, il pourrait agir contre les bactéries par l'interaction avec des cibles intracellulaires, comme cela a été décrit pour plusieurs membres du groupe des AMPs riches en proline (Kragol *et al.*, 2001; Otvos, 2002). Ainsi, nous pouvons proposer que la combinaison de *Cg*-BPI avec *Cg*-Defs ou *Cg*-Prp contre les bactéries à Gram-négatif, permette un meilleur accès au peptidoglycane pour *Cg*-Defs, ou à la cible intracellulaire pour *Cg*-Prp **(Fig. 16)**. De ce fait, cette action combinée des antimicrobiens réduirait la concentration de peptide requise pour une activité inhibitrice. Des travaux complémentaires seront nécessaires pour

déterminer les mécanismes moléculaires à la base des activités synergiques entre les variants du même AMP, ou entre *Cg*-Defs et *Cg*-Prp contre les bactéries à Gram-négatif.

Rôle des effecteurs antimicrobiens dans la réponse immunitaire de l'huître

L'objectif de la troisième partie de mon travail de thèse consistait à étudier l'implication respective des différents effecteurs antimicrobiens dans la réponse immunitaire de l'huître suite à une infection par un *Vibrio* pathogène. Pour cela, l'étude de l'expression des gènes de *Cg*-Defs, de *Cg*-Prp et de *Cg*-BPI, la localisation des effecteurs correspondant et la caractérisation *in vitro* de leur activité biologique ont été réalisées. Une grande diversité de profils d'expression des différents effecteurs a été mise en évidence dans les populations hémocytaires circulantes ainsi que dans les tissus de l'huître. Nos résultats ont révélé que les différents effecteurs seraient susceptibles d'interagir entre eux du fait de leur colocalization dans certains hémocytes mais également au travers de différents comportements migratoires des populations hémocytaires déclenchés en réponse à une infection.

Propriétés chimiotactiques des hémocytes: un élément principal dans la réponse antimicrobienne de l'huître?

Nous avons confirmé les résultats d'expression des transcrits de *Cg-BPI* et de *Cg-Defs* obtenus précédemment par notre groupe (Gueguen *et al.*, 2006b; Gonzalez *et al.*, 2007a; Gonzalez *et al.*, 2007b). *Cg-BPI* est l'unique effecteur modulé dans les hémocytes circulants en réponse à une stimulation par des bactéries non pathogènes ou pathogènes et il est constitutivement exprimé dans les épithéliums de nombreux organes. *Cg-Defhs* et *Cg-Defm* sont constitutivement exprimés dans les hémocytes et le manteau, respectivement, et ils ne sont pas modulés. Dans le cadre de notre étude, nous avons observé une augmentation du nombre de transcrits de *Cg-Defhs*, mais pas de *Cg-BPI*, au niveau du site d'injection des bactéries, ce qui suggère des propriétés chimiotactiques différentes entre les hémocytes qui expriment respectivement ces deux effecteurs.

En revanche, dans nos infections expérimentales (injection de bactéries vivantes pathogènes), les profils d'expression de *Cg-Prp* en qPCR diffèrent de ceux observés précédemment par hybridation *in situ* à partir d'huîtres stimulées par balnéation dans une eau riche en bactéries tuées (Gueguen *et al.*, 2009). Une augmentation de nombre de transcrits de *Cg-Prp* dans les hémocytes circulants et dans les hémocytes qui infiltrent les tissus contigus à l'intestin avaient été mis en évidence. Dans notre étude, l'injection des huîtres avec *V. splendidus* produit une diminution du nombre de transcrits de *Cg-Prp* dans les hémocytes circulants, sans augmentation dans la branchie ou dans le site d'injection. Cette diminution du nombre de transcrits de *Cg-Prp* pourrait être liée à une inhibition de l'expression de ce gène par le pathogène, comme démontré dans d'autres

espèces (Islam *et al.*, 2001; Salzman *et al.*, 2003a). Cette diminution de transcrits peut également s'expliquer par une migration des hémocytes qui expriment *Cg-Prp* vers d'autres tissus non analysés dans notre étude. Des analyses complémentaires apparaissent donc nécessaires pour répondre à ces questions et pour déterminer notamment l'influence des méthodes de stimulation sur les profils d'expression de *Cg-Prp* (balnéation *versus* injection et bactéries tuées *versus* bactéries pathogènes). Nos résultats mettent en évidence l'importance des propriétés chimiotactiques des hémocytes dans la réponse immunitaire de l'huître. L'expression constitutive des AMPs dans les hémocytes et l'existence de différentes propriétés chimiotactiques des hémocytes ont également été décrites chez la moule, *Mytilus galloprovincialis* (Mitta *et al.*, 2000c). Chez ce bivalve, les hémocytes sont les principaux producteurs des AMPs (défensines, mytilines et myticines). L'infection expérimentale des moules n'entraine pas d'augmentation du nombre de transcrits de défensines ou de mytilines, mais de nombreux hémocytes qui expriment les mytilines migrent et s'accumulent autour du site d'injection (Mitta *et al.*, 2000a; Mitta *et al.*, 2000b). Une distribution différentielle des hémocytes exprimant les mytilines et les défensines a clairement été établie (Mitta *et al.*, 1999b; Mitta *et al.*, 2000d). Ainsi, alors que de nombreux hémocytes exprimant les défensines infiltrent les épithéliums des diverticules digestifs, les hémocytes qui expriment les mytilines et les myticines sont beaucoup moins ou pas représentés dans ces épithéliums alors qu'ils sont bien représentés dans les branchies. De plus, les mytilines et les défensines sont observées colocalisées dans quelques hémocytes granulaires. Ainsi, comme chez la moule, les antimicrobiens de l'huître semblent être exprimés dans différentes populations hémocytaires qui transportent les divers AMPs dans différents organes et tissus de l'animal en réponse à une infection et/ou une blessure.

Rôle des antimicrobiens dans la défense épithéliale de l'huître

Outre les hémocytes, différents tissus de l'huître semblent exprimer des antimicrobiens (Gueguen *et al.*, 2006b; Gonzalez *et al.*, 2007b). De manière intéressante, dans tous les tissus que nous avons examinés, des effecteurs antimicrobiens avec des spectres d'activité complémentaires ont été détectés par qPCR. Ainsi, dans le manteau, *Cg-Defm* est co-exprimé avec la protéine *Cg*-BPI. Il est connu que la première ligne de défense chez les bivalves est déterminée par des barrières protectrices comme le manteau ou les branchies, et par le mucus (Glinski and Jarosz, 1997). Le rôle des AMPs dans la réponse épithéliale locale contre les infections a été décrit chez plusieurs vertébrés et invertébrés. Chez les mammifères, les cellules épithéliales de la peau et des voies respiratoire, alimentaire, et urinaire synthétisent et libèrent des AMPs (Ganz, 2002; Howell *et al.*, 2003; Zasloff, 2006). Chez les insectes, plusieurs AMPs sont exprimés de manière constitutive dans des tissus et sont modulés en réponse à une infection (Uvell and Engstrom, 2007). Chez la drosophile, différents AMPs peuvent être induits dans des épithéliums de façon tissu spécifique, et tous les épithéliums

expriment au moins un de ces AMPs (Tzou *et al.*, 2000). Chez l'huître, le manteau et les branchies sont des tissus impliqués dans des fonctions physiologiques essentielles, comme la sécrétion de la coquille et un rôle sensoriel pour le manteau, et l'acquisition d'aliments et l'échange gazeux pour les branchies. Ces tissus sont fortement exposés aux microorganismes dans le milieu aquatique. Ainsi, l'expression et la colocalization d'effecteurs antimicrobiens dans ces tissus/organes exposés suggère que **les AMPs pourraient avoir une participation locale dans la première ligne de défense d'huître.**

En raison des résultats obtenus concernant les forts taux de transcrits de *Cg-Defhs* observés dans les branchies, nous avons développé une méthode de purification d'antimicrobiens cationiques à partir de ces tissus. Dans plusieurs espèces d'invertébrés un grand nombre d'AMPs ont été caractérisés par des approches biochimiques (Bulet *et al.*, 1992; Destoumieux *et al.*, 1997; Mitta *et al.*, 1999a; Boulanger *et al.*, 2004; Cytrynska *et al.*, 2007). Nous avons identifié un mélange d'antimicrobiens qui appartiennent au groupe des AMPs dits «non conventionnels» à savoir, des histones et des protéines ribosomales, mais nous n'avons pas détecté de *Cg*-Defhs comme attendu. Ainsi, d'après cette approche il apparaît que l'huître peut disposer de protéines et de fragments de protéines, autres que des AMPs conventionnels, qui pourraient contribuer à la protection immunitaire épithéliale. La réponse immunitaire dans les épithéliums est fortement liée à la réparation des blessures. La surface épithéliale est régulièrement soumise à des risques d'abrasion, et les histones et protéines ribosomales pourraient permettre de lutter contre les pathogènes lors des dommages des cellules épithéliales. Ces résultats préliminaires sur l'implication de ces AMPs non conventionnels doivent être approfondis. Il sera notamment nécessaire de confirmer leurs activités à partir de peptides purifiés et/ou synthétiques et de déterminer s'ils sont présents dans d'autres tissus/organes et de façon constitutive ou induite en réponse à des infections.

Les antimicrobiens et la microflore endobionte de l'huître

Les huîtres vivent en contact permanent avec l'environnement marin, et par conséquence elles sont continuellement exposées à une grande variété de microorganismes incluant des pathogènes potentiels. Un grand nombre des bactéries trouvées dans cet environnement appartient à la microflore endobionte de l'huître et au cours de l'évolution, les huîtres ont pu développer un système immunitaire efficace leur permettant de discriminer les microorganismes qui leur sont bénéfiques et ceux qui peuvent être pathogènes. L'expression constitutive des AMPs dans des épithéliums contribue probablement au contrôle de la microflore endobionte, en conférant une première ligne de défense contre les pathogènes (Zasloff, 2006). Chez les mammifères, l'expression constitutive des AMPs dans des tissus exposés contribue à la régulation du nombre et du type de microorganismes et ainsi au maintien d'un environnement antimicrobien qui prévient une forte colonisation (Salzman *et al.*, 2007; Duerkop *et al.*, 2009). Par ailleurs, la microflore endobionte

contribue à la régulation de l'homéostasie de l'hôte et à la défense contre les pathogènes, en étant en compétition avec les pathogènes pour les sites d'accrochage et les substances nutritives, et par la synthèse de bacteriocines (Bevins, 2006). Chez le moustique *Anopheles*, la microflore intestinale stimule l'expression des AMPs dans l'épithélium, ce qui permet de lutter plus efficacement contre le parasite *Plasmodium falciparum* (Dong *et al.*, 2006b). L'huître pourrait ainsi probablement maintenir sa microflore endobionte à un niveau basal lui permettant de contrôler le développement de bactéries pathogènes de l'environnement.

Hypothèse sur le rôle des antimicrobiens dans la réponse antimicrobienne de l'huître

De l'ensemble de nos connaissances, il apparaît que la réponse antimicrobienne de *C. gigas* diffère de celles décrite chez d'autres espèces d'invertébrés. Nous avons discuté précédemment que des réactions de migrations sont observées chez l'huître comme chez la moule en réponse à une stimulation ou infection. Chez la moule, *M. galloprovincialis*, les AMPs sont constitutivement exprimés dans les hémocytes où ils sont stockés. Chez l'huître, l'expression de *Cg-Prp* et *Cg-BPI* apparaissent modulés. Les hémocytes de moule qui contiennent la mytiline migrent rapidement vers le site d'injection bactérienne où elles peuvent exercer une activité microbicide par phagocytose, mais les mytilines sont également abondamment libérées dans l'environnement immédiat des hémocytes par exocytosis (Mitta *et al.*, 2000b). En effet, des infections expérimentales ont démontré l'augmentation de la concentration plasmatique de mytilines et de défensines. Nous n'avons jamais mis en évidence de libération de peptides dans le plasma des huîtres, ce qui pourrait être du également à un défaut de détection lié à leur trop faible concentration. D'après leurs résultats, Mitta *et al.* ont proposé que les granulocytes qui contiennent seulement des mytilines et les granulocytes qui contiennent des mytilines et des défensines soient impliqués dans différentes phases de la réponse antimicrobienne. Les premiers seraient impliqués dans une phase de réponse précoce caractérisée par une migration vers les sites d'infection et par la phagocytose des microorganismes. Les granulocytes à mytilines et défensines seraient impliqués dans une étape postérieure de réponse systémique caractérisée par la libération des peptides (Mitta *et al.*, 2000c).

Des réponses systémiques ont été observées chez différentes espèces. Chez la drosophile, une infection bactérienne déclenche l'expression de gènes des AMPs qui sont rapidement synthétisés par le corps gras et immédiatement sécrétés dans l'hémolymphe, contribuant à une réponse systémique (Hoffmann, 2003). Chez la limule, *Tachypleus tridentatus*, les AMPs sont produits et stockés dans les hémocytes qui dégranulent et libèrent des AMPs dans le plasma suite à une infection (Iwanaga, 2002). Chez la crevette, les penaeidines sont produites et stockées dans des

hémocytes granulaires, et elles sont libérées dans le plasma et les tissus en réponse à une infection par *Vibrio* par un phénomène de degranulation intracellulaire suivi de la lyse hémocytaire (Destoumieux *et al.*, 2000; Munoz *et al.*, 2002; Munoz *et al.*, 2004). De plus, la stimulation de processus d'hématopoïèse en contribuant à une invasion massive des tissus des crevettes par des hémocytes exprimant des pénaieidines conduit à une réponse systémique à l'infection.

Chez l'huître, nous n'avons aucune évidence d'une réponse systémique. Après une infection bactérienne, nous n'avons jamais observé d'augmentation des AMPs dans le plasma ni de prolifération hémocytaire, et les concentrations dans les tissus et hémocytes semblent assez réduites. En revanche, chez l'huître, **la réponse antimicrobienne semble fortement liée aux processus de phagocytose des hémocytes.** Les AMPs d'huître pourraient avoir un rôle intracellulaire dans la réponse antimicrobienne, mettant en jeu des synergies entre antimicrobiens. Ces réactions se feraient de façon coordonnée avec d'autres réactions microbicides, liées aux enzymes lysosomiales et aux espèces réactives d'oxygène (ROS). En parallèle, l'expression et la colocalization de l'ensemble des AMPs dans des tissus exposés de l'huître suggèrent qu'ils pourraient avoir aussi une participation locale dans la réponse antimicrobienne.

Notre hypothèse est renforcée par des travaux de pathologie, qui semblent montrer que les réactions hémocytaires de l'huître seraient la cible de la pathogenicité de *V. splendidus* et *V. aestuarianus* (Schott *et al.*, 2003; Labreuche *et al.*, 2006) (*Duperthuy et al., cf. Annexes*). Ces deux pathogènes, associés aux mortalités estivales, semblent être capables d'échapper aux réactions hémocytaires. *V. splendidus* envahit les hémocytes d'huître par des propriétés adhésives de la protéine majoritaire de la membrane externe, OmpU. Puis, cette bactérie conserve sa viabilité à l'intérieur des hémocytes par l'inhibition de la formation du phagolysosome et par l'atténuation de la production d'espèces réactive oxygénées (ROS), deux mécanismes impliqués dans l'élimination de bactéries après phagocytose (*Duperthuy et al., cf. Annexes*). Par ailleurs, l'échappement de *V. aestuarianus* à la réponse immunitaire de l'huître fait intervenir l'inhibition de la phagocytose par la sécrétion de produits extracellulaires (Labreuche *et al.*, 2006). Ainsi, ces deux pathogènes ont développé différentes stratégies qui réduiraient la probabilité de rencontrer les AMPs de l'huître à l'intérieur des hémocytes. De plus, de nombreux travaux révèlent une grande variété de mécanismes de résistance aux AMPs (Yeaman and Yount, 2003; Kraus and Peschel, 2006; Peschel and Sahl, 2006) et nous avons pu montrer que les *Vibrios* présentent une forte résistance *in vitro* aux AMPs d'huître (Duperthuy *et al.*, 2010 ; Schmitt *et al.*, 2010b). Cependant, les antimicrobiens de l'huitre possèdent des activités vis-à-vis d'un large spectre de bactéries pouvant appartenir à sa microflore, suggérant leur implication potentielle dans le maintien de l'homéostasie d'huître par le contrôle et la régulation de sa microflore.

Perspectives

Les résultats obtenus au cours de cette thèse représentent une contribution à la connaissance des bases moléculaires de l'immunité de l'huître *C. gigas*, par la caractérisation de la diversité de trois effecteurs antimicrobiens et par l'étude du rôle de cette diversité dans la réponse antimicrobienne de l'huître. A partir des résultats obtenus, nous avons progressés sur la participation des AMPs et le rôle de leur diversité dans la réponse immunitaire de l'huître. Néanmoins, de nombreuses questions restent à élucider.

Fonction immunitaire des antimicrobiens d'huître

Une des difficultés majeures rencontrées pour caractériser la réponse antimicrobienne a été notre impossibilité de détecter et localiser les peptides et protéines antimicrobiens dans les tissus de l'huître. Une attention particulière doit ainsi être portée à la détection et à la localisation ultrastructurale des antimicrobiens dans les tissus d'huître en réponse à des stimulations et infections par des pathogènes. De telles données aideront considérablement à clarifier les différents comportements migratoires des populations hémocytaires. Nos résultats mettent en évidence que la caractérisation des populations ou des lignées hémocytaires, encore jamais vraiment élucidée chez *C. gigas*, reste de prime importance.

Des progrès ont été réalisés concernant la réponse antimicrobienne de l'huître par la caractérisation de nouvelles familles d'antimicrobiens, appartenant au groupe des AMPs non conventionnels (peptides dérivés d'histones et de protéines ribosomales). Des études complémentaires devront être mises en place afin de mieux comprendre leur rôle dans la réponse immunitaire de l'huître et leur interaction avec les AMPs conventionnels dans la lutte contre les pathogènes.

Dialogue entre les antimicrobiens et la microflore de l'huître

Dans de nombreuses espèces, il existe des preuves indiquant que les AMPs interagissent avec la microflore endobionte (Dong *et al.*, 2006b; Duerkop *et al.*, 2009). Cependant, nous connaissons relativement peu l'organisation des communautés microbiennes qui composent la microflore de l'huître et encore moins les facteurs impliqués dans la régulation des bactéries associées aux huîtres. Chez cette espèce qui vit en contact permanent avec une grande variété de microorganismes, il est particulièrement important de comprendre l'interaction des AMPs avec la microflore et les mécanismes permettant de maintenir l'homéostasie de l'huître et de discriminer les bactéries pathogènes des commensales.

Rôle des AMPs dans l'élimination des *Vibrios* pathogènes

Plusieurs questions restent à élucider, notamment la réelle implication des AMPs dans la capacité des huîtres à résister aux infections, notamment aux Vibrioses. Ceci pourra être clarifié par le développement de la technique d'interférence ARN (RNAi). Cette technique a été utilisée avec succès chez l'huître pour démontrer l'implication du gène *vasa* (*oyvlg*) dans le développement des cellules germinales (Fabioux *et al.*, 2009). En ce qui concerne des AMPs, le rôle des crustines dans la protection de la crevette contre l'infection par *V. penaeicida* a été mise en évidence par cette même technique (Shockey *et al.*, 2009). La technique du RNAi devrait également permettre de comprendre le rôle des AMPs dans le maintien de l'homéostasie et son dialogue avec la microflore endobionte.

Rôle potentiel des AMPs dans la résistance de l'huître aux mortalités estivales.

Nous avons maintenant l'évidence que *Cg*-Defs et *Cg*-Prp sont des familles multigéniques d'AMPs qui présentent une grande variation du nombre de copies de gènes. Les variations du nombre de copies des gènes de l'immunité pourraient contribuer aux différences de capacités de défense observées entre des individus(Ballana *et al.*, 2007). Cependant, contrairement à ce que l'on pourrait imaginer, un plus grand nombre de copies n'est pas forcement lié à une meilleure protection. En effet, une augmentation du nombre de copies des gènes des α- et β-défensines humaines a été associée à une plus grande susceptibilité à la septicémie sévère et au psoriasis (Hollox *et al.*, 2008; Chen *et al.*, 2010). Par conséquence, comme une base génétique semble exister dans la susceptibilité des huîtres aux mortalités estivales, il pourrait être intéressant de déterminer la variation de nombre de copies de gènes d'AMPs entre des populations d'huîtres de différents génotypes associés aux capacités de survie. Dans ce contexte, nous pouvons proposer de comparer des huîtres appartenant à des lignées « R » résistances et « S » sensibles aux mortalités estivales qui ont été sélectionnées dans le cadre du projet MOREST (Boudry *et al.*, 2007). Les données sur le nombre de copies des gènes codant des AMPs entre ces deux familles pourraient apporter un éclairage sur le rôle des AMPs dans les capacités de survie des huîtres aux mortalités estivales.

Enfin, les données obtenues récemment au laboratoire suggèrent une importante variabilité inter-individuelle du polymorphisme de séquences de certains des antimicrobiens, incluant parfois la présence ou l'absence de groupes complets de variants d'AMPs au niveau de l'ADN génomique. Ainsi, l'analyse de la diversité des AMPs réalisée à un niveau individuel donnera probablement de nouvelles pistes pour comprendre le rôle de cette diversité dans la défense de l'huître. Par ailleurs, une grande variabilité individuelle des niveaux d'expression des gènes codant les AMPs a également été observée entre des huîtres. Ces nouvelles données renforcent donc l'hypothèse que la présence ou absence de certains variants d'AMPs pourrait avoir un impact sur la résistance des huîtres.

Il apparaît aujourd'hui que les travaux sur la réponse antimicrobienne des huîtres doivent être menés à un niveau individuel. La prise en compte de la forte variabilité inter-individuelle devrait permettre de comprendre les mécanismes physiologiques et/ou génétiques qui contribuent à la résistance aux maladies et à la survie de l'huître *C. gigas* dans les conditions d'élevage.

General Introduction

Among the 15 oyster species cultivated worldwide, the Pacific oyster *Crassostrea gigas* represents 98 % of total world production with an output of 4.23 million tons in 2008. Nearly 3.9 million tons were produced in China that year, making this country the world's biggest producer. South Korea, Japan and France are also important oyster producing nations, with a production ranging from 116 000 to 314 000 tons (FAO, 2009). In France, the rearing of this species is the main aquaculture activity. It was introduced in the country in the early 1970s, replacing the cultivation of the Portuguese oyster (*Crassostrea angulata*), which was seriously affected by a viral disease. The importation took place from 1971 to 1975, with stocks from Canada and Japan (Grizel and Heral, 1991).

Aquaculture is subjected to the continual emergence of new diseases, which can be difficult to control due to the susceptibility of animals according to their developmental or maturation stage, and the diversity of and permanent contact with pathogens. In parallel, as aquatic animals in intensive culture, oysters are under continuous stress, which increases their susceptibility to a broad variety of infectious diseases and/or opportunistic parasites.

Over the past 40 years, the French oyster culture has regularly suffered from events of summer mortality. Summer mortalities have been known for decades in a number of different countries and are observed with more or less intensity from one year to another. However, since 2008 in France, mortalities have become a serious threat for the economic sustainability of oyster aquaculture. Over the three past 3 years, mortality events have been exceptional in terms of i) extent, all oyster cultures having lost 60 to 80 % of their spat stock, ii) death of juveniles (up to 18 months), which massively died independently of their origins, and iii) synchronized occurrence, all the French water coasts and ecosystems being affected in a similar period of time. Thus, the current mortalities are the most serious since the introduction of *C. gigas* in France.

The mortalities before 2008 have been studied in a multidisciplinary research program called MOREST, for MORTalités ESTivales (summer mortalities). The conclusions were that summer mortalities result from multiple factors, including oyster genetics, elevated water temperature, physiological stress associated to reproductive maturation, aquaculture practices, pathogens (the OsHV1 herpes virus, as well as the bacterial pathogens *Vibrio splendidus* and *V. aestuarianus*) and pollutants (Samain and McCombie, 2007). Mortalities since 2008 are somewhat different, because they clearly appear dominated by both viral (OsHV-1, and a recently identified variant, OsHV-1 µvar) and bacterial (*V. splendidus*) infectious agents. Bacteria were isolated from moribund oysters during summer mortalities events and their virulence has been confirmed by oyster experimental infections (Gay *et al.*, 2004a; Gay *et al.*, 2004b). Over the three past years, mortality events were much higher than during the 2001-2005 crises, displaying 60-100% death rates.

Because of the lack of adaptive immune system based on antibody production in invertebrates, vaccination is unfeasible in oysters. Strategies to improve the resistance of oysters must use other routes, such as prevention and selective breeding. This requires an increased knowledge of the molecular bases of their immunity. Research efforts have been made over the past years in that sense. In this context, the research team of Evelyne Bachère "Immune Response Aquaculture, Environment" has identified several effectors of the oyster immune response including antimicrobial peptides and proteins.

The objectives of my thesis have been to identify and characterize the molecular diversity of the *C. gigas* antimicrobials and to understand the importance of this diversity in oyster immunity. For this, I have studied three families of antimicrobial peptides (AMPs) and proteins previously identified in *C. gigas*: *Cg*-Defensins (*Cg*-Defs) (Gueguen *et al.*, 2006b; Gonzalez *et al.*, 2007a), *Cg*-Proline Rich peptides (*Cg*-Prp) (Gueguen *et al.*, 2009), and a Bactericidal Permeability Increasing protein, *Cg*-BPI (Gonzalez *et al.*, 2007b). The work presented here was performed in the Lagoon Ecosystems laboratory, a Research Unit (UMR 5119) affiliated to the IFREMER (Institut Francais de Recherche pour l'Exploitation de la Mer), the CNRS (Centre Nationale de Recherche Scientifique), the IRD (Institut de Recherche pour le Développement) and the University of Montpellier II, directed by Marc Troussellier.

In the **first chapter** of my thesis (state of art), I present an updated review of (i) oyster aquaculture, infectious agents and epizooties (ii) the biology and immune defense of oysters (iii) antimicrobial peptides in the innate immune response and (iv) molecular diversity in immune systems. In **chapter 2**, I present the results obtained during my graduate studies, divided into three sections. The **first section** deals with the results obtained on the molecular diversity of *C. gigas* antimicrobials and the underlying mechanisms that generate this molecular diversity. In the **second section** our results on the biological activities and mode of action of oyster defensins against the Gram-positive bacteria *Staphylococcus aureus* are presented. The **third section** provides an overview of the possible role of these antimicrobials in protecting oysters against pathogenic infections through an *in vitro* analysis of the biological properties, transcript expression and localization of *Cg*-BPI, *Cg*-Prp and *Cg*-Defs after a *Vibrio* challenge. The third section finishes with complementary data on the identification of new antimicrobial peptides from gill tissues from oyster. The **chapter 3** of this thesis presents a general discussion of the results obtained and a presentation of some future prospects on the implication of antimicrobials in the oyster defense reactions. Finally, the **chapter 4** presents the materials and methods used in this work.

Chapter 1

State of Art

I. The pacific oyster *Crassotrea gigas*: aquaculture, infectious agents and epizooties

I.1. The aquaculture of *C. gigas* in France

I.1.1. History

The consumption and culture of oysters are not recent. Indeed, archaeological studies show their consumption on the European and French territory 2000 years ago (Schneider and Lepetz, 2007). As early as the first century, Romans collected and exported oysters to the Empire, being captured on boards of the Italian coast (Héral, 1989). The credit of having been the first to lay out artificial oyster beds is given to Sergius Orata at the end of the first century. He was a famed merchant and hydraulic engineer of the Roman Republic and he was well-known by his contemporaries because of the breeding and commercialization of oysters. Orata developed a system for breeding oysters, constructing channels and preys to control sea tides, as well as high vaults over Lucrine Lake, Italy, so that fresh oyster could be always available (Gunther, 1897).

In France, the exploitation of natural stocks was the major source through the Middle Age and the Renaissance. It was not until the 17th century that oyster culture really began, first in the pools of the salt marshes of the Atlantic coast and then in specially managed ponds. The history of French oyster culture is complex. It consists in a succession of developmental phases using different species, followed by collapses caused by diseases. The endemic species *Ostrea edulis* was first replaced first by *Crassostrea angulata*, then *C. gigas* **(Fig 1)**. The first species, harvested from time immemorial by dredging natural beds, was the native flat oyster *O. edulis*. Oyster beds have been overexploited since the 18th century, especially along the Atlantic coast, and despite a long series of restrictive regulations, landings became poor and irregular during the 19th century (Goulletquer and Héral, 1997).

Flat oyster culture was well developed at the turn of the 19th century, but a crisis hit oyster populations throughout Europe after 1920, when massive, unexplained mortalities were reported (Bouchet *et al.*, 1997). The species then disappeared from the Arcachon and Marennes regions. However, spat capture restarted later in southern Brittany and in the Mediterranean until 1950, when high mortalities occurred again, putting an end to production (Fauvel, 1985).

The first imports of the Portuguese cupped oyster *Crassostrea angulata* into Arcachon were recorded in 1860 and were made to compensate for the scarcity of flat oysters. The species then colonized the Atlantic coastline and both species, *O. edulis* and *C. angulata*, were produced concomitantly. The Portuguese cupped oyster replaced the endemic species in the main culture sites

after the 1920 mortalities. Production gradually decreased due to a viral disease caused by an iridovirus, which produced an outbreak in 1966 (Comps *et al.*, 1976). This disease spread throughout all culture zones, causing massive mortalities between 1970 and 1973, and leading to the total extinction of *C. angulata* in France.

The oyster industry reacted quickly to this crisis with the massive introduction of a new species, *C. gigas*, from the Pacific. After some small-scale trials (1966–1970), several hundred tonnes of *C. gigas* were imported from Canada between 1971 and 1973 (Grizel and Heral, 1991). The species became established and an abundant spat settlement in Marennes-Oléron allowed healthy, fast-growing oysters to be produced. Meanwhile, 10.000 tonnes spat were imported from Japan, and production increased quickly. Spat capture developed rapidly in Arcachon and Marennes, so that further spat imports became unnecessary (Buestel *et al.*, 2009). Hence, despite several crises, France has always produced large quantities of oysters.

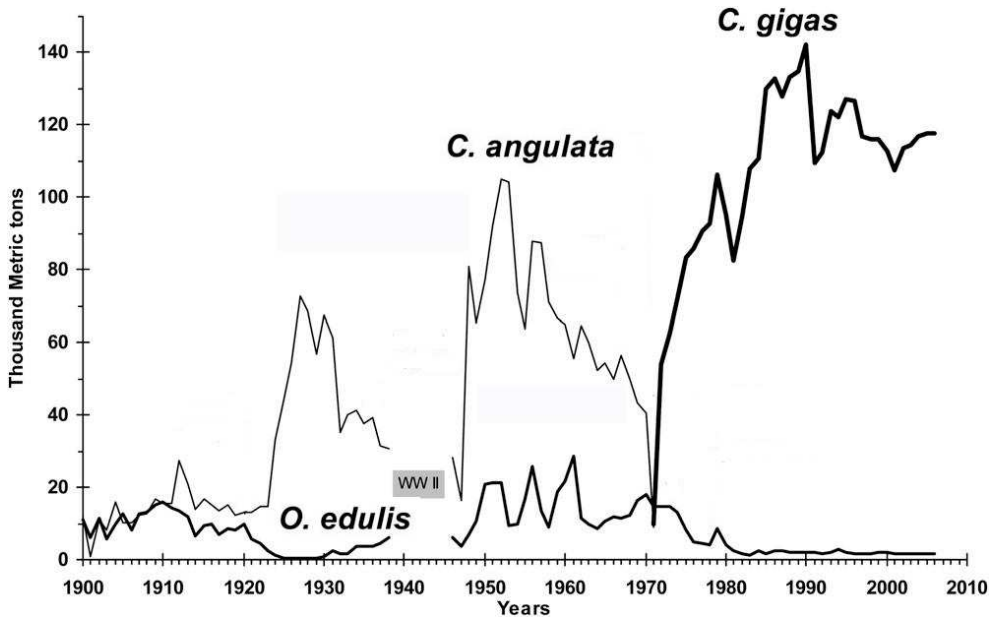

Figure 1. Historical trends of French oyster production. Adapted from Buestel *et al.*, 2009

Figure 2. Seven regions of oyster production along the French water coasts.

I.1.2. Oyster production and cultural practices

Nowadays, seven regions of oyster production with different characteristics can be distinguished along the French water coasts. These include (i) Normandy, (ii) North Brittany, (iii) South Brittany, (iv) Vendée, (v) Charente-Maritime (Ile de Ré and Marennes-Oléron), (vi) Arcachon and (vii) the Mediterranean sea (Thau, Leucate, and Corsica) **(Fig. 2)**.

C. gigas are produced all along the French coast from natural and hatchery spat. Various structures are used to collect spat from the wild. Over the past years, hatchery oyster spat has been increasingly used. Six French hatcheries dedicated to spat production transfer animals in a large-scale manner to the different farming zones (Buestel *et al.*, 2009). The spat production consists of diploid, and increasingly, triploid oysters, with three base genomes corresponding to 30 chromosomes per cell, which are now commonly produced in hatcheries. Triploid oysters are obtained by crossing female diploid oysters and male tetraploid oysters (Guo *et al.*, 1996). Successful tetraploid induction has been achieved in *C. gigas* by chemical treatment on a breeding of a fertile triploid female with a

diploid male (Guo and Allen, 1994), or by cytochalasin B inhibition of polar body 2 expulsion in diploid females bred with tetraploid males (McCombie *et al.*, 2005). Triploidy essentially confers a quicker growth rate and near sterility to Pacific oysters, endowing the animals with a more consistent quality (Gagnaire *et al.*, 2006).

After a growing-on period, oysters are cultivated by three main methods, namely (i) on-bottom culture in the intertidal zone or in deep water, (ii) off-bottom culture in plastic mesh bags in the intertidal zone, or (iii) suspended culture on ropes in the open sea (Buestel *et al.*, 2009). Over their lifespan, oysters are moved from one place to another to satisfy various production and quality criteria. Transfer of spat stock between basins are commonly performed to obtain a local-level geographical origin, such as the Indication Geographique Protegee (or IGP, an European Union label) or "Appellation d'Origine Controlée" (or AOC, granted by the French Minister of Agriculture). Thus, there is significant product variability depending on transfers and the associated environmental conditions (Buestel *et al.*, 2009). By the time they are available for consumers (about two years old), oysters have often be subjected to 4 to 6 transfers along the French farming zones.

I.1.3. Infectious agents and diseases in oyster species

Altogether, the development of intensive cultures, the degradation of environmental conditions, the development of high density hatcheries and the large-scale transfers of animals among the farming zones, facilitate the proliferation and the transmission of pathogens and as a consequence, the appearance of diseases. Oysters are filtering animals that need high volumes of water for their nutrition and respiration. Consequently, they are in permanent interaction with the marine environment and are in continuous contact with abundant microorganisms, which can develop diseases. Oyster pathogens include protozoans, viruses, and bacteria, which affect all stages of production, from larvae to juveniles and adult oysters. The following infectious agents have been isolated from different oyster species.

I.1.3.1. Protozoans

Several parasitic diseases produced by protozoans have been described in oyster culture. *Mikrocytos mackini* parasites the connective tissue and causes lethal infection of hemocytes and tissue necrosis, producing high mortalities of *C. gigas* in North America (Quayle, 1961; Farley *et al.*, 1988). *Marteilioides chungmuensis* causes the ovary enlargement disease of the Pacific oyster (Comps *et al.*, 1986). Although the latter disease was not reported to cause high mortalities, infected oysters develop abnormal gonads, resulting in a disfigured appearance and poor market appeal (Itoh *et al.*, 2004).

In *C. virginica*, the protozoan *Perkinsus marinus* is responsible for the disease commonly referred as “Dermo” or “Perkinsosis”, which produces high mortalities in North America and in the Mexican gulf (Andrews, 1988). The effects of infection in *C. virginica* range from pale appearance of the digestive gland, reductions in condition index, impaired gametogenic development, and altered hemolymph protein concentrations and lysozyme activity, to severe emaciation, gaping, retarded growth and occasionally the presence of pus-like pockets (Ford and Tripp, 1996).

Another protozoan that causes mortalities in *C. virginica* is *Haplosporidium nelsoni*, which generates the multinucleated sphere unknown disease (MSX) and progressively destroys the epithelia of digestive diverticula (Barber *et al.*, 1991).

In *O. edulis*, two protozoans have been identified as responsible for oyster rarefaction: *Marteilia refringens* and *Bonamia ostreae* (Figueras and Montes, 1988). *B. ostreae*, in conjunction with earlier epizootics caused by *M. refringens*, caused a drastic drop in the French production of *O. edulis* (Boudry *et al.*, 1996). Although many infected oysters appear normal, others may have yellow discoloration and/or perforated ulcers in the connective tissues of the gills, mantle and digestive gland. The pathology appears correlated with hemocyte destruction and diapedesis due to proliferation of *B. ostreae* (Balouet *et al.*, 1983). The Pacific oyster, *C. gigas* could not be naturally nor experimentally infected with *B. ostreae* and this species did not appear to act as a vector for this protozoan (Culloty *et al.*, 1999).

I.1.3.2. Viruses

Irido-like viruses have been associated to infections in various oyster species. One irido-like virus produces the oyster velar virus disease (OVVD), which attacks the velar epithelium of *C. gigas* midstage umbo larvae and can cause severe hatchery losses (nearly 100%) in affected tanks in North America (Elston and Wilkinson, 1985). Another disease produced by an irido-like virus is the gill disease of Portuguese oyster or gill necrosis virus disease (GNVD). This disease has been identified in France, Portugal, Spain, and Great Britain and attacks *C. angulata*. Gill disease also affects the Pacific oyster, but to a lesser extent: gill lesions were always reduced, suggesting partial resistance in this species (Comps, 1988). Gill disease produces extensive gill erosion, which correspond to high mortalities. The disease is considered to have been one factor that has contributed to the elimination of *C. angulata* from important culture areas on the Atlantic coast of France (Comps and Duthoit, 1979). Another irido-like virus produces the hemocytic infection virus disease (HIVD), reported in France and Spain. This disease caused mass mortalities of *C. angulata* in France from 1970 to 1973, followed by a report of a similar type of virus infection *in C. gigas* in France in 1977, long after the disappearance of *C. angulata* from this area. This virus produces atrophy and weakness of the adductor muscle and grayish discoloration of the visceral mass in *C. gigas* (Renault and Novoa, 2004).

Oyster Herpes Virus type 1 (OsHV-1) from *C. gigas* in France has been described and represents a major class of herpes viruses that differ from those in vertebrates (Davison *et al.*, 2005). The first description of this type of virus was reported in *C. virginica* (Farley *et al.*, 1972). In 1991, viruses belonging to the herpes-like virus group were associated with high mortality rates of hatchery-reared larval *C. gigas* in France (Nicolas *et al.*, 1992) and in New Zealand (Hine *et al.*, 1992). Since 1992, intermittent high mortalities of larval *C. gigas* have been regularly observed in some European hatcheriesocurring each year during summer in association with a herpes-like virus (Renault *et al.*, 1994). From 2001 to 2005, the OsHV-1 herpes-like virus has been identified, although not systematically, during summer mortality events in France (Friedman *et al.*, 2005). Its prevalence suddenly increased over the past years. In 2008, OsHV-1 has been detected in 76% of the analyzed oyster groups. Besides, the OsHV-1 genomic DNA sequencing allowed the identification of a new variant of OsHV-1 virus, the OsHV-1 µvar and this particular genotype has been detected in 49% of the analyzed groups the same year. In 2009, OsHV-1 virus has been detected in 96% and the new variant OsHV-1 µvar was found in 100% of the groups of analyzed oysters that died in 2009 (IFREMER, 2009).

Oyster herpes viral infections have also been reported in Pacific oyster seeds in North America and Mexico (Friedman *et al.*, 2005) and in spat and larvae of the European flat oyster*, O. edulis*, in France (Comps and Cochennec, 1993). Infected larvae show reducted feeding and swimming activities (Hine *et al.*, 1992; Nicolas *et al.*, 1992).

Papova-like or papilloma-like virus infections have been reported from the gonadal epithelia of *C. virginica* in Canada (McGladdery and Stephenson, 1994) and in North America (Meyers, 1981). This virus produces the viral gametocytic hypertrophy infection (VGH), which causes a massive hypertrophy of individual gametes and the gametogenic epithelium as the virus replicates in the host nucleus. The level of infection is generally low, with no indication of associated mortality (Choi *et al.*, 2004). Virus-like particles with characteristics of papilloma-like virus are also reported in the golden-lip pearl oyster, *Pinctada maxima* (Norton *et al.*, 1993).

I.1.3.3. Bacteria

Most bacterial diseases in oysters are produced by Gram-negative bacteria, mainly of the *Vibrio* genus. Nevertheless, a few Gram-positive bacteria are also involved in diseases. Bacterial diseases affect oysters differently according to their life stage and are commonly described in larval stages, in which they are associated with high mortalities in hatcheries (Lauckner, 1983; Sindermann, 1990). The difficulties encountered in identifying pathogenic bacteria are due to the normal accumulation of a very rich bacterial commensal microbiota composed of different species in bivalves. They belong to the genus *Vibrio, Pseudomonas, Flavobacterium, Cytophaga, Bacillus* and

Micrococcus, among others (Lauckner, 1983; Paillard *et al.*, 2004). Some of these bacteria, mainly opportunistic and pathogenic, eventually colonize and invade the host, depending on the environmental factors influencing host-bacteria interactions (Paillard *et al.*, 2004).

Disease symptoms in moribund larvae were first described in *C. gigas* by Tubiash e*t al*., who named them "bacillary necrosis", attributing the disease to bacteria from the *Vibrio* and *Aeromonas* genera (Tubiash *et al.*, 1965). Following this study, several strains belonging to the *Vibrio* genus were identified as pathogens of *C. gigas* larvae (Di Salvo *et al.*, 1978; Jeffries, 1982; Garland *et al.*, 1983; Sugumar *et al.*, 1998; Lambert *et al.*, 2001).

Three bacterial diseases have been reported to affect *C. gigas* juveniles, including hinge ligament erosion disease (Dungan and Elston, 1989), chronic abcess syndrome (Elston *et al.*, 1999) and recently, the oyster summer mortality syndrome in France (Lacoste *et al.*, 2001). Only the chronic abscess syndrome specifically affects juveniles (Elston *et al.*, 1999). The others can also affect adult bivalves. Except for summer oyster mortalities in *C. gigas*, the clinical signs of these diseases all include lesions of the mantle, shell organic matrix and ligament. The ligament disease is characterized by a destruction of the hinge ligament caused by *Cytophaga* sp.-like gliding bacteria (Elston, 1993). In summer mortalities, different bacteria, mostly *Vibrio*, have been involved. However, with exception of larvae, it remains unclear whether these bacteria act as primary pathogens or as opportunists (Paillard *et al.*, 2004).

Oyster mortalities are frequently associated to the presence of bacterial pathogens of the *Vibrio* genus, particularly *V. splendidus* and *V. aestuarianus* (Le Roux *et al.*, 2002; Garnier *et al.*, 2008). These *Vibrio* strains have been isolated from moribund oysters during summer mortalities and their virulence has been confirmed by oyster experimental infections (Gay *et al.*, 2004a; Garnier *et al.*, 2008). Besides, some of these *Vibrio* species are capable of acting in combination to improve their pathogenicity against *C. gigas* (Gay *et al.*, 2004b). The genomes of *V. splendidus* and *V. aestuarianus* have been sequenced and the complete genome of *V. splendidus* LGP32 is now accessible (Le Roux *et al.*, 2009).

Another bacterial disease referred to as Pacific Oyster Nocardiosis (PON) is produced by a bacterium belonging to the genus *Nocardia* (Friedman and Hedrick, 1991). PON is characterized by few to no external signs of infection in naturally or experimentally infected oysters. Diseased animals showed some mantle lesions with focal areas of discoloration in light infections and had large internal nodules in most tissues in heavy infections (Friedman and Hedrick, 1991).

Finally, some cases of infections produced by bacteria from the genus *Rickettsia* and *Chlamydia* have been described (Azevedo and Villalba, 1991; Renault and Cochennec, 1995). These are characterized for being intracellular and were detected in gills, digestive tract and mantle

epithelial cells. Nevertheless, level of infection is generally low with no indication of associated mortality in adult oysters (Renault and Cochennec, 1994).

I.2. Principal phenomena of summer mortalities

C. gigas summer mortalities have been reported from several decades. These episodes began along the Japanese Pacific coast in 1945 and continued along the west coast of United States in the late 1950s (Glude, 1975; Koganezawa, 1975). In France, summer mortalities were first reported in the 1980s, where they caused mass mortality of up to 90% of stocks in Arcachon and Marennes-Oléron Bays (Maurer and Comps, 1986; Bodoy *et al.*, 1990). Since then, the French oyster farms have experienced periodic mass mortalities (Saulnier *et al.*, 2009). In France, mortality events among adults generally occur during spring, while those among juveniles are more prevalent during summer (Fleury *et al.*, 2001), but mortality patterns are also characterized by a high inter-annual and inter-stock variability (Maurer and Comps, 1986).

I.2.1. The summer mortalities and the MOREST program

In order to better understand this complex phenomenon, the IFREMER (Institut Français de Recherche pour l'Exploitation de la Mer) created a multidisciplinary research program from 2001 to 2005 called MOREST, for MORTalités ESTivales (summer mortalities). Results of this program showed that summer mortalities cannot be explained by a single factor, but that several interacting factors contribute to the phenomenon. The project identified several factors involved in these events through a combination of field and experimental studies. They showed that (i) 45% of the observed variance in field mortality was due to **genetic variation among oyster families**, selecting two stocks of oysters on the basis of the highly heritable survival character: **Resistant' (R) or 'Sensitive' (S) oysters** to summer mortality (Degremont *et al.*, 2005); (ii) mortality risk factors included the **reproductive maturation status** of the oysters, which is dependent on environmental conditions, principally the **increase of water temperature and food availability**. Other risk factors were the proximity of the oysters to the sediments in the culture area (Gay *et al.*, 2004a) and (iii) the **presence of several infective agents**, in particular, strains belonging to the bacteria ***V. aestuarianus*** **and** ***V. splendidus***. Another infective agent, **OsHV-1 herpes-like virus**, has also been identified, but not systematically in all mortality episodes (Friedman *et al.*, 2005; Sauvage *et al.*, 2009). Consequently, the MOREST project finally established that *C. gigas* summer mortality is a result of multiple factors, including a significant genetic component for spat survival, elevated water temperature, physiological stress of oysters probably associated with sexual maturation, aquaculture practices, pathogens and pollutants **(Fig. 3)** (Samain and McCombie, 2007)**.**

I.2.2. The recent summer mortalities

Over the past three years, the general characteristics of summer mortality events in France differed from previous crises. The mortalities are dominated by infectious agents, both viral (OsHV-1, and a recently identified variant OsHV-1 µvar), and bacterial (*V. aestuarianus* and *V. splendidus*), but showing different prevalence rate over years.

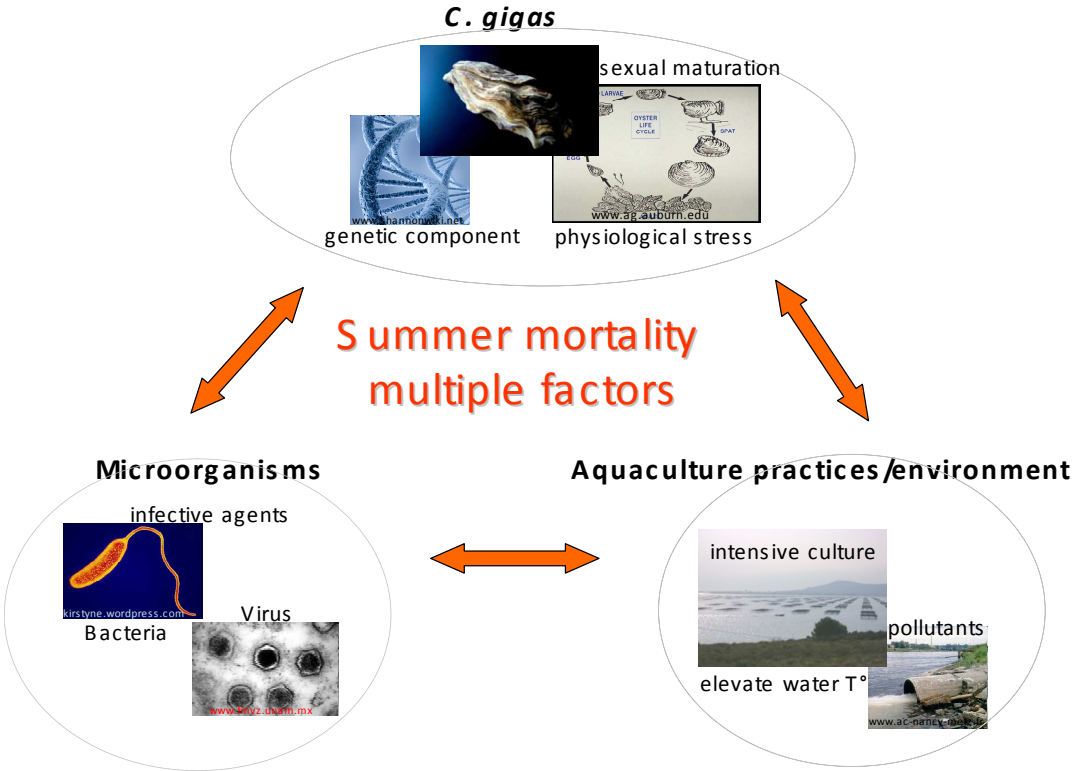

Figure 3. Interactions of factors involved in the 2001-2005 summer mortalities phenomena.

The IFREMER recently reported its findings and observations on the 2008-2009 mortalities (IFREMER, 2009). In 2008, mortality events were much higher than during 2001-2005 crises, displaying 60-100% death rate. Epidemiological studies showed that mortalities have affected the totality of cultures with the exception of various isolated zones (Corse, Prévost, Mediterranean lagoon, various sectors in North Brittany). Mortalities occurred as three major peaks over time and the spat stock aged of less than one year displayed the highest mortality rate. The development of diagnostic tools that specifically detect the virus OsHV-1, *V. splendidus* and *V. aestuarianus,* allowed the detection of the OsHV-1 virus in 76% and of *V. splendidus* in 50% of analyzed groups. Other bacterial species have been also detected but with weak prevalence (*V. aestuarianus* and *V. harveyi*). Besides, the OsHV-1 genomic DNA sequencing allowed the identification of a new variant of OsHV-1

virus, OsHV-1 µvar and this particular genotype has been detected in 49% of the analyzed groups. Finally, experimental infections transmitted from moribund oysters demonstrated the infectious and transmissible character of this disease.

In 2009, the general characteristics of summer mortality events differed from 2008, beginning earlier in the spring season. One peak was evidenced at the end of April in the Mediterranean, which then affected all the French oyster cultures, showing a progression from the South towards the North, from May in the Atlantic region and from June in the North region. Mortalities occurred very rapidly. In 2009, mortalities occurred at a sea water temperature of 16-17°C, lower than that identified in previous episodes (19°C). Like in 2008, 60-100 % of the spat stocks of less than one year died. In 2009, OsHV-1 virus has been detected in 96% of analyzed groups, *V. splendidus* in 46% and *V. aestuarianus* in 10.4%. Other bacterial species have also been detected. Regarding OsHV-1 virus, analyses from experimental groups demonstrated a positive correlation between the mortality observed and the viral load in oysters, as determined by quantitative PCR. Besides, the new variant OsHV-1 µvar was found in 100% of the groups of oysters that died in 2009. In a retrospective manner, groups of oysters collected in 1995, 2002, 2003, 2004, 2005, 2006 and 2007, from France, United States, Japan and Chine showed the complete absence of the µvar variant, supporting the infectious argument and the emergence of a new virus genotype. Nevertheless, to date, the virulence of the µvar variant has not been compared to that of OsHV-1 characterized in 2005. In 2010, mortalities events have already affected the French coasts, but the general characteristics of this phenomenon are being currently studied.

II. Biology and immune defense of oyster

II.1. The biology of oysters

II.1.1. Anatomical and physiological characteristics

The Pacific oyster *Crassostrea gigas* is a metazoan protostome belonging to the phylum Mollusca, class Bivalvia. An anatomic description of the oyster was published by Kennedy and co-authors (Kennedy *et al.*, 1996). Briefly, oysters are marine invertebrates characterized by a smooth body compressed laterally and enclosed in a **calcareous shell** consisting of two asymmetric valves articulated by a hinge and supported by an adductor muscle. The **valves** are asymmetrical and individuals are fixed onto a substrate by the convex left valve. The shell is secreted by the **mantle**, a fleshy fold of tissue which covers internal organs of the oyster. The mantle also has a sensory role and is in continuous contact with the valves although not attached to them. The large central cavity bounded by the mantle lobes is the **pallial cavity**, which contains the labial palps and gills on the ventral side and the rectum on the dorsal side. This animal is acephalic and the head is only represented by the labial palps and mouth **(Fig. 4).**

The **digestive system** starts at the mouth, an inverted U-shaped slit from where the esophagus begins directly. The **esophagus** is a dorsoventrally compressed tube that enters the anterior chamber of the **stomach**, a large sac-like organ, divided into anterior and posterior chambers. The anterior chamber gives rise to the anterior and posterior caeca, as well as to two primary ducts that lead to the digestive diverticula and the digestive gland. The posterior chamber of the stomach is more complex and it gives rise to two ducts: (i) a primary duct that leads to the digestive diverticula and (ii) an elongated outpounching duct called the **style-sac midgut**, which produces the crystalline style and rotates it against the gastric shield, releasing the carbohydrases within into the lumen of the posterior stomach. The ascending limb of the intestine arises at the common posterior chamber of the style sac and midgut and runs by an abrupt flexure anteriorly and dorsally in the visceral mass. The descending intestine opens into the rectum, which runs dorsally over the adductor muscle and ends in the anus that is located in the cloacal chamber. The **gonad** is situated in the visceral mass, between the digestive gland and the mantle. It spreads from the esophagus to the pericardial zone, forming two lobes that surround the **digestive gland**.

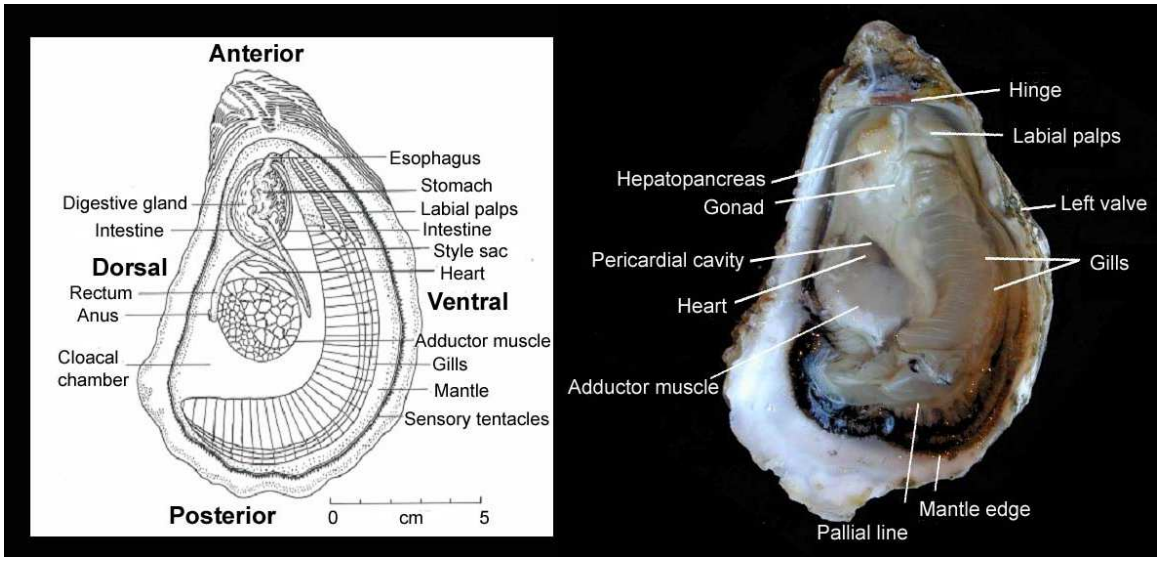

Figure 4. *C. gigas* anatomy. **A.** Schema of internal view of the left valve from an adult oyster. **B.** Photography of the same view. Adapted from (Kennedy *et al.*, 1996).

The **adductor muscle** is a dominant feature of the oyster, being placed in a posterior dorsal position. The adductor muscle consists of two types of muscle, one faster-acting than the other. Most of the adductor muscle of the Pacific oyster consists of translucent, fast muscle that is obliquely striated. To one side of the translucent muscle there is a smaller, crescent-shaped portion of opaque smooth muscle which contracts more slowly than the translucent part.

Oysters possess two **gills**, which constitute the largest organ of the oyster's body. Gills function as a site for food acquisition and gaseous exchange. Each gill is composed of inner and outer demibranchs. Each demibranch has an ascending and descending fold, termed a lamella. The dorsal margin of the descending lamellae is attached to the visceral mass at the gill axis. The ascending lamellae of both outer demibrachs are fused to the mantle. The ascending lamellae of inner demibranchs are fused to each other and the visceral mass along the central axis of the gill. These fusions divide the pallial cavity into an inhalant and exhalant cavity.

Oysters present a **semi-open circulatory system**. The systemic heart located in the cardial cavity beside the adductor muscle, pumps **hemolymph** into arteries that branch and ultimately open into sinuses; the latter are contiguous intercellular spaces filled with fluid and collectively represent the hemocoel. As a result of the periodic contraction of the muscular cardial wall and the tissues surrounding the sinuses, whole hemolymph is expelled from the body by diapedesis (Cheng, 1981). Oysters do not have respiratory pigments either dissolved in the hemolymph or contained in the **hemocytes**, their circulating blood cells.

II.1.2. The hemocytes

The classification of hemocyte populations in bivalve mollusks has been studied for several years (for review see (Cheng, 1981; Hine, 1999)). According to the classification of Hine (1999), the simple division of these cells into granular and agranular hemocytes of Cheng (1981) can be further subdivided into several sub-groups. Whereas granulocytes form a distinct group, composed of both basophilic and eosinophilic granulocytes (Bachère *et al.*, 1988), agranular hemocytes are heterogeneous in appearance and ultrastructure, characterized by the absence or the presence of few cytoplasmic granules. Three types of agranular hemocytes have been identified: blast-like cells, basophilic macrophage-like cells, and hyalinocytes. Blast-like cells present a high nucleocytoplasmic ratio and free ribosomes, suggesting that they could be precursor cells. In fact, agranular cells could correspond to a continuous series of morphologically and functionally related cells. According to their activity, they may evolve from young cells with a high nucleocytoplasmic ratio into large hemocytes with a small nucleus and a cytoplasm rich in vesicles **(Fig. 5)** (Bachère *et al.*, 2004).

The origin, life cycle and life span of bivalve hemocytes has not yet been completely elucidated. A hematopoietic organ has not been identified until now. The generally accepted belief is that hemocytes arise from differentiation of connective tissue cells, with division of blast-like hemocytes and subsequent development of immature, poorly differentiated hemocytes in the hemolymph (Cheng, 1981). However, Tirape *et al.* (2007) proposed that hematopoietic cells could instead derive from vessel endothelia cells. Indeed, *Cg-tal*, which belongs to the Tal1/SCL family transcription factors, is essential for the generation of hematopoietic cells in early embryos in vertebrates (Green, 1996) and was detected by Tirape *et al.* only in hemocytes attached to blood vessel endothelium (Tirape *et al.*, 2007).

Besides their essential role in defense (see below), hemocytes play a fundamental role in oyster vital functions like wound repair, shell recalcification, nutrient transport, digestion and excretion processes (Sminia and Van der Knaap, 1987; Cheng, 1996). Indeed, hemocytes transport calcium and proteins for shell repair, nutrients and defense molecules (McCormick-Ray and Howard, 1991; Cheng, 1996). Oyster hemocytes are found circulating in the hemolymph or infiltrating tissues. Due to the open circulatory system, infiltrating-hemocytes are present in great proportion in all cavities and tissues in oyster body.

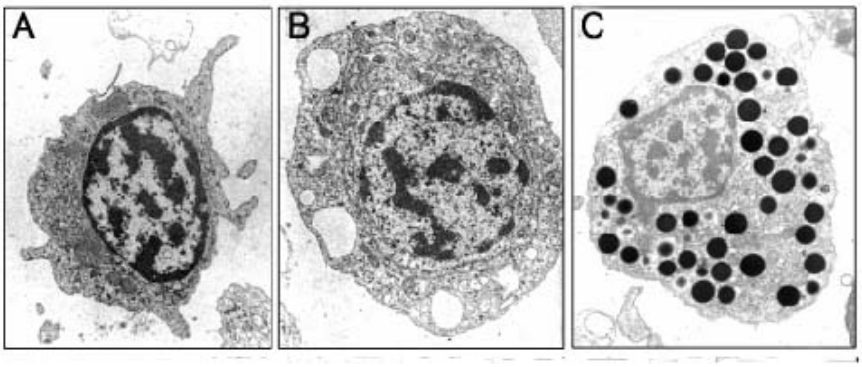

Figure 5. Types of hemocytes in *C. gigas*. **A.** Small cell, probably young hemocyte. **B.** Hyaline basophilic hemocyte. **C.** Granular hemocyte containing electron dense granules. Electronic transmission microscopy x 10800. Adapted from Bachère *et al.*, 2004.

II.2. The immune defense of oysters

II.2.1. Overview

Oysters, like all invertebrates, have a defense system based on innate mechanisms only, which consists of cellular and humoral components that interact to recognize and eliminate pathogens. They lack the acquired immunity present in higher vertebrates, which creates immunological memory to recognize specific pathogens more efficiently by the production of antibodies. Nevertheless, invertebrates have developed effective systems to discriminate advantageous microorganisms from pathogenic ones.

The innate immune system protects organisms through layered defenses of increasing specificity. The first line of defense is determined by physical protective barriers that prevent damage of the underlying tissues, body fluid losses and infections by pathogenic microorganisms. In oysters, the main physical barriers are the shell, epithelial tissues like mantle or gills, and mucus (Glinski and Jarosz, 1997). If a pathogen breaches these barriers, the innate immune system provides an immediate response with another line of defense, determined by the hemolymph, which passes out the open end of arteries, bathes all organs and returns to the heart by way of the sinuses and gills.

The hemocytes and soluble factors in the hemolymph operate in a coordinated way to provide protection (Rinkevich and Muller, 1996; Pruzzo *et al.*, 2005). Hemocytes, the immunocompetent cells, are capable of non-self recognition through lectins and chemotaxis, encapsulation and active phagocytosis (Cheng, 1981; Bachère *et al.*, 2004). They are also implicated in cytotoxic reactions such as the production of lysosomal enzymes, antimicrobial peptides and oxygen metabolites (Hine, 1999; Canesi *et al.*, 2002).

II.2.2. Non-self recognition

Non-self recognition is essential for the triggering of various innate immune responses upon exposure to microorganisms. In invertebrates, recognition relies on the binding of pattern recognition receptors or proteins (PRRs or PRPs) to pathogen-associated molecule patterns (PAMPs) (Janeway *et al.*, 2005). Since PAMPs are absent from host cells, they can serve as a means to discriminate between self and non-self. Pattern recognition molecules can be soluble in the hemolymph (PRPs) or associated with the hemocyte membranes as receptors (PRRs). PRRs and PRPs recognize PAMPs directly. They are also referred to as lectins when the recognized PAMP moiety is a carbohydrate. Their agglutination and opsonisation properties allow the immobilization of microorganisms and the activation of phagocytosis or encapsulation. Among substances that the innate immune system can recognize as PAMPs, we can mention (i) the lipopolysaccharides (LPS), toxic macromolecular complexes abundant on the external membrane of Gram-negative bacteria; (ii) lipoteichoic acid, a major constituent of the cell wall of Gram-positive bacteria; (iii) peptidoglycan, a major component of the bacterial cell wall, (iv) β-1,3-glucans, polysaccharides which constitute the cell wall of fungi; and (v) double stranded RNA (dsRNA) of replicating viruses (Beutler, 2004). PAMPs are often essential for survival of the microbes, meaning that these targets of innate immunity cannot be discarded by microbes in an effort to evade recognition by the host.

Over the past decade, several PRPs and PRRs, belonging to several classes, were identified in *C. gigas*: Lipopolysaccharide-binding proteins (Gonzalez, 2005; Gonzalez *et al.*, 2005), peptidoglycan recognition proteins (Itoh and Takahashi, 2008), β-glucan-binding proteins (Tanguy *et al.*, 2004; Itoh *et al.*, 2009), and lectins (Gueguen *et al.*, 2003; Yamaura *et al.*, 2008).

II.2.2.1. Lipopolysaccharide (LPS)-binding proteins

Two plasmatic proteins with LPS-binding properties have been found in *C. gigas*: *Cg*-SOD and *Cg*-LBP, both of which were originally isolated from oyster plasma by their binding to heat-killed *Escherichia coli* (Gonzalez, 2005; Gonzalez *et al.*, 2005).

Cg-SOD sequence was first evidenced in *C. gigas* by suppression subtractive hybridization from mantle and gonad tissue (Boutet *et al.*, 2004). *Cg*-SOD was the major protein of oyster plasma, expressed by hemocytes. The plasmatic *Cg*-SOD was shown to bind to hemocyte β-integrin (Gonzalez *et al.*, 2005). Besides its LPS-binding properties (shown by surface plasmon resonance), *Cg*-SOD was identified as an extracellular Cu/ZnSOD. Superoxide dismutases are metalloenzymes that catalyze the dismutation of superoxide into oxygen and hydrogen peroxide. Therefore, they have an important antioxidant role in cells exposed to oxygen and represent an important line of defense against Reactive Oxygen Species (ROS), which can be harmful for the tissues at elevated concentrations. It

was shown that *Cg*-SOD displays such an antioxidant activity by the inhibition on the ROS production from oyster hemocytes (Gonzalez, 2005).

Contrary to *Cg*-SOD, only biochemical data are available for *Cg*-LBP. The N-terminal amino sequence of this protein shares no sequence homology with the LPS-binding proteins already isolated, but the purified protein showed an LPS-binding ability comparable to that of known LPS-binding proteins (Tan *et al.*, 2000). To date, nothing is know about it tissue expression.

II.2.2.2. Peptidoglycan recognition proteins (PGRP)

PGRPs are pattern recognition receptors that specifically bind to peptidoglycan, the major component of the bacterial cell wall. In *C. gigas*, several full length cDNA sequences presenting homologies to these proteins were identified, referred to as *Cg*PGRP-S1S, -S1L, -S2 and -S3 (Itoh and Takahashi, 2008). Homology and domain searches classified these *Cg*PGRPs as short-type PGRPs for extracellular PGN recognition. Although phylogenetic analysis indicated that *Cg*PGRPs are closely related to each other, they showed different tissue expression patterns; *Cg*PGRP-S1S in the mantle and the gill, -S1L in the mantle, -S2 in the hemocytes and -S3 in the digestive diverticula, suggesting that *Cg*PGRPs might survey bacterial invasion in specific tissues. Interestingly, defensin-like domains were found in *Cg*PGRP-S1S and -S1L, suggesting that they may also function as antimicrobial proteins. In addition, a novel PGRP cDNA sequence was identified, referred to as *Cg*PGRP-L. A conserved domain search identified amidase_2/PGRP and goose-type (g-type) lysozyme domains in its structure. Catalytic residues for PGRP and g-type lysozyme are well conserved, suggesting that *Cg*PGRP-L may have both binding and lytic functions against bacteria (Itoh and Takahashi, 2009).

II.2.2.3. β-glucan-binding proteins (βGBP)

Recognition proteins belonging to this group have been identified by different approaches in various marine bivalve species (Su *et al.*, 2004; Zhang *et al.*, 2007; Jayaraj *et al.*, 2008) including oysters (Tanguy *et al.*, 2004; Itoh *et al.*, 2009). In *C. gigas*, *Cg*-βGBPs present a signal peptide domain, motifs for polysaccharide binding, glucanase, β-glucan recognition and catalytic residues, which are typical features of invertebrate βGBPs. The further characterization of two variants (*Cg*-βGBP-1 and *Cg*-βGBP-2) revealed that they have different structural and functional features (Itoh *et al.*, 2009). While *Cg*-βGBP-1 presents two possible integrin recognition sites, *Cg*-βGBP-2 does not. In addition, these variants are expressed in different tissues; *Cg*-βGBP-1 is expressed in the hemocytes and *Cg*-βGBP-2 is expressed in the digestive gland. Both variants show β-glucan binding ability. In addition, by testing both variants against hemocytes under presence of laminarin as modulator, authors identified the enhancement of phenoloxidase (PO) activity only for r*Cg*-βGBP-2, which was interpreted as a possible difference in physiological functions.

II.2.2.4. Other lectins

In *C. gigas*, two additional lectins, gigalin E and H, have been characterized. They display agglutination properties for mammalian erythrocytes and bacteria including *Vibrio anguillarum* (Olafsen *et al.*, 1992). Two other types of lectins, named ficolins and galectins, have been identified by expressed sequence tag (EST) analysis (Gueguen *et al.*, 2003; Roberts *et al.*, 2009). Ficolins are lectins with collagen-like and fibrinogen-like domains, and might operate as opsonins (Teh *et al.*, 2000). Galectins are found in both vertebrates and invertebrates and are characterized by a specific affinity for β-galactoside sugar structures. Galectins are synthesized and stored in the cytoplasm, but upon infection or tissue damage, they are either passively released by dying cells or actively secreted by inflammatory activated cells, acting as PRR and as immunomodulators in the innate response (Sato *et al.*, 2009). Several studies indicate that galectins may be involved in various immune-related phenomena including adhesion, wound repair and encapsulation reactions (Vasta *et al.*, 1999; Sato *et al.*, 2002). Furthermore, an increase of galectin transcript abundance was evidenced in *C. gigas* hemocytes in response to immune challenge (Gueguen *et al.*, 2003: Yamaura, 2008 #620).

II.2.3. Cellular communication

Recognition of pathogens by soluble PRP or cellular PRR triggers a series of cellular and humoral responses (exposed above) to circumvent the infection. While such a triggering is usually direct when PRRs are involved, it requires cellular communication events when soluble PRPs mediate the response. Those events may involve cell surface receptors such as integrins and soluble communication molecules such as cytokines.

II.2.3.1. β-Integrins

Integrins are cell adhesion receptors that are evolutionary old and that play important roles during developmental and pathological processes (for review see (Barczyk *et al.* 2010)). They are transmembrane receptors consisting of non-covalently associated αβ-heterodimers, which mediate the attachment of cells to the extracellular matrix (ECM) but that also take part in specialized cell-cell interactions. Integrins are responsible for the transmission of signals into the cytoplasm that are capable of activating cell responses against microorganisms (Hynes, 1992). In humans, only a subset of integrins recognizes the RGD (Arg-Gly-Asp) sequence in the native ligands. In some ECM molecules, such as collagen and certain laminin isoforms, the RGD sequences are exposed upon denaturation or proteolytic cleavage, allowing cells to bind these ligands by using RGD-binding receptors. In mollusks, as well as other invertebrates, integrins are involved in adhesion, spreading (Davids and Yoshino, 1998; Ballarin *et al.*, 2002), degranulation, phagocytosis (Johansson and Soderhall, 1989; Wittwer and Wiesner, 1996) and encapsulation (Pech and Strand, 1995). In *C. gigas*,

a β-Integrin from hemocytes, named β_{CGH}, was identified by a genomic approach (Terahara *et al.*, 2006). Based on immuno-colocalization experiments, it was proposed that the oyster β-integrin could recognize the extracellular Cg-SOD via its RGD motif (Gonzalez *et al.*, 2005). β_{CGH} expression patterns was shown to differ between hemocyte subpopulations according to their phagocytic ability (Terahara *et al.*, 2006). The authors proposed that the phagocytosis of hyalinocytes is regulated by an integrin-dependent mechanism and that the phagocytosis of granulocytes is elicited by other functional receptors.

II.2.3.2. Toll-like receptors

Unlike the mammalian Toll-like receptors (TLRs), which are PRRs capable of recognizing PAMPs, TLRs of invertebrates may require the use of a host cytokine to be activated. This was unambiguously demonstrated in *Drosophila melanogaster*, the organism from which Toll receptor was originally characterized. Indeed, in this invertebrate, Toll is activated by Spatzle, a cytokine activated by cleavage after recognition of fungal and bacterial PAMPs (Ashok, 2009). Thus, the *Drosophila* Toll receptor is not a PRR *per se*. Although TLRs have been identified by our group in *C. gigas* as expressed by hemocytes (unpublished data from a serial gene expression analysis SAGE), no functional characterization has been performed to date.

Data from mollusks are mostly related to the snail *Biomphalaria glabrata* or, when referring to marine bivalves, the scallops *Chlamys farreri* and *Argopecten irradians irradians*. In *Chlamys farreri*, an EST sequence corresponding to a Toll-like receptor (CfToll-1) was identified. The predicted amino acid sequence comprised an extracellular domain with a potential signal peptide, 19 leucine-rich repeats (LRR), two LRR-C-terminal (LRRCT) motifs, and a LRR-N-terminal (LRRNT), followed by a transmembrane segment and a cytoplasmic region containing the Toll/IL-1R domain (TIR). The analysis of the tissue-specific expression of the CfToll-1 gene by quantitative PCR showed that the transcripts were constitutively expressed in hemocytes, muscle, mantle, heart, gonad and gill, and the CfToll-1 expression in hemocytes was regulated by LPS in a dose-dependent manner (Qiu *et al.*, 2007).

II.2.3.3. Cytokine-like proteins and receptors

In vertebrates, cytokines are soluble mediators of the immune response to exogenous and endogenous stimuli, of wound healing and restoration of tissue homeostasis. In recent years, a wide variety of studies have led to the identification of several putative functional analogues of cytokines in invertebrates (Habicht and Beck, 1996; Ottaviani *et al.*, 1997; Caselgrandi *et al.*, 2000).

In *C. gigas*, a homologue of the transforming growth factor-β super family, named *Cg*-TGF-β/activin, was isolated (Lelong *et al.*, 2007). *Cg*-TGF-β is constitutively expressed in adult tissues and

was transcriptionally stimulated after bacterial infection. Since all TGF-β members function as extracellular signaling factors, the authors proposed that *Cg*-TGF-β/activin could behave as an immune cytokine in oyster. In addition, Herpin *et al.* had previously cloned and characterized a type I TGF-β/activin receptor gene named *Cg*-ALR1 (Herpin *et al.*, 2002). Furthermore, a gene homologous to that of the vertebrate interleukin IL-17 was identified in *C. gigas* (Roberts *et al.*, 2008). Its transcript abundance in hemocytes increased in response to immune challenge, suggesting that it may be involved in the stimulation of other immune genes after bacterial infection.

II.2.4. Signaling pathways

Upon PAMP recognition and activation of cellular receptors, signal cascades are initiated from the cell membrane to the nucleus to trigger the production of immune effectors. In recent years, several studies have revealed remarkable similarities in the signaling pathways used by vertebrates and invertebrates to activate their innate immune responses. One of the most conserved signaling pathways from *Drosophila* to humans is the Nuclear Factor-κB (NF-κB) pathway (Hoffmann and Reichhart, 2002). In *Drosophila*, the NF-κB are multi-component pathways that regulate the expression of many genes responsible for the development of the early embryo (formation of dorsal-ventral polarity), the immune response (*e.g.* expression of some antimicrobial peptides), and hematopoiesis. In *Drosophila*, the extracellular events initiating the NF-κB pathway is the binding of the spatzle cytokine to the Toll receptor. Although the receptor involved is not conserved between *Drosophila* and vertebrates, the downstream events are remarkably similar (Minakhina and Steward, 2006). In both cases, the activation of the intracellular signaling cascade involves a Rel/NF-κB family of transcriptional activator proteins, composed of a set of structurally related and evolutionarily conserved DNA-binding proteins (Silverman and Maniatis, 2001; Hayden *et al.*, 2006).

From a *C. gigas* hemocyte EST library, four cDNAs were identified that are highly homologous to adaptor molecules and transcription factors of the Rel/NF-κβ pathway. They include the adapter proteins MyD88, ECSIT and TRAF, and the *Drosophila* homolog to IκB (Cactus) (Gueguen *et al.*, 2003). In addition, the identification and characterization of a Rel/NF-κB transcription factor structurally related to insect Rel proteins (Cg-Rel), an oyster IκB kinase-like protein (oIKK) and an inhibitor of NF-κB (Cg-IκB) strongly suggest the existence of a highly conserved NF-κB signaling pathway in *C. gigas* **(Fig. 6)** (Escoubas *et al.*, 1999; Montagnani *et al.*, 2004; Montagnani *et al.*, 2008).

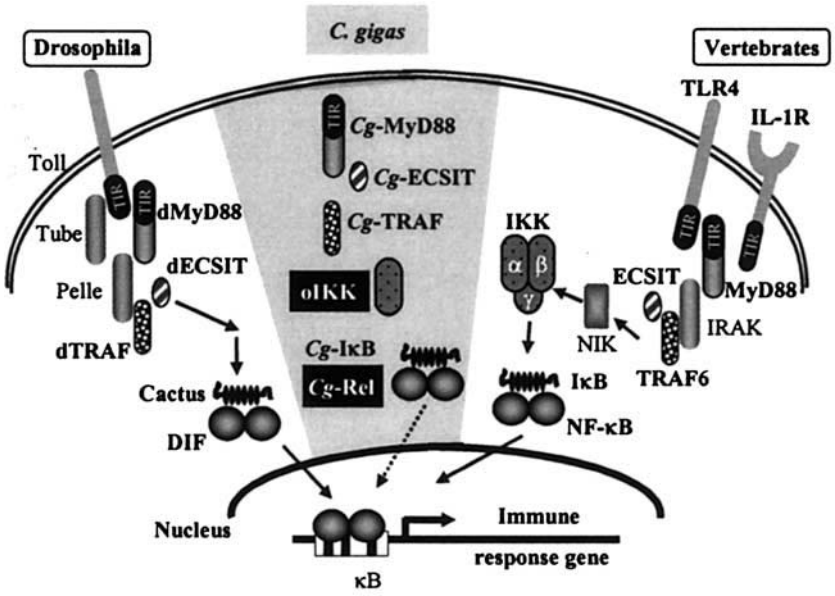

Figure 6. Conservation of Rel/NF-UB pathways in vertebrates, *Drosophila* and oyster (from Montagnani *et al.*, 2004).

II.2.5. Defense reactions in oysters

Upon activation (PAMP recognition), hemocytes undergo various functions in the oyster immune defense. They are both key effectors of the cellular response, and the initiators of various humoral reactions. They migrate towards sites of infection or injury, where they transport defense molecules and aggregate to restrict the infection or injury (McCormick-Ray and Howard, 1991; Cheng, 1996). The first phase corresponds to an infiltration of hemocytes to the site, followed by a phagocytic activity. This is one of the essential functions for the elimination of unnecessary elements, either endogenous (dead cells) or exogenous (pathogens, residual food and gametes). Among their best known properties, hemocytes contain hydrolytic enzymes (Cheng and Rodrick, 1975) and produce reactive oxygen species (ROS) (Bachère *et al.*, 1991; Lambert *et al.*, 2007), as well as antimicrobial peptides/proteins (Gueguen *et al.,* 2006b; Gonzalez *et al.*, 2007a), which play a key role in pathogen degradation.

II.2.5.1. Infiltration, phagocytosis and encapsulation

Infiltration leads to an increase in the number of hemocytes in an infection or injury site, suggesting a recruitment process at the site and a possibility for cells to migrate from the circulatory system to connective tissues (Fisher, 1986; Cochennec-Laureau *et al.*, 2003). The origin of infiltrating hemocytes is not clear. In addition to the recruitment of circulating hemocytes through diapedesis, Ford *et al.* suggested that hemocytes could proliferate *in situ* (Ford *et al.*, 1993).

Phagocytosis is a cellular process by which hemocytes engulf particles via the cell membrane to form an internal phagosome. It is the main mechanism used to remove bacteria, dead tissue cells

and small mineral particles. Phagocytosis is made up of the following series of events: (i) the recognition of the foreign or unwanted particle, (ii) attachment, (iii) pseudopod formation, (iv) ingestion in the phagosome, (v) degradation by oxygen-dependent or oxygen-independent mechanisms involving the fusion of the phagosome with lysosomes and (vi) the elimination of the degraded products (Bayne, 1990) **(Fig. 7)**. In *C. gigas*, granulocytes show high phagocytic activity against several species of bacteria and yeast cells, whereas agranulocytes show little phagocytosis against these foreign particles. However, agranulocytes were reported to be more phagocytically active than granulocytes against formalinized erythrocytes and latex beads (Takahashi and Mori, 2000), suggesting that differences in phagocytosis between granulocytes and agranular hemocytes may be related to the type of phagocytosed particles involved, rather than differences in phagocytic ability (Hine, 1999). It was recently proposed that the phagocytosis of hyalinocytes could be integrin-dependent (see β-integrins section) while the phagocytosis of granulocytes would be elicited by other functional receptors (Terahara *et al.*, 2006). The intracellular degradation involves the hydrolytic effect of different lysosomal enzymes in degradation of pathogenic organisms inside the hemocytes (Cheng, 1996). Another essential mechanism of intracellular degradation are the oxidative metabolic events known as the respiratory burst, which are characterized by the production of ROS (Halliwell and Gutteridge, 1984). ROS are composed of superoxides and peroxides with strong microbicidal activities, which in some cases have toxic effects (Beutler, 2004). The production of lysosomal enzymes and ROS has been well described in *C. gigas* (Bachère *et al.*, 1991; Xue and Renault, 2000; Lambert *et al.*, 2003), as well as genes coding for lysozyme (Matsumoto *et al.*, 2006).

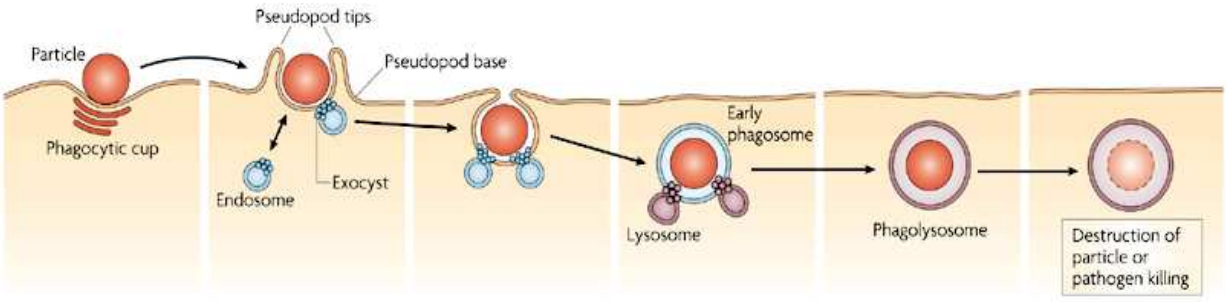

Figure 7. Schematic representation of phagocytic process of hemocytes (from Stuart and Ezekowitz, 2008)

Encapsulation allows the elimination of particles larger than hemocytes by surrounding them with multicellular capsules and subsequent release of cytotoxic products. These particles can include parasites (trematodes, cestodes and large protozans) or fungal hyphae (Ratcliffe, 1985). This

phenomenon has not been very well studied in *C. gigas*, but several studies in other mollusks suggest that this process is regulated by a complex interaction between non specific electrostatic forces, cellular and humoral plasma opsonic factors (Cheng, 1996; Smolowitz *et al.*, 1998; Wootton *et al.*, 2006). In contrast to bacterial phagocytosis, encapsulation requires the coordinated interactions of hemocytes, not only with the parasite (or other foreign body) and their secreted product but also with other hemocytes, to form the multicellular capsule that effectively eliminates the foreign microorganism (Yoshino *et al.*, 2001).

II.2.5.2. Coagulation

The coagulation is a relatively universal reaction throughout the animal kingdom. It contains the hemorrhage, allowing an improved management of blood fluidity following vascular injury. This ancient complex process, by which cells and blood molecules develop solid clots, controls blood loss from damaged vessels, as part of hemostasis.

Coagulation in higher invertebrates is generally mediated by a combination of cells and clottable proteins. The best-characterized system is that of the horseshoe crab (Iwanaga *et al.*, 1994; Theopold *et al.*, 2004). Coagulation in horseshoe crabs is initiated by the aggregation of circulating hemocytes. The cellular plug is quickly reinforced by a protein gel. In this reaction, a clottable protein (coagulogen) is cleaved and the resulting products cross-linked to one another to form a coagulin clot (Kawasaki *et al.*, 2000). Cleavage of coagulogen is mediated by the serine proteases. The reaction takes place very quickly and therefore the enzyme and substrate cannot circulate together. Rather, they are sequestered in circulating cells and are released only upon stimulation, resulting in clot formation.

Contrary to the well described coagulation of arthropods (Theopold *et al.*, 2004), no coagulation has been described in the oyster. Some phenomena of cell aggregation are observed, but they are still poorly characterized. Besides, the plasmatic coagulation seems to be absent, even though the presence of anticoagulants have been evidenced in bivalve mollusks (Dietrich *et al.*, 1985; Cesaretti *et al.*, 2004; Jung *et al.*, 2007).

II.2.5.3. The phenoloxidase system

The enzyme phenoloxidase is a critical component of the immunological defense of invertebrates. Phenoloxidase activity has been described in many arthropods; including crustaceans, insects, and chelicerates (Liu *et al.*, 2007; Soderhall *et al.*, 2009). Phenoloxidase circulates in the hemolymph within hemocytes in an inactive form, called prophenoloxidase. The conversion of the proenzyme to the active form is an extensively studied system of enzymatic checks and balances known as the phenoloxidase cascade, which regulates the release and activation of phenoloxidase at

the appropriate time and place (Soderhall and Cerenius, 1998). The phenoloxidase cascade is a reaction triggered by PAMPs (*e.g.* peptidoglycans, lipopolysaccharides or β-1,3-glucans) upon recognition by host proteins. This initiates the liberation of molecules from hemocytes and a serine proteinase cascade. This cascade leads to the conversion of zymogenic prophenoloxidase (proPO) into catalytically active phenoloxidase, generates antimicrobial compounds derived from quinones and produces the melanisation process associated to the encapsulation of the pathogen (Terwilliger and Ryan, 2006).

Some studies gave evidences of phenoloxidase activity in oyster hemolymph (Hellio *et al.*, 2007; Butt and Raftos, 2008; Thomas-Guyon *et al.*, 2009) and the presence of quinones in oyster hemocytes (Kuchel *et al.*, 2010). Nevertheless, the prophenoloxidase gene seems to be absent from the *C. gigas*, due to its absence in the existing transcriptomic libraries.

II.2.5.4. Protease inhibitors

Protease inhibitors are proteins that regulate the catalytic activity of proteases. They are important in a large variety of physiological processes including coagulation, digestion and immunity. One of the functions of protease inhibitors is to avoid the excessive activation of proteolytic cascades in the cells that generate toxic compounds and contribute to tissue damage in the host (Hibbetts *et al.*, 1999). On the other hand, they also act as inhibitors of bacteria and protozoan proteases. Pathogen proteases often are factors of virulence that allow pathogens to enter and proliferate inside the host (Faisal *et al.*, 1998). Protease inhibitors are classified as either non-specific protease inhibitors or class-specific protease inhibitors. Non-specific protease inhibitors consist exclusively of the α2-**macroglobulins**. Class-specific protease inhibitors are sub-classified as **serine protease inhibitors** (serpins), **aspartic protease inhibitors**, **tissue inhibitors of metalloproteases** (TIMP) and **cysteine protease inhibitors** (for review see (van Eijk *et al.*, 2003)).

The **α_2-macroglobulins** react by a unique mechanism that involves the physical entrapment of the target protease within the folds of their molecule. This entrapment process involves a reorganization of α_2-macroglobulin molecule, initiated by its proteolytic cleavage by the target protease and culminating with the protease molecule imprisoned within an internal pocket in the α_2-macroglobulin molecule (Armstrong and Quigley, 1999). This mechanism defines their non-specific action and establishes them as universal protease inhibitors. In *C. gigas*, cDNA sequences from an EST analysis showed high homologies to an α2-macroglobulin and its receptor, supporting the evidence of the existence of these type of protease inhibitors in the oyster (Gueguen *et al.*, 2003).

Serpins are a superfamily of proteins, most of which function as serine proteinase inhibitors. They are single chain proteins which participate as active regulators of inflammation, blood coagulation, fibrinolysis, and complement activation in vertebrates. Several serpins have been

isolated and characterized in arthropods, with roles in protection against microbial proteases, regulation of endogenous proteases, hemolymph coagulation and phenoloxidase activation (Kanost, 1999). In *C. gigas*, cDNA sequences encoding serpins were identified by EST analysis, but their functions have not yet been assigned (Gueguen *et al.*, 2003; Roberts *et al.*, 2009).

Tissue inhibitors of metalloproteases (TIMPs) are secreted proteins that play essential roles in the regulation of extracellular matrix (ECM) metabolism. They act as inhibitors of matrix metalloproteases (MMPs), a family of zinc-dependent endopeptidases (Gomez *et al.*, 1997). In invertebrates, TIMPs could operate as direct effectors in defense mechanisms because the virulence of numerous pathogens is due to their proteases and metalloproteases (Lepore *et al.*, 1996). This class of protease inhibitors is also found in *C. gigas*. By an mRNA differential display approach (DD-RT-PCR), Montagnani *et al*. identified a cDNA sequence coding a protein that presented high homology with vertebrate TIMPs (Montagnani *et al.*, 2001). *Cg*-TIMP is only expressed in hemocytes and *Cg*-TIMP mRNA accumulates in response to shell damage and bacterial challenge, suggesting that *Cg*-TIMP may be an important factor in wound healing and defense mechanisms. Additionally, the *Cg-timp* promoter contains κB-motifs of the Rel/NF-κB family of transcription factors, suggesting also that *Cg-timp* gene expression might be controlled by the oyster Rel/NF-κB pathway (Montagnani *et al.*, 2007).

II.2.5.5. Hydrolytic enzymes

Immune effectors with direct antimicrobial activity include **hydrolytic enzymes, antimicrobial peptides** and **antimicrobial proteins**. **Lysozymes** are probably the best known antimicrobials. They are cationic hydrolytic enzymes that are wildly distributed in the animal kingdom and found in a variety of cells, tissues and secretions from bacteria to humans (Ito *et al.*, 1999). The biological function of lysozymes is self-defense from bacterial infection, because they induce bacterial cell lysis by cleaving the glycosidic bond between two amino sugars, N-acetylmuramic acid and N-acetylglucosamine, of the peptidoglycan that makes up bacterial cell walls (Bachali *et al.*, 2002). Lysozymes of several bivalve mollusks have been purified and characterized mostly from the digestive system, such as the crystalline style and viscera (McHenery and Birkbeck, 1982; Nilsen *et al.*, 1999; Olsen *et al.*, 2003). In *C. gigas*, lysozyme activities were reported from the gills, mantle and digestive diverticula (Takahashi *et al.*, 1986). Later, using genomic approaches, cDNA sequences for two different lysozymes were identified from *C. gigas* digestive diverticula. Oyster lysozyme genes *CGL-1* and *CGL-2* are expressed in digestive cells and their presence and expression of multiple lysozymes in one specific tissue is propose to reflect complementary roles (Matsumoto *et al.*, 2006; Itoh and Takahashi, 2007).

II.2.5.6. Antimicrobial peptides and proteins

Antimicrobial peptides (AMPs) are ancient host defense effectors present in virtually all organisms across the evolutionary spectrum (Yeaman and Yount, 2003). They display complex and multifunctional roles in immunity. The most recognized of these is the killing of microbes, but they can also boost specific innate immune responses and exert selective immunomodulatory effects on the host (Nicolas and El Amri, 2009).

In mollusks, they were first isolated from mussels, in which they are abundant. The isolated AMPs are **cysteine-rich peptides**, including *M. galloprovincialis* defensins (MGDs), myticins, mytilins and mytimycin. First, Charlet *et al.* (1996) isolated antibacterial and antifungal peptides from the blood of immune-challenged *M. edulis* (Charlet *et al.*, 1996). Two AMPs, defensins A and B, were characterized, showing high homologies to arthropod defensins. Soon after, the defensins MGD-1 and MGD-2 were isolated from the plasma of *M. galloprovincialis* (Hubert *et al.,* 1996; Mitta *et al.*, 1999b). MGD-1 and -2 are expressed within a larger precursor molecule, which is excised into active AMPs in hemocytes. Mytilins A and B were isolated and characterized from *M. edulis* (Charlet *et al.*, 1996), and mytilin isoforms B, C, D, and G1 were isolated from *M. galloprovincialis* (Mitta *et al.*, 2000d). In addition, an antifungal peptide named mytimycin from *M. edulis* was shown to delay the growth of the fungi *Neurospora crassa* and *Fusarium culmorum* (Charlet *et al.*, 1996). In parallel, another AMP class, named myticins, was isolated from *M. galloprovincialis* hemocytes and plasma. Myticins are also cysteine-rich peptides, but they present a different cysteine array from that of previously characterized cysteine-rich AMPs (Mitta *et al.*, 1999a). Myticins A and B were reported to antibacterial activity against Gram-positive bacteria, myticin B being also against the fungus *Fusarium oxysporum* and the Gram-negative bacteria *E. coli*. A third isoform, myticin C, was identified by genomic approaches (Pallavicini *et al.*, 2008). Although only one isoform has been described for myticins A and B, a total of 74 different isoforms have been recently reported for myticin C (Pallavicini *et al.*, 2008).

Defensin-like peptides also have been identified and characterized **in oysters.** The first defensin was purified from acidified gill extract of the American oyster *C. virginica*. The peptide termed *Cv*-Def had 38 amino acid residues with 3 intramolecular disulfide bridges (Seo *et al.*, 2005). A second defensin (*Cg*-Defm) was identified from the oyster *C. gigas* mantle by EST approaches. Recombinant *Cg*-Defm was active *in vitro* against Gram-positive bacteria, but showed limited to no activity against Gram-negative bacteria and fungi (Gueguen *et al.*, 2006b). Two additional defensins named *Cg*-Defhs (*Cg*-Defh1 and *Cg*-Defh2) were then characterized from hemocytes by a genomic approach (Gonzalez *et al.*, 2007a). Each of the defensins from *C. gigas*, *Cg*-Defm and *Cg*-Defhs, appears to be continuously expressed respectively in mantle and hemocytes.

A mollusk AMP equivalent to a big defensin, first characterized in chelicerates(Kawabata *et al.*, 1997) and designated AiBD, was identified from the hemocyte cDNA library of the bay scallop *Argopecten irradians*. The biological activity assay with the recombinant AiBD showed a broad antimicrobial activity spectrum, which included both Gram-positive and Gram-negative bacteria and certain fungi (Zhao *et al.*, 2007). Big defensin was also isolated and characterized from the plasma of the clam *Ruditapes philippinesis*, exhibiting strong inhibition on the growth of Gram-negative and Gram-positive bacteria (Wei *et al.*, 2003). In the oyster *C. gigas*, a sequence showing homologies to a big defensin was identified by EST and SAGE (Serial Analysis of Gene Expression) approaches (Fleury *et al.*, 2009) (Rafael Da Rosa, pers. comm).

Another AMP identified in *C. gigas* by genomic approaches is the **proline-rich peptide** *Cg*-Prp (Gueguen *et al.*, 2009). This peptide presents homologies to proline-rich antimicrobial peptides. *Cg*-Prp synthetic variants showed limited antimicrobial activity when tested alone, but showed strong synergistic antimicrobial activity with *Cg*-Defm, the oyster defensin from mantle, suggesting their implication in the oyster antimicrobialresponse.

A member of the **Bactericidal/Permeability-Increasing protein** (BPI) family has been characterized in *C. gigas* by a screening of a hemocyte EST library, and the deduced 128-amino acid sequence is homologous to the *C*-terminal domain of mammalian LBP/BPI proteins (Gonzalez *et al.*, 2007b). BPI antimicrobial proteins are found in mammals, and *C. gigas* BPI resembles human BPI, not only due to its structural and biological properties, but also because of the localization of gene expression and its expression profile in response to bacterial challenge. The recombinant protein was found to display bactericidal activity against the Gram-negative bacteria *E. coli,* and to permeabilize its inner and outer membrane. By *in situ* hybridization, the expression of the *Cg-bpi* was found to be induced in oyster hemocytes following bacterial challenge and to be continuous in various epithelia of unchallenged oysters (Gonzalez *et al.*, 2007b).

III. Antimicrobial peptides in the innate immune response

Several *in vitro* and *in vivo* studies support the role of antimicrobial peptides (AMPs) in the immunity of vertebrates (Morrison *et al.*, 2002; Morrison *et al.*, 2003; Salzman *et al.*, 2003b) and invertebrates (Blandin *et al.*, 2002; Shockey *et al.*, 2009; Han-Ching Wang *et al.*, 2010). Besides their main antimicrobial function, many studies have now shown that they are multifunctional host defense peptides. For instance, human α and β-defensins have immunomodulatory activity, being chemotactic for immature dendritic cells and memory T cells (Yang *et al.*, 1999; Biragyn *et al.*, 2001).

III.1. Expression of AMPs in host cells and tissues

III.1.1. Gene-encoded AMPs.

The vast majority of known AMPs is encoded by one single gene, and derives directly from the traduction of the nucleotide sequence without any major processing event. AMPs are synthesized as larger precursors that are proteolytically cleaved to form a mature and active peptide. The most common maturation steps of the precursor polypeptide are the elimination of a signal peptide and of an anionic propiece (Bulet *et al.*, 2004) **(Fig. 8).** The anionic propiece could have a neutralizing function over the cationic charges of the mature peptide, which are essential for electrostatic interactions with the negatively charged cell surface molecules of bacteria. Thus, the anionic propiece could inhibits the cytotoxicity of mature AMPs, as demonstrated for HNP-1 defensin (Valore *et al.*, 1996).

Various tissues express AMPs, among which blood cells and epithelia appear to play a major role. However, specialized tissues may express AMPs according to the biology and physiology of the animal. In mammals, AMPs are expressed in leukocytes in which they are stored within phagocytic vacuoles or secretory granules. They are also found in blister fluids, and epithelia (Ganz, 2002). In epithelia, they are either constitutively expressed and stored in the granules of specialized cells (the Paneth cells) or induced by infection and immediately secreted (Ouellette and Bevins, 2001; Ganz, 2003). In vertebrates, AMPs have also been reported in amphibian skin secretions, gastric mucosa, or intestinal epithelia (Simmaco *et al.*, 1998; Zasloff, 2002).

In invertebrates, AMP expression also follows distinct pathways according to the species and tissues. In insects, AMPs are produced by hemocytes and certain epithelia, but also and mainly by the fat body, a functional equivalent of the mammalian liver, before being secreted into the hemolymph (Hoffmann, 2003). In marine invertebrates (shrimp and mollusks), AMPs are found in hemocyte cytoplasmic granules (Destoumieux *et al.*, 2000; Mitta *et al.*, 2000c). For example, in the mussel *M. galloprovincialis*, different AMPs are found both in different and in identical hemocyte types, and

eventually in the same granule (Mitta *et al.*, 2000c). Antimicrobial peptides and proteins have also been found in epithelial cells, like in gill tissues from oyster (Gonzalez *et al.*, 2007b; Seo *et al.*, 2009)

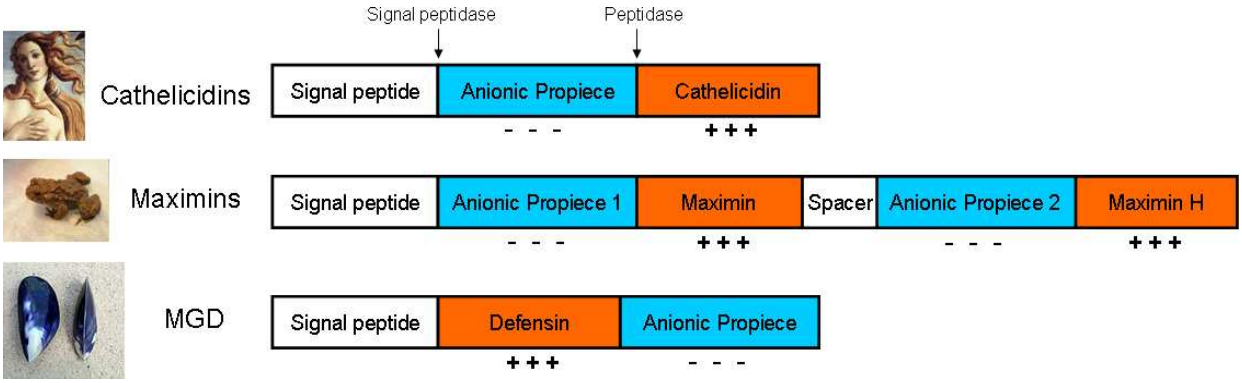

Figure 8. Precursor structure of three different AMPs.

III.1.2. AMPs derived from large protein precursors.

Some cationic AMPs are generated upon **cleavage of a large precursor protein** endowed with a distinct biological function. These include the pepsinogen-derived bPaAP and bPcAP from *Rana catesbeiana* (Minn *et al.*, 1998), the lactoferrin-derived lactoferricin (Dionysius and Milne, 1997), and peptides derived from respiratory pigments, like bovine hemoglobin (Fogaca *et al.*, 1999) and shrimp hemocyanin (Destoumieux-Garzon *et al.*, 2001; Lee *et al.*, 2003). Among this class of AMP, we can found several peptides derived from histones, in particular the N-terminal fragment of histones (Luders *et al.*, 2005). Three AMPs derived from the N-terminus domain of histone H2A have been purified and characterized from fish and toad: the histone H2A-derived buforin (Park *et al.*, 1996), parasin (Park *et al.*, 1998b) and hipposin (Birkemo *et al.*, 2003). In invertebrates, there are reports about the antibacterial activity of N-terminal H2A in *Litopenaeus vannamei* (Patat *et al.*, 2004) and in the scallop *Chlamys farreri* (Li *et al.*, 2007). Additionally, the American oyster, *C. virginica,* expresses a potent AMP derived from the histone H2B (Seo *et al.*, 2009). The immune relevance of this processing was demonstrated in crustaceans, where the peptide generation from precursor was shown to occur in response to microbial challenge (Destoumieux-Garzon *et al.*, 2001; Lee *et al.*, 2003). To our knowledge, no AMP belonging to this group has been described in *C. gigas*.

III.2. Classification of AMPs

More than 1200 different AMPs have been identified to date and their sequences are available in the AMSDb database (http://www.bbcm.univ.trieste.it/). AMPs are a diverse group of molecules divided into different classes on the basis of their amino acid composition and structure (Boman, 1995; Vizioli and Salzet, 2002). Most AMPs are cationic, meaning that they have a positive net charge at physiological pH, due to the presence of a higher content in Arginine and Lysine amino acids than in Aspartic acid and Glutamic acid. This cationic character is sometimes reinforced by a C-terminal amidation. In addition to their cationic property, AMPs can adopt an amphiphilic structure with opposite hydrophobic and hydrophilic faces (Bulet *et al.*, 2004). Both the cationic and amphiphilic character of the AMPs facilitates their interaction with the anionic phospholipid membranes of microorganisms (Oren and Shai, 1998). AMPs present broad antimicrobial spectra against Gram-negative and Gram-positive bacteria, fungi, protozoa and enveloped viruses. In addition, some AMPs can be highly hemolytic. These characteristics are believed to be essential for their mechanisms of action, like the widely accepted model of membrane disruption (see below).

Cationic AMPs can be classified into three major groups according to their physicochemical properties and origin. These include **(i) linear peptides that can form amphipathic α–helices, (ii) peptides that contain cysteine residues and form disulphide bonds, and (iii) peptides with one or two over-represented amino acids** (Brogden, 2005) **(Fig. 9).** Although not as common, an increasing number of anionic AMPs have been described, but their functional characterization is still poorly documented.

III.2.1. Amphipatic α-helical peptides

Found in a large variety of organisms quite evolutionarily distant (e.g. arthropods, amphibians, fishes, rabbits and humans), α-helical AMPs vary remarkably in their sequence. However, it is possible to fit them into distinct groups on the basis of sequence homologies, which clearly follow taxonomic lines (Tossi *et al.*, 2000). Most of these peptides are below 30–40 residues in length, rich in lysine and arginine residues, and C-terminally amidated. Many are unstructured in solution, and adopt an α-helical conformation upon contact with biological membranes. Some examples of α-helical peptides include cecropins, ceratotoxin and melittin from insects; magainin, dermaseptin, bombinin, and buforin II from amphibians, and LL37 from humans.

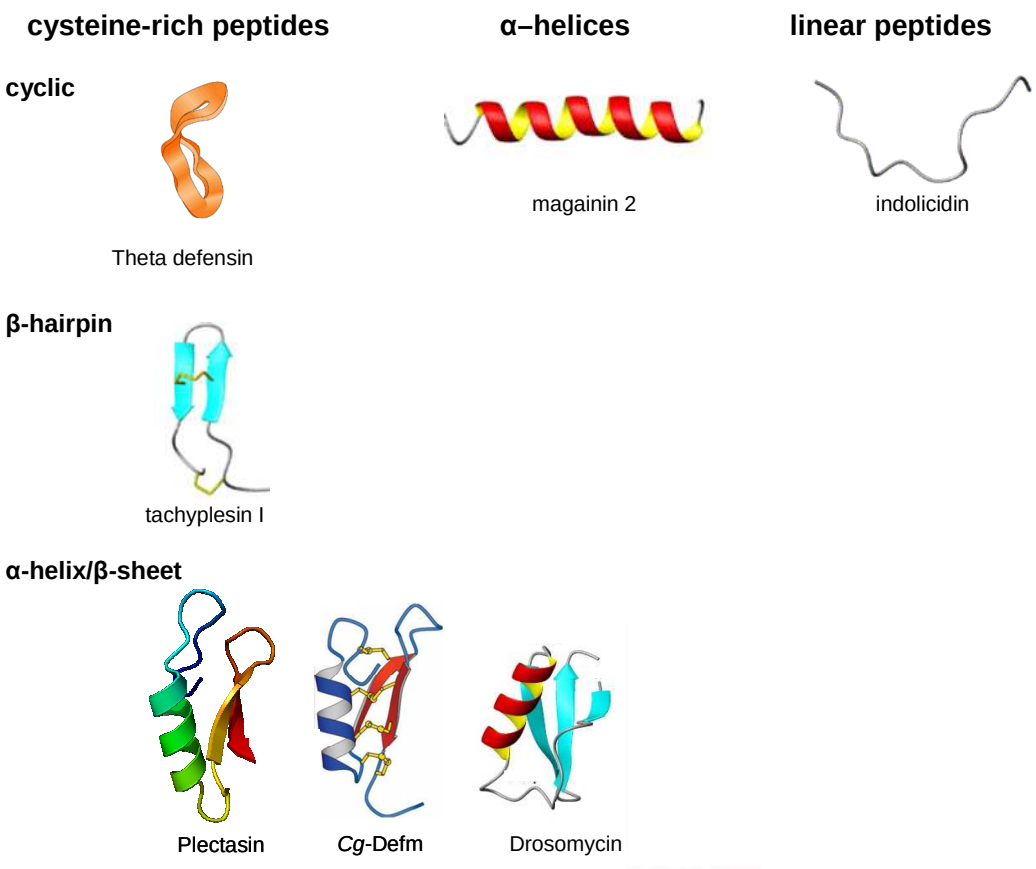

Figure 9. Structural classes of antimicrobial peptides. Linear, α-helical and cysteine rich peptides are indicated with representative peptides for each class. Cysteine-rich peptides are divided in 3 subgroups depending on the number of cysteine residues and their pairing. Sheet structures are indicated by flat ribbons and arrows. Protein Data Bank ID: Theta defensin [1HVZ], Tachyplesin [1WO0], plectasin [1ZFU], *Cg*-Defm [2B68], Drosomycin [1MYN], Magainin 2 [PDB 2MAG]

III.2.2. Cationic peptides that contain cysteines and form disulphide bonds

This is a large group of AMPs that contains pairs of cysteine residues oxidized to form internal disulfide bridges. These cysteine-containing peptides are often open-ended, but there also exists a cyclic cysteine-containing peptide from the rhesus monkey named θ-defensin (Tang *et al.*, 1999). This group gathers peptides (i) with 1 disulphide bond, such as brevinins from amphibians (Basir *et al.*, 2000); (ii) with 2 disulphide bonds, such as protegrins from pigs and tachyplesins from horseshoe crabs (Kokryakov *et al.*, 1993), (iii) with 3 disulphide bonds, including α-defensins and β-defensins from humans (Lehrer, 2004), θ-defensin from the rhesus monkey (RTD-1) (Tang *et al.*, 1999), big-defensins from horseshoe crab (Kawabata *et al.*, 1997), plectasin from fungus (Mygind *et al.*, 2005), and insect defensins like defensin A (Cociancich *et al.*, 1993); and finally, (iv) with 4

disulphide bonds, like drosomycin from *Drosophila*, plant antifungal defensins (Fehlbaum *et al.*, 1994) and mollusk defensins (Yang *et al.*, 2000 . Gueguen, 2006 #23). Depending on the number of cysteine residues and their pairing, they may adopt a β-sheet conformation with triple strands (most of the vertebrate defensins), a β-hairpin-like structure (like tachyplesin from horseshoe crab or brevinins and esculentins from amphibians) or a mixed α-helix/β-sheet conformation, as observed for arthropod and mollusk defensins, for plant defensins and for some mammalian defensins. Despite structural variations, the cysteine-containing AMPs have been shown to all display one conserved three-dimensional feature referred to as the γ-core. It was proposed that this motif, which approximately corresponds to the tachyplesin 3D-structure (β-hairpin), is a key determinant of the cysteine-containing AMPs conserved throughout evolution (Yount and Yeaman, 2004).

III.2.3. Peptides with one or two over-represented amino acids

They are peptides rich in certain amino acids, lacking cysteine residues, and linear, although some can form extended coils. These peptides normally show an overrepresentation of one or two amino acids, such as proline/arginine, proline/histidine or proline/lysine-containing peptides. Among these, proline-rich antimicrobial peptides are a group of linear peptides, isolated from mammals and insects, which are characterized by their high content (up to 50%) of proline residues (Gennaro *et al.*, 2002). Proline-rich antimicrobial peptides were first isolated from honeybees (Casteels *et al.*, 1989) and cattle (Gennaro *et al.*, 1989) and subsequently found in other arthropod and mammalian species (Schnapp *et al.*, 1996; Bulet *et al.*, 1999; Otvos, 2000). They are generally cationic, due to a high content in arginine or, (less frequently) lysine residues. Members of this group are predominantly active against Gram-negative bacteria, which they kill without bacterial membrane lysis, in contrast with the majority of known AMPs.

III.2.4. Anionic AMPs

This is one exceptional class of AMPs. Structural characterization shows that they generally range from - 1 to - 7 in net charge, and from 5 to 70 residues in length. For a number of these peptides, post-translational modifications are essential for antimicrobial activity. In some cases, antimicrobial activity appears to be a secondary role for these peptides, other biological activities constituting their primary role (Harris *et al.*, 2009).

In vertebrates, anionic AMPs are small peptides (721.6–823.8 Da), rich in glutamic and aspartic acids and found in amphibians, sheep, cattle and humans. In humans, they are present in surfactant extracts, bronchoalveolar fluid and airway epithelial cells. They are produced in millimolar concentrations, require zinc as a cofactor for antimicrobial activity and are active against both Gram-positive and Gram-negative bacteria (Lai *et al.*, 2002; Brogden, 2005).

In invertebrates, larger cysteine-rich anionic AMPs (8.5 to 10 kDa) have been characterized from the leech *Hirudo medicinalis* (theromacin and theromyzin) (Tasiemski *et al.*, 2004) and the shrimp *Litopeaneus stylirostris* (stylicins) (Rolland *et al.*, 2010). Stylicins are expressed by shrimp hemocytes and are very active (MIC = 2.5 µM) against the shrimp fungal pathogen *Fusarium oxysporum,* while theromacin and theromyzin are expressed in large fat cells and epithelia, and are active against gram-positive bacteria. Two additional anionic AMPs (4.8 and 6.9 kDa) were also recently described in the insect *Galleria mellonella*. However, their activity against gram-positive and fungal strains remains rather poor (MIC = 90 µM) (Cytrynska *et al.*, 2007).

III.3. Mechanisms of action of AMPs

Cationic AMPs have the basic capability to interact with membranes. Some are membrane active, but others are not (Brogden, 2005). New evidence points to intracellular targets as being fundamental in the antimicrobial mechanisms of these peptides (Yeaman and Yount, 2003).

III.3.1. Interaction with biological membranes

The initial mechanism by which AMPs target microbes occurs via **electrostatic interactions**. Cationic AMPs and negatively charged lipid membranes of bacteria are mutually and vigorously attracted. The mechanism by which electrostatic attractions drive peptide-membrane interactions have been examined in numerous studies (Vaz Gomes *et al.*, 1993; Hancock, 1997; Matsuzaki *et al.*, 1997; Dathe *et al.*, 2001). In the case of Gram-negative organisms, Hancock suggested a mechanism of peptide interaction with membranes, termed self-promoted uptake (Hancock, 1997). This mechanism proposes that the initial action of the peptide involves a competitive displacement of the LPS-associated divalent cations that stabilize the outer membrane of Gram-negative bacteria. In comparison, Gram-positive organisms lack an outer membrane or LPS, but their cell envelopes are enriched in negatively charged teichoic and teichuronic acids (Yeaman and Yount, 2003). After the initial peptide-membrane electrostatic interaction, the following events differ depending on the specific mode of action.

III.3.2. Membrane-disruptive mechanisms

The mechanisms by which peptides may permeabilize and traverse microbial membranes are not entirely clear and probably vary between peptides. A number of models for antimicrobial peptide membrane permeabilization have been proposed but, given the variability in microbial membrane ultrastructure, a peptide may act via different mechanisms in distinct membrane environments. Three models are generally accepted, although these were established from results obtained with individual peptides against artificial membrane systems and there is still no consensus among

investigators. These models are the toroidal pore, the barrel-stave and the carpet-like mechanism **(Fig. 10)**.

III.3.2.1. The Toroidal Pore model

In this model, peptides in the extracellular environment adopt an α-helical structure as they interact with the charged and hydrophobic bacterial membrane **(Fig. 10a)**. Helices are initially oriented parallel to the membrane surface and the hydrophobic residues of peptides shift the polar head groups, creating a break in the hydrophobic region and inducing positive curvature strain in the membrane. When peptides reach a threshold concentration, they become perpendicularly orientated to the membrane and helices may begin to self-associate because their polar residues are no longer exposed to the membrane hydrocarbon chains. Thus, lipids are intercalated with the peptides in the transmembrane channel. Upon disintegration of the pore, some peptide becomes translocated to the cytoplasmic leaflet of the membrane (Uematsu and Matsuzaki, 2000). Magainins, melittin and protegrins are examples that appear to induce transmembrane pores matching the toroidal model (Yang *et al.*, 2001).

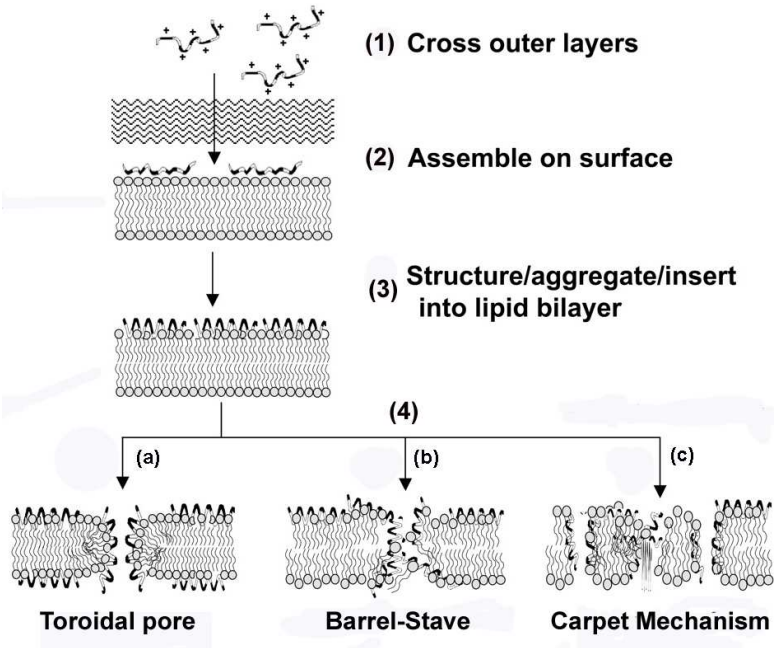

Figure 10. Models for pore formation by AMPs. (1).Outer membrane in Gram-positive bacteria, thick peptidoglycan in Gram-negative bacteria; (2) electrostatic attraction to anionic bacterial membrane surface; (3) formation of an amphiphilic structure capable of membrane insertion; (4) possible permeabilization mechanisms, which depend on peptide sequence/concentration and membrane characteristics. Adapted from Sahl *et al.*, 2005.

III.3.2.2. The Barrel-Stave model

In this model, a variable number of channel forming peptides are positioned in a "barrel-like" ring around an aqueous pore. In this mechanism, the hydrophobic surfaces of an AMP face external toward the acyl chains of the membrane, whereas the hydrophilic surfaces form the pore lining (Breukink *et al.*, 1999) **(Fig. 10b)**.The initial step in barrel-stave pore formation involves peptide binding at the membrane surface, most likely as monomers. Upon binding, the peptide may undergo a conformational phase transition and the hydrophobic portion of the peptide is inserted into the membrane. Positioning of the positively charged amino acids near the phospholipid head groups facilitates this process.

When the bound peptide reaches a threshold concentration, peptide monomers self-aggregate and insert deeper into the hydrophobic membrane core. Continued accumulation of peptide monomers results in further expansion of the membrane pore (Yeaman and Yount, 2003). This model has been proposed, for example, for alamethicin, a fungal AMP (Yang *et al.*, 2001).

III.3.2.3. The Carpet-like Mechanism

Models of nonspecific membrane permeabilization by AMPs traditionally include diffuse effects that have been compared to detergents. In this sense, some peptides may act against microorganisms in a relatively diffuse manner, termed the carpet mechanism **(Fig. 10c)**. In the carpet model, a high density of peptides accumulates on the target membrane surface. Phospholipid dislocation changes membrane fluidity and/or reduces the barrier properties of membrane, and therefore leads to membrane disruption. As in other models, peptides initially bind to the membrane mainly via electrostatic interactions, carpeting the phospholipid bilayer (Shai, 2002). Cecropin P1, an AMP from *Cecropia* moths, appears to target microorganisms in this manner (Sitaram and Nagaraj, 1999).

III.3.3. Non membrane-disruptive mechanisms

There is widespread acceptance that cationic AMPs, apart from their membrane-disrupting properties, also operate through interactions with intracellular targets, and subsequent impairment of key cellular processes. Although most cationic AMPs act at high concentrations by disrupting the microbial membrane, recent studies have reported several peptides that affect microbial viability at low to moderate concentrations through interaction with one or more intracellular targets. Examples of intracellular activity include **inhibition of DNA and protein synthesis, inhibition of chaperone-assisted protein folding** and **inhibition of cell wall synthesis** (Brogden, 2005; Nicolas, 2009) **(Fig. 11)**.

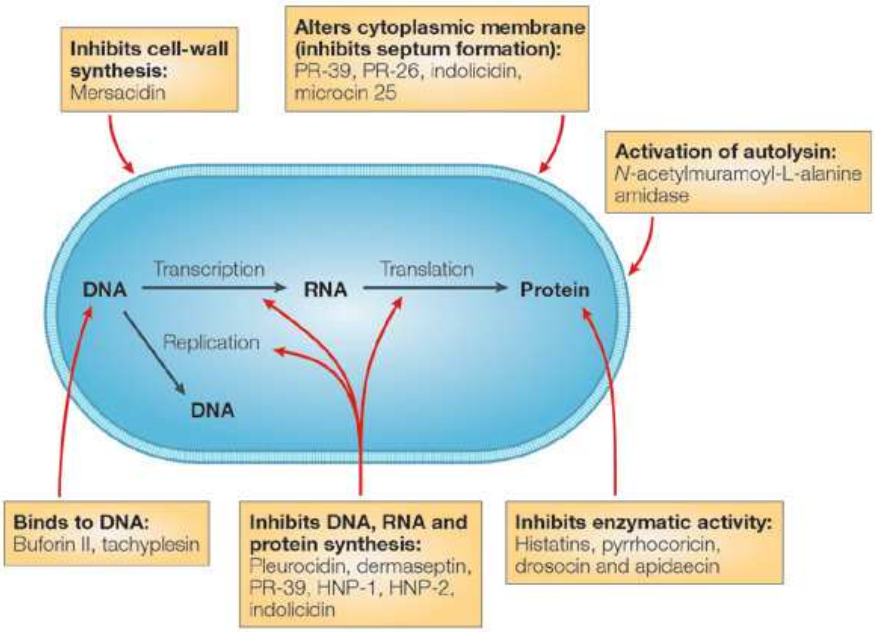

Figure 11. Mode of action of intracellular AMPs by non membrane disruptive mechanisms (from Brogden, 2005)

III.3.3.1. Inhibition of DNA and protein synthesis

Among the AMPs which **inhibit DNA and protein synthesis**, there is a great variety of structure and origin. The histone H2A-derived buforin II and the cyclic β-sheet tachyplesin appear to bind to DNA (Yonezawa *et al.*, 1992; Park *et al.*, 1998a). α-helical peptides (pleurocidin and dermaseptin), proline- and arginine-rich peptides (PR-39 and indolicidin) and human neutrophil defensin (HNP-1) block (^{3}H)thymidine, (^{3}H)uridine and (^{3}H)leucine uptake in *E. coli*, showing that they inhibit DNA, RNA and protein synthesis (Lehrer *et al.*, 1989; Boman *et al.*, 1993; Subbalakshmi and Sitaram, 1998; Patrzykat *et al.*, 2002). Indolicidin, a mammalian proline/tryptophan-rich AMP, completely inhibits DNA and RNA synthesis in *E. coli* but does not have any effect on protein synthesis (Subbalakshmi and Sitaram, 1998). Histatin, a human α-helical peptide, binds to a receptor on the fungal cell membrane, enters the cytoplasm and induces the nonlytic loss of ATP (Kavanagh and Dowd, 2004). The lack of membrane permeabilizing effects of these AMPs shows that the inhibition of DNA and protein synthesis is not due to the interruption of bacterial respiration process.

III.3.3.2. Inhibition of chaperone-assisted protein folding

This mechanism has been identified for several proline-rich AMPs. For example, insect pyrrhocidin, drosocin and apidaecin enter the target cells and specifically bind to DnaK, a heat shock protein involved in chaperone-assisted protein folding. Pyrrhocidin inhibits the ATPase activity of

DnaK, which results in the accumulation of misfolded proteins and subsequent cell death (Kragol *et al.*, 2001).

III.3.3.3. Inhibition of cell wall biosynthesis

In contrast to the great variety of AMPs implicated in the inhibition of DNA, RNA and protein synthesis and folding, **inhibition of cell wall biosynthesis** is mostly described for peptides produced by Gram-positive bacteria, the lanthionine-containing peptide antibiotics (lantibiotics) (Bauer and Dicks, 2005).

The **peptidoglycan biosynthesis cycle** consists of two lipid-bound intermediates, lipid I and lipid II, synthesized at the cytosolic side of the plasmatic membrane. Lipid I is formed by the transfer of the phospho-*N*-acetyl muramic acid (MurNAc)-pentapeptide group from the uridine 5'-diphosphate (UDP)-activated amino sugar to the membrane-bound lipid carrier undecaprenyl-pyrophosphate (C55P). Lipid II results from coupling of a second UDP-activated amino sugar *N*-acetyl-d-glucosamine (GlcNAc) to the MurNAc group of lipid I. Lipid II is subsequently transported to the exterior side of the membrane by a flippase. The two amino sugars and the pentapeptide are then coupled to the cell wall and converted into peptidoglycan. The cycle is completed once the remaining undecaprenyl-pyrophosphate is transported back to the cytosolic side of the membrane, where it is dephosphorylated to undecaprenylphosphate (Breukink and de Kruijff, 1999; Bauer and Dicks, 2005) **(Fig. 12)**.

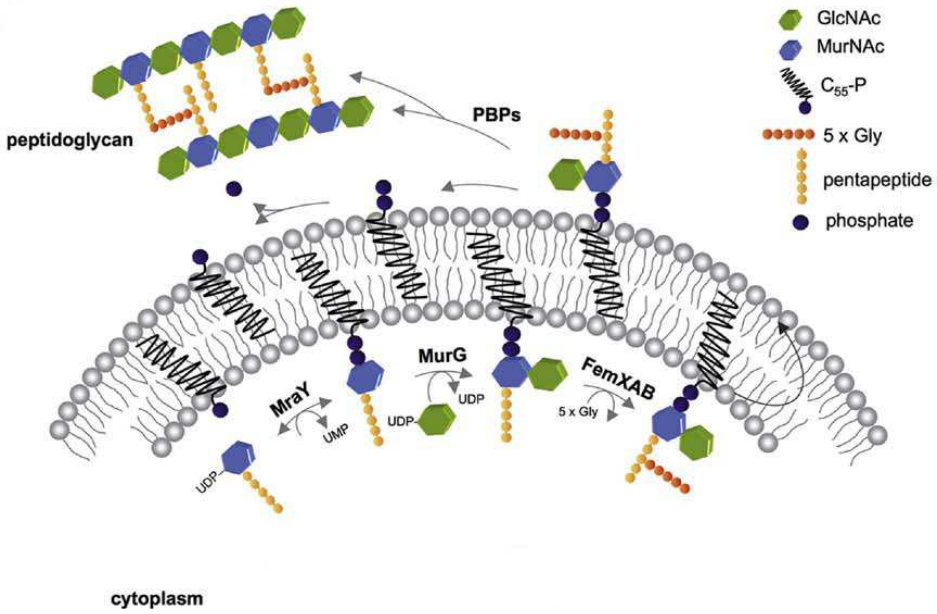

Figure 12. Schematic representation of cell wall biosynthesis in *S. aureus* (Schneider and Sahl, 2010)

The first identified lantibiotic showing inhibition of cell wall biosynthesis was nisin Z, which uses the membrane-anchored cell wall precursor lipid II as a receptor (Breukink and de Kruijff, 1999). Nisin is the oldest known and most extensively studied lantibiotic. It is active in nanomolar concentrations against Gram-positive bacteria, a quality which results from its ability to use lipid II as a docking molecule and subsequent pore formation. The lantibiotics gallidermin, epidermin, mersacidin and plantaricin C also target lipid II, inhibiting peptidoglycan biosynthesis by interfering with membrane-associated transglycosylation and in some cases, producing pore formation (Brotz *et al.*, 1998; Bonelli *et al.*, 2006). The cyclic lipopeptide friulimicin has a different target and a unique mode of action. It interrupts the cell wall precursor cycle through the formation of a Ca^{2+}-dependent complex with the undecaprenyl-phosphate carrier C55P. Since C55P also serves as a carrier in teichoic acid biosynthesis and capsule formation, it is likely that friulimicin blocks multiple pathways that are essential for a functional Gram-positive cell envelope (Schneider *et al.*, 2009). Because activities of lantibiotics are based on combined killing mechanisms, it has become evident that the *in vivo* activity of lantibiotics cannot be assigned to a single factor. It appears rather that cell wall synthesis inhibition and pore formation may contribute differently to the antimicrobial activity depending on the target strain (Wiedemann *et al.*, 2006).

IV. Molecular diversity in immune systems

IV.1. Host-pathogen interactions and the co-evolution theory

The continuous battle between hosts and pathogens has been a major subject of study for evolutionary biologists. Because hosts and pathogens live in strong relationship with each other, their interconnected fight for existence is both continual and rapid. At the molecular level, the evolutionary response is reflected in mutations being under continual selection pressures (Holmes, 2004). Natural selection (see below) may act strongly on immune systems since hosts adapt to novel, diverse and co-evolving pathogens. Any effective host defense system must have the capacity to recognize a potential pathogen, activate the immune response and kill the infectious agent. In parallel, from the perspective of the pathogen, it is essential to avoid the host immune defense. This places the pathogen under strong evolutionary pressure to generate mechanisms to evade, resist or suppress host immunity. Every successful advance by the pathogen, however, creates selective pressure on the host to evolve so as to re-establish immunity (Lazzaro, 2008). **Therefore, the general hypothesis of co-evolution or evolutionary "arms race" proposes that pathogens/parasites evolve continuously to escape from the immune response of host and, consequently, the immune system of the host evolves to improve barriers against pathogens/parasites.** Consequently, such interactions might provide rapid evolution in genes involved in host-pathogen interactions. This adaptation of the Red Queen hypothesis (Van Valen, 1974) predicts that in an "arms race", both host and pathogen/parasite develop mechanisms that generate diversity and polymorphism of molecules that play key roles in their interplay. Thus, it is not surprising that genes involved in immunity frequently show high levels of genetic diversity and present some of the best examples of positive selection reported to date (Yang and Bielawski, 2000).

IV.2. Natural selection and molecular adaptation

Natural selection is an important process by which the evolution of organisms takes place. It is a key mechanism that constitutes one of the bases of modern biology. The term was introduced by Darwin in his pioneering book *The Origin of Species* (1859), and remains the primary explanation for adaptive evolution. It is defined as the process by which heritable traits that increase the probability of an organism to survive become more common in a population over successive generations (Hughes, 1999a). Consequently, at the genomic level, different types of selective forces could shape evolution. On one hand, new alleles that confer a higher fitness to an individual tend to increase in frequency over time until they reach fixation, thus replacing the ancestral allele in the population.

This evolutionary process is called **positive selection or adaptive evolution**. Conversely, new mutations that decrease the fitness tend to disappear from populations through a process known as **negative or purifying selection**. Finally, it may happen that a mutation is advantageous only in heterozygotes but not in homozygotes. Such alleles tend to be maintained at an intermediate frequency in populations by way of the process known as **balancing selection**. Positive selection favors the fixation of beneficial mutations that lead to evolution of new traits. Therefore, a mutation is said to be adaptive if it performs a function that is in some way advantageous in the population. Negative selection favors the conservation of existing phenotypes or particular genes functionally constrained, playing an important role in maintaining the long-term stability of biological gene function (Kimura, 1983). However, the neutral theory of Kimura maintains that the most frequently observed molecular polymorphism is due to random fixation of neutral mutations by genetic drift(Kimura, 1983). Random fixation can be associated to balancing selection, which favor the existence of multiple alleles, rising polymorphism.

One of the most powerful approaches to detect selection pressures at the genomic level is by comparing the number of nucleotide non-synonymous substitutions per non-synonymous site (dN) with the number of synonymous substitutions per synonymous site (dS) (Kimura, 1983; Hughes and Nei, 1989). Mutation at a codon site that results in amino acid change is known as non-synonymous substitution. In contrast, a mutation which does not lead an amino acid change was known as synonymous substitution. If the protein-coding gene is functionally constrained, then the rate of non-synonymous change will be lower than the neutral rate. There are three different types of selective pressure that can be detected from dN and dS ratio, which are (i) $dN/dS < 1$, and the gene is considered to be subjected to strong purifying selection. Alternatively, if non-synonymous mutations are beneficial then the average rate of non-synonymous changes is expected to be higher than the neutral rate, resulting in (ii) $dN/dS > 1$, and the gene is considered to be subjected to positive selection. When (iii) $dN/dS = 1$, it indicates neutral evolution. Several tests of neutrality are used to reject strict neutrality and they can be applied to complete coding sequences or at codon level, by using codon-based maximum likelihood methods. These models are useful to identify amino acid sites under diversifying selection (Yang and Bielawski, 2000).

IV.3. Mechanisms of genetic diversity

One major solution for increasing diversity is to make more final products than the number of inherited genes that one individual could account for. In general, genetic diversity of immune receptors and effectors could be generated by different processes: (i) gene duplication, (ii) exon shuffling, (iii) polymorphism, (iv) recombination, (v) somatic hypermutation or (vi) alternative splicing (Pasquier, 2006).

In all domains of life, genomes probably have been generated by **gene duplication.** Gene duplication can result from: unequal crossing over, retroposition or chromosomal (or genome) duplication. Unequal crossing over usually generates tandem gene duplication. Retroposition occurs when an mRNA is retrotranscribed to complementary DNA and then inserted into the genome; it is usually unlinked to original gene, because the insertion of cDNA into the genome is more or less random. Chromosomal or genome duplication probably occurs by a lack of disjunction among daughter chromosomes after DNA replication (Zhang, 2003).

The existence of duplicate genes is occasionally beneficial just because extra amounts of protein or RNA products are provided and two paralogous genes can maintain the same function by gene conversion. Under gene conversion, two paralogous genes will have very similar sequences and functions, and this mode of evolution is referred to as concerted evolution (Li *et al.*, 2001). Alternatively, strong negative selection can also prevent duplicated genes from diverging. In parallel, two duplicate genes can also be maintained when they differ in their functions (Nowak *et al.*, 1997) and this can occur by **subfunctionalization**, in which each gene adopts part of the functions of the original gene, or by **neofunctionalization**, which is one of the most important outcomes of gene duplication and represents the origin of a novel function by adaptive evolution (Hughes, 1994).

Exon shuffling leads to the increase of protein structure diversity. By this mechanism, individual structural or functional units can be put together in various combinations to create many different proteins. According to the exon shuffling theory, exons in genes correspond to functional modules in the encoded proteins. Duplication, permutation and rearrangement of these exons would result in novel genes that express proteins with diverse functional modules (Gilbert, 1978).

Other way to increase the diversity is to add **polymorphism** to the multiple gene copy number. This can be done any time during the history of a gene family. As it changes with time, polymorphism provides allelic diversity that offers flexibility within populations as a function of environmental variation and pathogens (Pasquier, 2006 392).

Genetic recombination is a process by which a molecule of DNA is broken and then joined to another DNA molecule. Recombination can occur between similar DNA molecules, as in homologous recombination, or dissimilar DNA molecules, as in non-homologous end joining. When this process is associated with polymorphism, recombination moves the mutation into different genetic backgrounds, which could be associated with functional variation of selective importance (Spencer *et al.*, 2006). Furthermore, the observed positive correlation between recombination rate and genetic diversity suggests that many loci showing recombination have been subject to adaptive evolution (Nachman, 2001).

Somatic hypermutation consists mainly of a programmed process of single nucleotide changes with rare insertions and deletions. Such changes would be introduced during error-prone

repair of lesions involving single-strand DNA breaks or, more likely, double-strand DNA breaks (Wu *et al.*, 2003a). Somatic hypermutation diversifies the receptors used by the immune system to recognize antigens and allows the adaptation of its response to new threats during the organism lifetime (Janeway *et al.*, 2005).

Alternate splicing is the mechanism by which a single primary mRNA transcript can be transformed into a number of different mature mRNAs encoding different proteins. This is accomplished by post-transcriptional control of gene expression. After the removal of the introns, some exons may also be removed. The result is a family of related proteins, formed by the joining of different combinations of exons, although the exons always remain in the same order as in the primary transcript (Jensen *et al.*, 2009).

All these mechanisms can act alone or simultaneously to produce the diversification of immune receptors and effectors of the immune system. Whatever the mechanism used, these molecular processes have the same goal: creating a variety of molecules that are adequately diverse to discriminate between the wide spectra of potential pathogens.

IV.4. The adaptive immune response in vertebrates

In vertebrates, the immune system is composed by the innate immune response and the adaptive immune response. The adaptive response provides the vertebrate immune system with the ability to recognize and remember specific pathogens. This system offers two major arms, the immunoglobulins (Ig) (the humoral response) and T cells (the cellular response), both of which set down an immunological specificity and memory for future defense (Holmes, 2004). Thus, two major features characterize the adaptive immune system of vertebrates: **specificity** and **memory** (Zinkernagel *et al.*, 1996; Jiang and Chess, 2009). **Specificity** is characterized by a great diversity of immune effectors directed against a variety of antigens. Specificity is based on the receptors of immune cells, called the T and B lymphocytes, presenting Ig, T cell receptors (TCR) and molecules of the major histocompatibility complex (MHC) (Cresswell, 1994; Engelhard, 1994; McHeyzer-Williams and Davis, 1995). **Memory** relies on the selection of these specific molecules in lymphocytes. Once the infection is over, these cells will remain in the organism to constitute a cellular memory that they will allow a more rapid response when a second infection by the same pathogen occurs.

IV.4.1. The paradigm shift between adaptive and innate immunity

Invertebrates do not possess an adaptive immune system and have therefore been assumed to lack diverse and specific immune defenses. Nevertheless, immunology has recently confronted a major paradigm shift. Recent evidence has demonstrated that some **invertebrates have complex**

immune responses with high levels of immune diversity and specificity. The old notion that invertebrates have a simple innate immune system that recognize broad groups of pathogens but lack the capacity for precise discrimination of individual pathogens is actually out of date. Some works also suggested the existence of **memory** in invertebrate immunity (Kurtz and Franz, 2003; Little *et al.*, 2003; Pham *et al.*, 2007) but it is still controversial. Several authors sustains that conclusions based only on phenomena such as survival or reproductive capacity of organisms is not sufficient, and that real evidence can only be upheld if supported by descriptions of the underlying mechanisms (Hauton and Smith, 2007).

Although invertebrates include more than 95% of all species in the animal kingdom, only a small number of species, mostly arthropods, have been investigated at the molecular level to establish the mechanisms of defense and the nature of the molecules produced. This is also true for the second largest invertebrate phylum, the Mollusca. Nevertheless, we can find important studies on model species that provide evidence of how invertebrates generate diverse innate immune molecules.

The following examples in both adaptive and innate immune systems were chosen with two purposes: (i) to highlight the existing mechanisms of diversification of immune receptors and effectors in vertebrates and invertebrates and (ii) to show the most representative evidence of innate immune diversification that has supported this paradigm shift.

IV.5. Diversification of immune receptors

IV.5.1. In the adaptive response of vertebrates

The mechanisms underlying the generation of **highly variable receptors** in the adaptive immune system have been widely investigated. The recognition molecules, Immunoglobulins (Ig) and T cell receptors (TCR) are generated by a mechanism of **somatic gene rearrangement** and **hypermutation**, which randomly selects and assembles segments of genes encoding specific proteins by Recombination Activating Genes (RAG) enzymes. This site-specific recombination reaction generates a diverse repertoire of Ig and TCR molecules that are necessary for the recognition of diverse antigens. The product of this rearrangement is expressed clonally in lymphocytes and the antigen presentation process. This allows the selection of appropriate receptors depending on a set of enzymes and presenting molecules encoded in the Major Histocompatibility Complex (MHC) (Du Pasquier, 2004). It is believed that the Ig repertoire generated by somatic hypermutation can practically recognize an infinite number of non-self structures (Teng and Papavasiliou, 2007).

The MHC is another example of greatly diversified molecules, but the mutational mechanism underlying the diversity in MHC genes is still controversial (Reusch and Langefors, 2005). MHC

comprises a multigene family coding for transmembrane receptor glycoproteins, which bind antigens and present them to T lymphocytes that initiate the subsequent immune response by recognizing a foreign peptide (Brown *et al.*, 1993). The MHC genes contain some of the most polymorphic coding genes in the vertebrate genome (Bernatchez and Landry, 2003) and gene duplication, allelic diversity, inter and intralocus recombination and selection pressures have all been suggested as mechanisms of diversification (Reusch and Langefors, 2005; Schaschl *et al.*, 2006; Axtner and Sommer, 2007).

IV.5.2. In the innate response of invertebrates

The following examples of diversification of immune receptors in invertebrates contradict the paradigm of Medzhitov and Janeway, which established that receptor diversity of the innate immune system is limited and germline encoded (Medzhitov and Janeway, 1997). These key studies provide strong evidence that like vertebrates, invertebrates use not only germline-encoded receptors but also highly diversified receptors generated by somatic mechanisms to react rapidly to antigenic changes of pathogens (Kurtz and Franz, 2003).

IV.5.2.1. Germline-encoded diversity

Scavenger receptor cysteine-rich repeats (SRCR) proteins from sea urchin

The purple sea urchin *Strongylocentrotus purpuratus* expresses a complex set of transcripts belonging to scavenger receptor cysteine-rich repeats (SRCR) in hemocytes (Pancer, 2000). SRCR domains form part of a superfamily of proteins. SRCR proteins are implicated in development of the immune system of vertebrates and in the regulation of immune responses, mediating growth, differentiation and activation of immune cells as well as other cells of the body, most likely via protein–protein interactions (Resnick *et al.*, 1994). In some invertebrates, members of the SRCR superfamily have been shown to function as cell surface receptors (Dangott *et al.*, 1989; Blumbach *et al.*, 1998). Pancer highlighted a heterogeneous and transcriptionally dynamic set of SRCR-containing molecules which are expressed specifically in sea urchin hemocytes (Pancer, 2000). The heterogeneity of SRCR domains was demonstrated at genomic and transcriptional levels, and features a multigene family (150 genes) that encodes an estimated 1200 SRCR domains with specific patterns particular to each individual. However, the mechanisms by which SRCR-containing molecules diversify have not yet been clearly shown. Elucidation of the function of sea urchin SRCR gene products requires further research, but their importance has been illustrated in terms of gene numbers, complex genomic organization, and dynamic transcription profiles in this marine invertebrate.

Immunoglobulin (Ig)-type variable (V) region-containing chitin binding proteins (VCBPs) from amphioxus

The amphioxus *Branchiostoma floridae* presents a unique immune receptor repertoire of immunoglobulin (Ig)-type variable (V) region-containing chitin binding proteins (VCBPs). These proteins are soluble proteins consisting of two Ig-type V regions joined to a carboxy-terminal chitin-binding domain. Although their ligands are unknown, VCBPs are likely candidates for innate immune receptors and represent the only example of an innate receptor in which the functional unit is a hyperdiversified Ig-type V region. VCBPs are distributed across at least five families (VCBP 1-5) (Cannon *et al.*, 2002) and regionalized peptide sequence hypervariability was noted in the N-terminal V region of pooled amphioxus VCBP2 cDNAs (Cannon *et al.*, 2004). A very high degree of regionalized hypervariability was observed in VCBP2 at the level of the individual germline, which produces a unique repertoire of proteins at the individual level. A total of 43 different peptides were encoded across the amplified region of VCBP2 by only 13 different animals. Although the particular mechanism that gives rise to the observed genetic polymorphism in VCBP2 genes is unknown, Cannon *et al*. (2004) suggested that in species with large populations, such as amphioxus, extensive polymorphisms may compensate for the absence of somatic modification in the maintenance of immune receptor diversity (Cannon *et al.*, 2004).

IV.5.2.2. Somatically-generated diversity

Down syndrome cell adhesion molecules (Dscam) from insects

A member of the immunoglobulin superfamily (IgSF), the Down syndrome cell adhesion molecule (*Dscam*), in the fruitfly *D. melanogaster*, and its ortolog *AgDscam* in the mosquito *A. gambiae*, were found to be associated with pathogen resistance and to diversify, through alternative splicing, to generate more than 30 000 variants (Watson *et al.*, 2005; Dong *et al.*, 2006a). *Dscam* is a transmembrane molecule that contains extracellular domains of the Ig and fibronectin types, as well as a cytoplasmic domain (Schmucker *et al.*, 2000) and the gene is present as a single gene copy in both species. The extremely diversified *Dscam* repertoire in *D. melanogaster* is expressed in the hemocytes, and the modification of the expression of *Dscam* gene by RNA interference alters their phagocytic activity. These data suggested the implication of *Dscam* in *Drosophila* immunity. However, the latest and more important advance was evidenced in the work done by Dong *et al*. (2006). The authors demonstrated that, in *A. gambiae*, the immune response against *Plasmodium* does not increase the *AgDscam* transcript level, but produces a change in the representation of some changeable exons by alternative splicing. The different Ig domain-containing splice forms are over- or

under-represented depending on which pathogen the host cell is exposed to. Thus, challenge with different pathogens produces specific *AgDscam* splice-form repertoires with different adhesive characteristics.

Fibrinogen Related Proteins (FREPs) from mollusks

In mollusks, the diversification of the Fibrinogen Related Proteins (FREPs) has been reported in the pulmonate gastropod *Biomphalaria glabrata* (Zhang *et al.*, 2004). FREPs are soluble proteins and their genes are expressed in response to trematode parasites in hemocytes (Adema *et al.*, 1997). They are members of the immunoglobulin superfamily (IgSF) and consist of one or two amino-terminal IgSF domains and a carboxyl-terminal fibrinogen domain. In *B. glabrata*, 13 different subfamilies of FREP genes have been identified and they are coded by a variable number (1 to 8) of gene copies (Zhang *et al.*, 2004). The complete sequences of FREP 2, 3, 4 and 7 are already identified (Zhang and Loker, 2003; Zhang *et al.*, 2004) and a study focused on the characterization of the IgSF and fibrinogen domains of the subfamily FREP 3 evidenced a great diversity in these regions (Zhang *et al.*, 2004). From one single individual, 45 different sequences were isolated, but this number disagrees with the number of identified gene copies. As a result of further studies, this high level of diversification was proposed to be generated from a limited number of germline genes that are submitted to two types of mechanism: (i) point mutations, which allow the generation of new variants and (ii) somatic recombination using a limited set of source genes, which would lead to new mosaic genes. This study was the first proposing somatic recombination as a mechanism of diversification in organisms other than higher vertebrates.

An interesting example of co-evolution was proposed from the *B.glabrata*/*Schistosoma mansoni* model system. *B. glabrata* is an intermediate host for the human parasite *S. mansoni*, and if the arms race hypothesis is applicable, mechanisms of diversification based on polymorphism that permit the survival of species also have to exist in host parasites. The discovery of highly polymorphic mucins in *S. mansoni*, which are potential key factor proteins in the compatibility process of this parasite with *B. glabrata*, supports the hypothesis of an arms race taking place in this model (Roger *et al.*, 2008b). Mucins are known to play a key role in host–parasite interplay and are involved in immune evasion, host invasion and parasite protection (Hicks *et al.*, 2000; Theodoropoulos *et al.*, 2001). *S. mansoni* polymorphic mucins (*Sm*PoMucs) are differentially expressed in response to a compatible or an incompatible parasite strains (Roger *et al.*, 2008b) and each individual larva expresses a unique combination of glycosylated *Sm*PoMucs (Roger *et al.*, 2008a). It was found that *Sm*PoMucs are encoded by a multigene family, in which genes are transcribed in an individual-specific manner and create multiple splice variants for each gene. Global diversity is finally produced

by exon shuffling, gene conversion and frequent recombination, performed by functional genes or by pseudogenes. Finally, post-transcriptional regulation events, like alternative and aberrant splicing were shown to contribute to *Sm*PoMucs polymorphism (Roger *et al.*, 2008a).

Concerning the host/parasite interaction, the function of the FREPs family in *B. glabrata* is still not clear, although previous evidence suggested that FREPs, as pattern recognition receptors, are capable of binding molecules of foreign origin similar to *Sm*PoMucs (Adema *et al.*, 1997). In this context, both host and parasite could have created a system that generates a high degree of polymorphism to avoid, from the parasite side, the recognition by the host immune response and, from the host counterpart, to improve barriers against the parasite infection.

IV.6. Diversification of immune effectors

The mechanisms involved in the molecular diversification of immune effectors have been broadly described in several vertebrate and invertebrate taxa and a high number of these studies are related to AMP diversification (Hughes, 1999b; Lee *et al.*, 2005; Tennessen, 2005; Cohuet *et al.*, 2008). On the one hand, AMP diversification was studied from an evolutionary point of view, focusing on genetic mechanisms such as gene duplication, recombination, and allelic polymorphisms in both vertebrates and invertebrates. Several hypotheses have been proposed about the role of this diversification in the immune response. On the other hand, structure-activity relationship of AMP variants has been studied with the aim of designing novel antimicrobials (Powers and Hancock, 2003; Chandrababu *et al.*, 2009; Haney *et al.*, 2009). However, few studies have linked the evolutionary divergence of AMPs to functional analyses of their biological activity. The next examples of AMP diversification show those who have been characterized up to the biological significance of their diversity.

IV.6.1. Molecular evolution of AMPs and functional diversification

IV.6.1.1. Antimicrobial activities

Mammalian defensins provide one of the most striking examples of a diverse and evolving AMP gene family. As presented above, mammalian defensins are classified into three subfamilies based on the arrangement of the six cysteine motif: α-, β- and θ-defensins (Lehrer and Ganz, 2002), and α- and β-defensins have been extensively studied at evolutionary, structural and functional levels. A number of evolutionary studies showed that α- and β-defensins have a common ancestor, which has evolved by gene duplication and rapid sequence divergence (Maxwell *et al.*, 2003; Semple *et al.*, 2006; Hollox and Armour, 2008). It seems that they arose initially by unequal crossing over and tandem duplication (Hughes, 1999b; Morrison *et al.*, 2003). This mechanism of diversification means

that each subfamily presents a large degree of sequence variability and that subfamilies display low sequence similarity (Semple *et al.*, 2006). Additionally both α- and β-defensins have been subjected to allelic recombination between distinct genomic locations, which generated copy number diversity (Aldred *et al.*, 2005; Linzmeier and Ganz, 2005; Abu Bakar *et al.*, 2009).

Role of the anionic propiece. Mammalian α and β-defensins generally consist of three and two exons, respectively. The first exon encodes the signal and pro-region and the second (and third) exon encodes the mature peptide. Comparison of rates of synonymous and nonsynonymous nucleotide substitutions suggested that gene duplication has often been followed by the diversification of exons encoding the mature peptide by positive selection (Maxwell *et al.*, 2003). In many cases, it appears that amino acid changes in this region occurred in a balance manner between the mature peptide region and the propiece region (Hughes and Yeager, 1997). These data support the hypothesis of the neutralizing action of the propiece, which might be important to prevent intracellular interaction of peptides with membranes (Valore *et al.*, 1996).

Role of the cysteine scaffold. Despite the sequence variation in the mature peptide, the six cysteines are retained in all defensins and are under negative selection, implying that they are essential to the functional structure of the molecule. The importance of the six cysteines in the antimicrobial function of β-defensins has been challenged by the finding that different synthetic peptides of the human defensin 3 (hBD3), synthesized with different cysteine connectivities, exhibited similar antimicrobial activity (Kluver *et al.*, 2005). Similarly, a wide range of chemotactic activities was retained with these analogs. Interestingly, when the disulphide bridges were absent, the bactericidal activity remained unaffected but the chemotactic activity of linear hBD3 is altered (Wu *et al.*, 2003b). These results show that the cysteine pairing is required for the peptide chemotactic activities rather than antimicrobial activities. However, since the presence of the six cysteines at conserved positions is required for both the antimicrobial and chemotactic activity of defensins (Taylor *et al.*, 2008), the conservation of the cysteines during evolution reflects their involvement in both activities. Additionally, a mammalian defensin has recently been shown to have unexpected involvement in hair pigmentation, suggesting that mammalian AMPs may play roles outside immunity, which places them under different selective pressures (Candille *et al.*, 2007).

Role of charge distribution. There is experimental evidence that mammalian defensins are diverse in their potency against different pathogens. Functional differences have been observed *in vitro* between a number of β-defensin variants (Lehrer and Ganz, 2002). The correlation of the antimicrobial activity with the structural changes in hBD-3 reveals that the potency was determined by the distribution of positively charged and hydrophobic amino acid residues (Kluver *et al.*, 2005). Several evolutionary studies show evidence of positive selection in sites coding these residues, which can be linked to the functional diversification (Pazgier *et al.*, 2007). Concerning *α*-defensins, the

mouse neutrophil peptide NP-1 presents a positively charged arginine residue in the active peptide, which is under adaptive evolution (Lynn *et al.*, 2004) and it seems to be responsible for its antimicrobial activity against Gram-negative bacteria from genus *Actinobacillus* (Ouellette and Bevins, 2001). In humans, HNP1 and HNP3 differ only by one residue under positive selection at the N-terminus of the mature peptide and HNP1 exhibits potent activity against *Candida albicans* whereas HNP3 has little effect (Raj *et al.*, 2000; Lynn *et al.*, 2004). Therefore, small changes in the primary structure of these molecules can have significant effect on their activities, and positive selection can act at certain sites to increase their potency.

IV.6.1.2. Ecology

A remarkable example of adaptive evolution and functional divergence in vertebrate antimicrobials in relation with ecology is the case of **amphibian AMPs**. Frog species live in different ecological niches associated to a large spectra of potential pathogens, and every frog species are equipped with their own unique set of AMPs, constituting families from 2 to 100 closely related members (Mangoni, 2006). The dermal glands of frogs produce a great number of AMPs with remarkable levels of interspecific and intraspecific diversity (Nicolas *et al.*, 2003). The principal classes of amphibian AMPs include bombinins, magainins, dermaseptins, brevinins, esculentins and temporins (Mangoni and Shai, 2009). These principal classes of amphibian AMPs are genetically related, with a remarkable identity in signal and propiece sequences of their preprosequences. Evolutionary relationship between the conserved proregions has been analyzed, indicating that they all derived from a single family of precursor polypeptides, designated as the preprodermaseptins, which have been diverged to create several families of peptides that are structurally and functionally distinct (Vanhoye *et al.*, 2003) **(Fig. 13)**. This unusual pattern acting on the diversification of amphibian AMPs supports the hypothesis of an evolutionary strategy developed to confer ecological niche specialization, which arises by the encounter with new pathogens. Consequently, different species of frogs are equipped with different sets of peptides belonging to different families. The impressive divergence between and within frog species is such that no one identical peptide has been found between species at the amino acid level.

Evolutionary analyses showed that frog skin AMPs have arisen by gene duplication, focal hypermutation and diversifying selection. Each precursor results from transcription of a single genetic locus that encodes a single mature peptide. The contrast between the conserved preproregion and the hyperdivergent mature peptides is one of the most extreme examples observed to date for homologous gene products within a single order of organisms, with positive selection operating exclusively within their antimicrobial domain (Nicolas *et al.*, 2003). The combination of targeted hypermutation to generate great variation and the subsequent action of

positive selection might explain the hypervariation and large number of peptides per species (Nicolas *et al.*, 2003; Nicolas and El Amri, 2009).

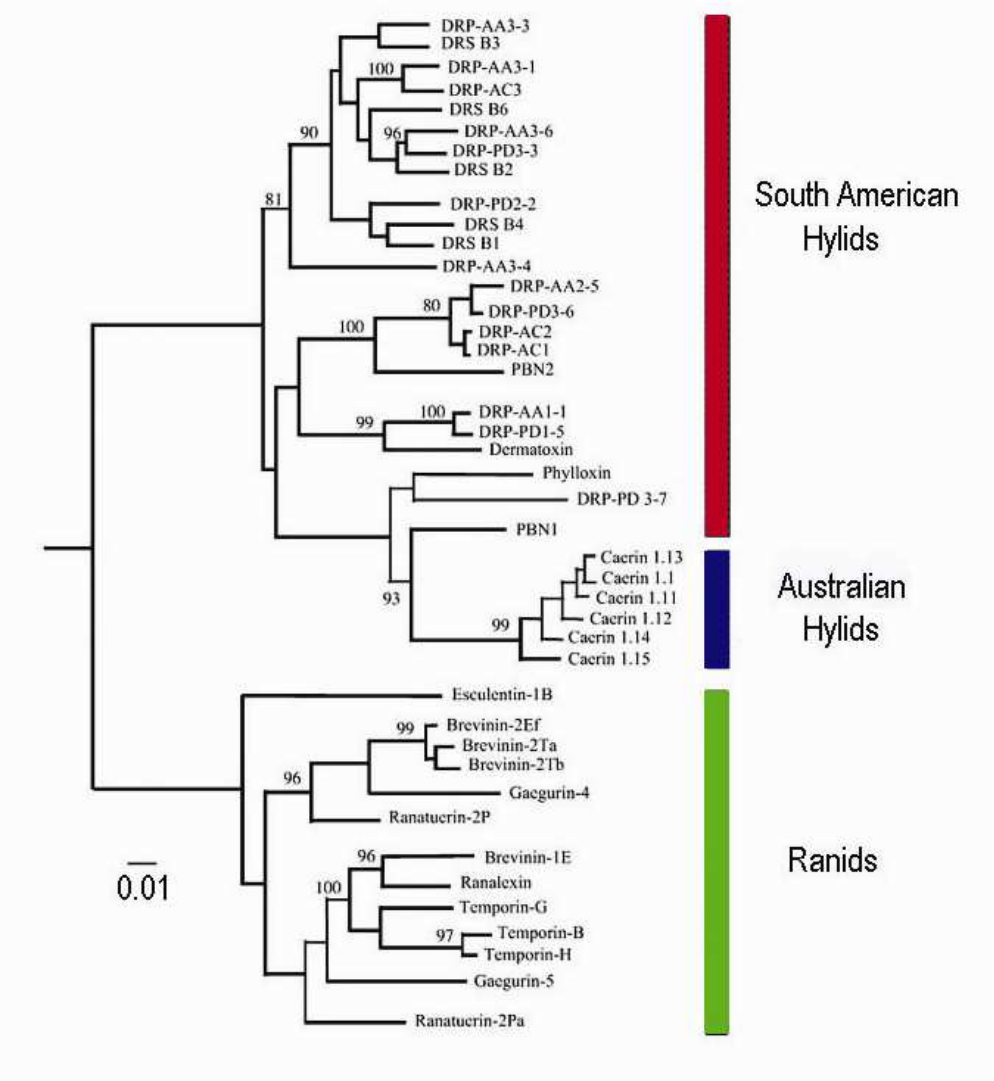

Figure 13. Molecular phylogeny of preprodermaseptins. Adapted from (Vanhoye *et al.*, 2003)

IV.6.1.3. In invertebrate AMPs

The molecular evolution of invertebrate AMPs has been mainly studied in model species, but studies that include their evolutionary history up to their gene function have not been as strongly developed as in vertebrates. Nonetheless, particular patterns of evolution in invertebrate AMPs have been evidenced in arthropods.

In the Pacific white shrimp, *Litopenaeus vannamei*, where three distinct classes of penaeidins, named PEN2, PEN3, and PEN4, are expressed in the hemocytes (Destoumieux *et al.*, 1999; Cuthbertson *et al.*, 2002). Multiple isoforms, generated by substitutions and deletions, have

been reported at the mRNA level for all three classes of penaeidins. Each penaeidin class is encoded by a unique gene and isoform diversity is generated by allelic polymorphism within each gene family (O'Leary and Gross, 2006; Padhi *et al.*, 2007). The multiple copies of penaeidins have evolved by gene duplication and adaptive evolution is the likely cause of accelerated rate of amino acid substitutions (Padhi *et al.*, 2007). A feature of the penaeidin family is intra-class diversity, particularly for class 3, where multiple unique isoforms have been reported between species and within an individual organism (Destoumieux *et al.*, 1997; Cuthbertson *et al.*, 2002; Gueguen *et al.*, 2006a). The antimicrobial spectra of the synthetic penaeidins from classes 3 and 4 were compared. Penaeidin class 3 has a broader range of microbial targets and is more potent against certain bacterial species than class 4 (Destoumieux *et al.*, 1999; Cuthbertson *et al.*, 2005), but the antimicrobial activity of class 4 appears to be more specifically directed against certain microbial strains (Cuthbertson *et al.*, 2008). Thus, it seems that diversification of an AMP family like penaeidins could produce a functional divergence of isoforms to enlarge the antimicrobial spectra.

At the present, diversification of AMPs in both vertebrates and invertebrates seems to present various common patterns, like gene duplication, allelic polymorphisms and, in some cases, evidence of adaptive evolution at certain codons. Furthermore, the biological significance of this diversification has been evidenced at different levels, in the acquisition of synergistic activities, microbial target specificity or extending the antimicrobial spectrum. However, there are still a lot of unresolved issues concerning AMP evolution, such as intraspecies AMP diversity. More research at the genetic level may answer many of these questions, including specific selective pressures, strength of selection and causes of divergence or conservation. Those who study the genetic selection to improve resistance to pathogens or find therapeutic uses of AMPs should attempt to identify the AMP families in which selection has produced high variation in antimicrobial properties. Or, alternatively, they should attempt to identify AMP families that do not appear to be under constant diversifying selection, perhaps because pathogens cannot easily evolve resistance to them. We therefore need to do further research to identify the underlying mechanisms of diversification of these molecules in order to better understand their function in the antimicrobial response and to be able to use them for specific purposes.

Chapter 2

Results

I. Section 1. Molecular diversity of antimicrobials from *Crassostrea gigas*.

Publication N°1. Molecular diversity of antimicrobial effectors in the oyster *Crassostrea gigas.* ***BMC Evolutionary Biology*** **2010**, 10: 23

In recent years, high levels of sequence diversity were reported to be characteristic of several immune genes from invertebrates (for review see (Schulenburg *et al.*, 2007; Du Pasquier, 2009)). Thus, the paradigm that diversification of immune genes is an exclusive property of the vertebrate adaptive immunity is now obsolete. Nowadays, studies in invertebrate model organisms have given insights into the origin and evolution of innate immune genes, evidencing numerous mechanisms of diversification and directional selection pressures, related to adaptive molecular evolution (Flajnik and Du Pasquier, 2004; Lee *et al.*, 2005; Litman *et al.*, 2005; Pujol *et al.*, 2008). Therefore, new evidences demonstrate that the innate immunity system of invertebrates is more complex that we previously thought.

In this context, the rapid evolution of AMPs is well documented (Patil *et al.*, 2004; Jiggins and Kim, 2005; Semple *et al.*, 2006) and suggests that hosts exposed to diverse pathogens may evolve a broader repertoire of antimicrobials that enhance their defensive potential (Pujol *et al.*, 2008). The rapid evolution of AMPs has been related with the general hypothesis of co-evolution or "arms race". In this theory, pathogens evolve continuously to escape from the immune response of hosts and, consequently, the immune system of hosts evolves to improve new barriers against pathogens (Dawkins and Krebs, 1979).

In the Pacific oyster, *Crassostrea gigas,* several antimicrobials have been characterized. First, an antimicrobial peptide (AMP), member of the defensin family (*Cg*-Defm) was identified from the oyster mantle (Gueguen *et al.*, 2006b) and then, two additional defensins named *Cg*-Defhs (*Cg*-Defh1 and *Cg*-Defh2) were characterized from hemocytes (Gonzalez *et al.*, 2007a). Another peptide named *Cg*-Prp*,* which belongs to the Proline-rich AMP family, was also identified from hemocytes (Gueguen *et al.*, 2009). Additionally, a member of the Bactericidal Permeability Increasing protein (BPI) family was characterized in hemocytes and oyster tissues (Gonzalez *et al.*, 2007b). From those works, the existence of a diversity of sequences at amino acid level began to be evidenced, which motivated further investigation. Hence, the first part of my thesis work deals with the characterization of the molecular diversity of the oyster antimicrobials by genomic approaches.

Nucleotide sequences for the antimicrobials were obtained by specific PCR amplification from oyster cDNA and genomic DNA, and by *in silico* search of homologous sequences among the 29,745 unique sequences available at the Sigenae *C. gigas* EST database (http://public-contigbrowser.sigenae.org:9090/Crassostrea_gigas/index.html). In the light of the high diversity observed among the AMP sequences and the discovery of novel variants, we checked the mechanisms by which the diversity could be generated. We evidenced that the AMPs, namely *Cg*-Defs and *Cg*-Prp are all the product of multigenic families displaying a variety of gene structures and copy number variation. In contrast, *Cg-bpi* is encoded by a single copy gene. Moreover, we identified for both AMPs several genetic mechanisms of diversification such as recombination, parallel mutations leading to phylogenetic homoplasy and indel events. In addition, the non synonymous to synonymous substitutions ratio by codon (dN/dS) revealed several negatively and positively selected sites for both AMPs, suggesting that directional selection pressures have shaped their sequence variations. Besides, the diversity of the three antimicrobials has been compared with the diversity present in a non-immune related gene (*Cg-actin*), showing that variability is not a common characteristic for all oyster genes.

This is the first evidence a high level of variability in oyster AMP sequences. The results accumulated reveal that AMPs from *C. gigas* have been subject to distinct patterns of diversification and we evidence the existence of different evolutionary routes leading to such sequence variability. The results are detailed and discussed in publication N°1.

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Molecular diversity of antimicrobial effectors in the oyster *Crassostrea gigas*

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Abstract

Background: To gain insight into the molecular diversity of antimicrobial peptides and proteins in the oyster *Crassostrea gigas*, we characterized and compared the sequence polymorphism of the antimicrobial peptides (AMPs), *Cg*-Defensins (*Cg*-Defs) and *Cg*-Proline Rich peptide (*Cg*-Prp), and of the bactericidal permeability increasing protein, *Cg*-BPI. For that, we analyzed genomic and transcript sequences obtained by specific PCR amplification and *in silico* searches.

Results: High diversification among the three antimicrobial effectors was evidenced by this polymorphism survey. On the basis of sequence phylogenies, each AMP aggregates into clearly defined groups of variants and is the product of a multigenic family displaying a variety of gene structures. In contrast, Cg-*bpi* forms a single group and is encoded by a single gene copy. Moreover, we identified for both AMPs several genetic mechanisms of diversification such as recombination, parallel mutations leading to phylogenetic homoplasy and indel events. In addition, the non synonymous to synonymous substitutions ratio by codon (dN/dS) revealed several negatively and positively selected sites for both AMPs, suggesting that directional selection pressures have shaped their sequence variations.

Conclusions: This study shows for the first time in a mollusc that antimicrobial peptides and proteins have been subject to distinct patterns of diversification and we evidence the existence of different evolutionary routes leading to such sequence variability.

Background

The Pacific oyster, *Crassostrea gigas*, is a filter feeder bivalve mollusc which is continually exposed to microorganisms naturally present in the marine environment. Thus, the capacity to control the microflora and overcome infections is essential for the oyster survival. The hemocytes, immunocompetent cells, play a central role in innate antimicrobial response. Indeed, in molluscs, hemocytes infiltrate injured tissues, phagocyte microorganisms, produce antimicrobial peptides (AMPs) and release factors such as lectins and reactive oxygen species (ROS) [1-3].

In *C. gigas*, several antimicrobial effectors have been recently characterized. An AMP, member of the defensin family named *Cg*-Defm, was identified from the oyster mantle [4]. Then, two additional defensins named *Cg*-Defhs (*Cg*-Defh1 and *Cg*-Defh2)have been characterized from hemocytes [5]. Both *Cg-defm* and *Cg-defhs* appear to be continuously expressed in different tissues. More recently, a new peptide, *Cg*-Prp, which belongs to the proline-rich AMP family, has been found in hemocytes [6]. Whereas *Cg*-Prp displays alone a weak *in vitro* antimicrobial activity, it revealed synergistic activity with *Cg*-Defm against both Gram positive and negative bacteria [6]. Additionally, a member of the bactericidal/permeability-increasing protein (BPI) family has been characterized [7]. *Cg-bpi* expression is induced in hemocytes after oyster bacterial challenge and constitutive in various tissue epithelia of unchallenged oysters. Similar to vertebrate BPIs, the oyster BPI binds LPS, displays bactericidal activity against Gram negative bacteria and increases the permeability of the bacterial cytoplasmic membrane [7].

High levels of sequence diversity were reported to be a characteristic of several antimicrobial effectors pertaining to the innate immunity system of invertebrates [8].

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The functional significance of this diversity has been addressed in some cases. Multiple variants of lectins from the horseshoe crab *Carcinoscorpius rotundicauda* are able to recognize and differentiate bacteria and fungi [9]. The AMP drosomycin from *Drosophila melanogaster* presents six isoforms with different antifungal activities [10]. Thus, diversification of antimicrobial effectors by accumulation of multiple variations around an originally unique form may provide significant means of acquiring microbial target specificity [11], concerned in the evolutionary arms race between pathogens and their hosts.

In this work, we have investigated the diversity of three oyster antimicrobial effectors and shown the existence of a high diversity in the transcript and genomic sequences of three antimicrobials, two peptides (*Cg-prp* and *Cg-defs*) and one protein (*Cg-bpi*). We also evidence distinct phylogenies between them and propose the existence of a combination of different genetic mechanisms from which sequence variability arises.

Results

C. gigas antimicrobial peptides and BPI protein show distinct phylogeny conformations

To characterize the diversity of all the genes, i.e. the two antimicrobial peptides *Cg-defs* and *Cg-prp*, the antimicrobial protein *Cg-bpi* and the non immune gene *Cg-actin*, we performed an exhaustive sequencing of cloned PCR products and collected sequences in the Sigenae database http://www.sigenae.org/. Finally, we got a total of 256 cDNA sequences and 60 gDNA sequences that we aligned and used to build phylogenies (Table 1). Alignments are available as Popset in GenBank; cDNA sequences of *Cg-prp*, *Cg-defs*, *Cg-bpi* and *Cg-actin*: FJ669353-FJ669402, FJ669323-FJ669352, FJ669296-FJ669322, FJ669287-FJ669295 respectively and gDNA sequences of *Cg-prp* and *Cg-defs*: FJ669252-FJ669286 and FJ669403-FJ669423 respectively. We obtained for both AMPs, *Cg-prp* and *Cg-defs*, clearly structured phylogenies where sequences clustered in distinct groups, whereas *Cg-bpi* and *Cg-actin* sequences formed star-like phylogenies instead (Figure 1). On this basis, we identified three distinct and more or less distant groups of *Cg-defs* (Figure 1a, group I, II and III), each one composed by multiple variants of *Cg-defh1*, *Cg-defh2* and *Cg-defm*, respectively.

Furthermore, we uncovered two lengths of transcripts for *Cg-defm*, one corresponding to the coding sequence (CDS) of 195 bp previously described [4] and a longer one identified here for the first time with a CDS of 219 bp. Similarly, we identified two lengths of transcripts for *Cg-prp*, the one already described with a CDS of 183 bp [6] and a novel shorter one with an indel of 6 nucleotides in the C-terminal region. Likewise to *Cg-defs*, *Cg-*

Table 1 Number of nucleotide sequences and general polymorphism values of antimicrobial effectors and non immune gene

	Cg-prp	*Cg-defm*	*Cg-defhs*	*Cg-bpi*	*Cg-actin*
PCR gDNA	35	11	14	0	0
PCR cDNA	57	40	43	36	40
in silico	12	2	15	1	10
total	104	53	72	37	50
different*	50	18	12	28	9
Length (bp)	177/183	195/219	195	1431	1128
SNP ratio 3	4.06	12.9	5.2	16.8	66.3
Hd	0.983	0.857	0.454	0.987	0.093
Pi	0.044	0.009	0.022	0.009	0.002

*N° of sequences with a minimum of 1 nucleotide difference founded at least two times (PCR and/or *in silico* searches)
SNP ratio: ratio of the number of polymorphic sites to the length of CDS i.e. reflect the density of polymorphic sites
Hd: Proportion of different sequences

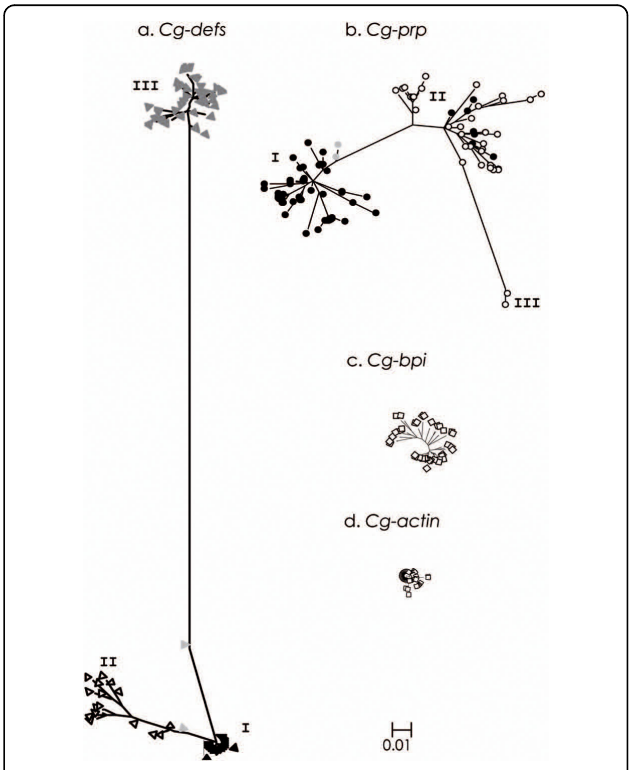

Figure 1 Phylogenies from CDS of three antimicrobial effectors and non immune gene from *C. gigas*. a. *Cg-defs*: I. *Cg-defh1* (black triangles); II. *Cg-defh2* (white triangles) and III. *Cg-defm* (dark gray triangles). Recombinant sequences between *Cg-defh1* and *Cg-defh2* are indicated (light gray triangles). **b**. *Cg-prp*: I. *Cg-prp* short forms (black circles), II. *Cg-prp* short and long forms, and III. *Cg-prp* long forms (white circles). *Cg-prp* recombinant sequences are indicated (gray circles). **c**. *Cg-bpi* (white rhombus) and **d**. *Cg-actin* (white squares). Construction of cladograms were performed with PHYML v2.4.4 using the models GTR+Γ for *Cg-defs*, HKY85+Γ for *Cg-prp*, TN93+I for *Cg-bpi* and F84+Γ for *Cg-actin* determined by the Akaike information criterion. For accurate comparison between the four genes, cladograms were drawn in the same scale.

Section 2. Mechanism of action of oyster defensins.

Publication N°2. Insight into invertebrate defensin mechanism of action: oyster defensins inhibit peptidoglycan biosynthesis by binding to lipid II. ***The Journal of Biological Chemistry.*** **2010** *in press.*

In the past years, antimicrobial peptides were generally assumed to kill microbes by non-specific pore formation and disruption of the membrane bilayer. Currently, the assumption that these peptides are uniform and indiscriminant membrane detergents is over. The recognition of sophisticated and thematic structure activity relationships causing distinct mechanisms of action is now entirely recognized (Yeaman and Yount, 2003). It is well known that AMPs must display an appropriate balance of hydrophobicity and net positive charge, since they are involved in their amphiphilic character and in their capacity to interact with bacterial membranes (Shai, 2002; Bulet *et al.*, 2004). Nevertheless, after this first contact, they could display a variety of antimicrobial mechanisms. AMPs can kill microbes by pore formation or by altering metabolic processes without membrane disruption, like inhibition of cell-wall biosynthesis, nucleic acid and protein synthesis or enzymatic activity (Brogden, 2005 89).

In the first part of this thesis, we characterized the molecular diversity of three oyster antimicrobials. Considering oyster defensins, results revealed that they have arisen through gene duplication, subjected to directional selection pressures and diverged in three distinct groups (*Cg*-Defh1, *Cg*-Defh2 and *Cg*-Defm), displaying a highly conserved mature peptide within each group. The evolution and divergence of AMP multigene families have been well described in vertebrates and invertebrates, (Cohuet *et al.*, 2008; Hollox and Armour, 2008; Lazzaro, 2008; Nicolas and El Amri, 2008; Padhi and Verghese, 2008), but these approaches lack experimental support to understand the biological consequence of divergence. Interestingly, the emergence of new AMPs variants by gene duplication, as well as the evidence of strong selective pressures driving evolution has been related to the functional diversification of these molecules (Lynch and Conery, 2000; Tennessen, 2005).

For these reasons, the diversity of oyster defensins led us to speculate on a functional divergence of variants, raising the question about the biological meaning of the existence of different isoforms. Therefore, three major variants representative of oyster defensin diversity (*Cg*-Defh1, *Cg*-Defh2 and *Cg*-Defm) were produced as recombinant peptides and their antimicrobial activities and mechanism of action were studied.

We found that oyster defensins display strong but different potency against Gram-positive bacteria and little to no activity against Gram-negative bacteria. Consequently, their mechanism of action was studied against the Gram-positive bacteria *Staphylococcus aureus*. We evidenced that

oyster defensins are a non forming pore AMP, which kill *S. aureus* through binding to the cell wall precursor lipid II, resulting in the inhibition of peptidoglycan biosynthesis. Interestingly, we identified a differential binding of the three defensin variants to lipid II, which correlated with their differential antibacterial activities. Altogether, these results support the hypothesis that selective pressures have directed oyster defensins towards the design of new variants displaying higher potency. Moreover, the lipid II-trapping, an essential step of oyster defensin antibacterial activity, would be under strong functional constraints.

The results are detailed and discussed in publication N°2.

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INSIGHT INTO INVERTEBRATE DEFENSIN MECHANISM OF ACTION: OYSTER DEFENSINS INHIBIT PEPTIDOGLYCAN BIOSYNTHESIS BY BINDING TO LIPID II

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Running head: Lipid II is a target for invertebrate defensins

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Three oyster defensin variants (*Cg*-Defh1, *Cg*-Defh2 and *Cg*-Defm) were produced as recombinant peptides and characterized in terms of activities and mechanism of action. In agreement with their spectrum of activity almost specifically directed against Gram-positive bacteria, oyster defensins were shown here to be specific inhibitors of a bacterial biosynthesis pathway rather than mere membrane active agents. Indeed, at lethal concentrations, the three defensins did not compromise *Staphylococcus aureus* membrane integrity but inhibited the cell-wall biosynthesis as indicated by the accumulation of the UDP-N-acetylmuramyl-pentapeptide cell wall precursor. In addition, a combination of antagonization assays, thin layer chromatography and surface plasmon resonance measurements showed that oyster defensins bind almost irreversibly to the lipid II peptidoglycan precursor, thereby inhibiting the cell-wall biosynthesis. To our knowledge, this is the first detailed analysis of the mechanism of action of antibacterial defensins produced by invertebrates. Interestingly, the three defensins, which were chosen as representative of the oyster defensin molecular diversity, bound differentially to lipid II. This correlated with their differential antibacterial activities. From our experimental data and the analysis of oyster defensin sequence diversity, we propose that oyster defensin activity results from selective forces that have (i) conserved residues involved in lipid II-binding, and (ii) diversified residues at the surface of oyster defensins that could improve electrostatic interactions with the bacterial membranes.

INTRODUCTION

Antimicrobial peptides (AMPs) are effector molecules of the innate immune system. Present in virtually all living organisms, they provide successful barriers against invading pathogens. Over the past decades, a wide variety of AMPs have been identified, giving evidence of a great diversity in terms of structure, size, and mode of action. Nevertheless, most AMPs are characterized by the prevalence of cationic and hydrophobic amino acids. It is well known that these peptides must display an appropriate balance of hydrophobicity and net positive charge, since their amphiphilic character is essential for their initial interaction with bacterial membranes (for review see (1,2)). Then, AMPs could display a variety of mechanisms of action, killing microbes by membrane disruption (*e.g.* pore formation) or by altering metabolic processes such as the septum

formation, or the cell-wall, nucleic-acid and protein syntheses (for review see (3 89).

Defensins are 3–5 kDa AMPs that contain three to four disulfide bridges (4). They are broadly distributed in the animal and plant kingdom (2). Mammalian defensins (α- and β-defensins) adopt a three stranded antiparallel β-sheet structure while the group of arthropod and plant defensins are composed of an α-helix linked to an antiparallel two-stranded β-sheet by disulfide bridges, making the so-called cysteine-stabilized α-helix/β-sheet motif (CSαβ). First evidenced in scorpion toxins, this motif is widespread in invertebrate defensins like arthropod and mollusk defensins (5-7), but is also found in plectasin, a defensin from the saprophytic ascomycete *Pseudoplectania nigrella* (8). CSαβ-containing defensins are mostly active against Gram-positive bacteria (7-9**).** Conversely, mammalian α- and β-defensins are usually both active against Gram-positive and Gram-negative bacteria (10).

The diverse spectra of activity of AMPs are believed to be indicative of different modes of action (5). However, the mechanisms of how defensins kill microorganisms are still incompletely understood. It is well established that the amphiphilic structure they adopt is crucial for the first interaction with the microbial surface (11). In addition, several defensins have been reported to damage bacterial and artificial membranes, including mammalian α- and β-defensins (12,13), as well as arthropod defensins (14,15). However, non membrane-disruptive mechanisms of action have also been proposed, as for the α-defensin HNP-1, which appears to transit across the cytoplasmic membrane with minimal disruption (13). Thus, over the past years, the debate has increased on how far membrane disruption accounts for the antimicrobial activity of defensins and other AMPs (16-18). Strictly antifungal defensins, which include defensins from plants and from lepidopteran insects, are not only membrane-disrupting agents but also interact with fungal glucosylceramides (19). Similarly, antibacterial defensins, which include mammalian, invertebrate (non lepidopteran) and fungal defensins, can be specific inhibitors of a bacterial biosynthesis pathway. For instance, the antibacterial activity of two mammalian and one fungal defensins has been recently shown to result from an inhibition of peptidoglycan biosynthesis (20-22).

We have performed here a comparative study of the mechanism of action of antibacterial invertebrate defensins, the cellular targets of which are still unknown. For that, we used as a model three defensin variants characterized in the oyster *Crassostrea gigas.* One was identified from the oyster mantle (*Cg*-Defm) (7), and two others from the immune cells, the hemocytes (*Cg*-Defh1 and *Cg*-Defh2) (23). The best studied is by far *Cg*-Defm. Like all oyster defensins, and mussel defensin (6), *Cg*-Defm exhibits four disulfide bonds. Nonetheless, its 3D-structure (7) is very similar to that of plectasin, which contains only three disulfide bonds (8). In a recent study, we showed that the diversity of oyster defensins has arisen through gene duplication and directional selection pressures, and that they cluster in three distinct groups of which *Cg*-Defh1, *Cg*-Defh2 and *Cg*-Defm are the representatives (24). As for other antibacterial invertebrate defensins, almost nothing is known at present about oyster defensin mechanism of action.

Here, we asked whether the oyster defensin diversity had a functional relevance in terms of spectrum of activity and mechanism of action. Three representatives of the oyster defensin diversity were therefore expressed as recombinant peptides and studied for their antimicrobial activities and mechanism of action. We found that oyster defensins are mainly active against Gram-positive bacteria, with *Cg*-Defh2 and *Cg*-Defm being noticeably more potent than *Cg*-Defh1 against several Gram-positive strains. By a combination of *in vivo* and *in vitro* assays, including UDP-MurNAc-pp accumulation assays, thin layer chromatography, surface plasmon resonance, and NMR, we showed that all oyster defensins inhibit peptidoglycan biosynthesis by binding to lipid II. We propose that the residues involved in lipid II-binding have been conserved through evolution, and we show that residues conferring improved antibacterial activity to oyster defensins by modifying their charge distribution are under diversifying selection.

Section 3. Role of antimicrobials in *Crassostrea gigas* immune response.

Transcript expression and localization of *Cg*-BPI, *Cg*-Prp and *Cg*-Defs in response to a *Vibrio* challenge and biological properties *in vitro*.

Publication N°3. Role of antimicrobial peptides and proteins in the immune response of the oyster *Crassostrea gigas.* ***To be submitted.***

Increasing knowledge on the antimicrobial response in *Crassostrea gigas* has been a major goal in the characterization of molecular processes involved in the defense reactions of this species. As already presented in this thesis, several families of antimicrobials, namely *Cg*-Defs, *Cg*-Prp and *Cg*-BPI, have been characterized from oyster hemocytes and various tissue epithelia. In a first time, we have shown that these antimicrobials display a high molecular diversity. In a second time, we determined the mechanism of action of *Cg*-Defs. Here, we aim to understand how these antimicrobials are involved in the oyster defense against pathogenic *Vibrios*. During the summer mortality outbreaks in France, several *Vibrio splendidus* strains have been isolated from oysters, and two strains (LGP31 and LGP32) were shown to cause mortalities when injected to oysters (Gay *et al.*, 2004a; Gay *et al.*, 2004b). We therefore monitored the expression and colocalization of *Cg*-Defs, *Cg*-Prp and *Cg*-BPI after a *Vibrio splendidus* LGP32 bacterial challenge.

For this, we analyzed the expression of genes encoding *Cg-Defhs, Cg-Defm*, *Cg-Prp* and *Cg-Bpi* by quantitative PCR (qPCR) in circulating hemocytes, gill, mantle and adductor muscle tissues (as the injection site) collected from oysters 12h after a *V. splendidus* challenge. The selection of the tissues was motivated by (i) the potential migration and infiltration of hemocyte populations from the hemolymph to exposed or infected tissues, and (ii) the previous evidence of epithelial expression of *Cg-Defm* (Gueguen *et al.*, 2006b) and *Cg-BPI* (Gonzalez *et al.*, 2007b). Besides, attention was paid to the colocalization of the antimicrobials in oyster circulating hemocytes by immunohistochemistry. To complete this study, the antimicrobial properties of these different immune effectors were analyzed together with potential synergic activities. For that, three recombinant variants of *Cg*-Defs, four synthetic variants of *Cg*-Prp and one recombinant *Cg*-BPI were produced.

We evidenced that oyster antimicrobials display a wide variety of expression profiles in hemocyte populations and tissues, some being constitutively expressed in specific tissues such as mantle (*Cg-Defm*) or hemocytes (*Cg-Defhs*), others being inducible (*Cg-BPI*) or apparently down-regulated (*Cg-Prp*) in hemocytes upon infection. In addition, *Cg-Defhs* expressing hemocytes probably migrate towards the infection site and it was highly expressed in gills tissues. As already shown for *Cg*-Defhs and *Cg*-Prp (Gueguen *et al.*, 2009), which are present in different hemocytes but

also inside same hemocytes, *Cg*-Defhs colocalizes also with *Cg*-BPI in some hemocyte populations. Finally we showed that oyster antimicrobials exhibit distinct spectra of activity that can be greatly extended through synergistic effects, between antimicrobials and between variants of the same AMP family. Altogether, these results suggest that the migratory properties of oyster hemocytes and the AMP basal expression in tissues could permit the encounter of these antimicrobials during a pathogenic infection, which might result in synergistic activities of antimicrobials to control diseases. We propose that such a synergy, which was evidenced both within and between families of antimicrobials, is essential during the oyster immune response, and that the high degree of sequence diversity in oyster antimicrobials compensates through synergic activities their low concentration in oyster tissues. The results are detailed and discussed in publication N°3.

Finally, we provide, as complementary data in this section, preliminary results obtained on the presence of cationic antimicrobials in *C. gigas* gills**.** Using a biochemical method that selectively enriches gill extracts in cationic molecules, we recovered a mixture of peptides that displayed antimicrobial activity against both Gram-positive and Gram-negative bacteria. By partial N-terminal sequencing and LC MS/MS, we identified several fragments of histones and ribosomal proteins. These new data evidence that the complete repertoire of antimicrobials present in oyster epithelial tissues remains to be further characterized in order to better understand their role in protecting oyster against diseases.

Role of antimicrobial peptides and proteins in the immune response of the oyster *Crassostrea gigas*

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To be submitted

Abstract

We have investigated here the implication of antimicrobial peptides and proteins in the oyster immune response against a pathogenic infection by *Vibrio splendidus.* Through an analysis of the transcript expression and immunolocalization of *Cg*-Defs, *Cg*-Prp and *Cg*-BPI, we found that oyster antimicrobials display a wide variety of expression profiles in hemocyte populations and tissues, some being constitutively expressed in specific tissues such as mantle (*Cg*-Defm) or hemocytes (*Cg*-Defhs), others being inducible (*Cg*-BPI) or apparently down-regulated (*Cg*-Prp) in hemocytes upon infection. Interestingly, hemocytes were found here to serve as vehicle for given peptides like *Cg*-Defhs, driving them to tissues exposed to microorganisms where other peptides like *Cg*-Defm or *Cg*-BPI are constitutively expressed. The migratory behavior of hemocytes that express the different antimicrobials was found to be of prime importance in response of oysters to a pathogenic *Vibrio* infection, contributing to colocalize antimicrobials that were shown here to have synergistic activities *in vitro.* We propose that such a synergy, which was evidenced both within and between families of antimicrobials, is essential during the oyster immune response, and that the high degree of sequence diversity in oyster antimicrobials compensates through synergic activities their low concentration in oyster tissues.

Introduction

Great efforts were made in the last years to characterize the molecular processes involved in the defense reactions of the oyster *Crassostrea gigas*. A particular attention has been paid to oyster antimicrobials, leading to the identification of various antimicrobial peptides (AMPs) and proteins by genomic approaches. First, a member of the defensin family (*Cg*-Defm) was identified from the oyster mantle [1], then two additional defensins named *Cg*-Defhs (*Cg*-Defh1 and *Cg*-Defh2) were characterized from hemocytes [2]. Both *Cg-Defm* and *Cg-Defhs* appeared to be constitutively expressed in different tissues. Another peptide named *Cg*-Prp*,* which belongs to the proline-rich AMP

family, was also identified from hemocytes [3]. Additionally, a bactericidal/permeability-increasing protein (BPI) was characterized, whose gene expression was shown to be induced in hemocytes from immune challenge oysters and constitutive in various tissue epithelia of challenged and unchallenged oysters [4].

In a recent study, the molecular diversity of these antimicrobials was investigated [5]. Phylogenetic analyses gave evidence of highly variable sequence diversity among *C. gigas* antimicrobials. *Cg*-Defs cluster in three distinct groups of which *Cg*-Defh1, *Cg*-Defh2 and *Cg*-Defm are the most representatives. In parallel, *Cg*-Prp presents a high number of variants with two lengths; the shorter peptide derived from that previously published [3] by a deletion of a conserved Proline-Arginine motif in the C-terminal region which corresponds to the putative mature peptide. In contrast to both AMPs, *Cg*-BPI is mostly represented by the sequence originally identified [5]. Thus, *C. gigas* possesses a variety of diversified antimicrobials.

Over the last 40 years in France, oyster culture has been regularly confronted to high summer mortality outbreaks. Summer mortalities are a phenomenon known from decades, observed worldwide with more or less intensity according to the years. During these mortality episodes in France, several *Vibrio splendidus* strains have been isolated from oysters, and two strains (LGP31 and LGP32) were shown to cause mortalities when injected to oysters [6 , 7].

The aim of this study was to understand how antimicrobials are involved in the defense against pathogenic *Vibrios*. We therefore monitored the expression and tissue colocalization of *Cg*-Defs, *Cg*-Prp and *Cg*-BPI protein after a *Vibrio splendidus* LGP32 bacterial challenge. We proposed here a global study based on analyses of the antimicrobial modulation of expression and their tissue localization in response to infection, at the level of transcripts and protein. For this, we considered the circulating hemocytes, gill, mantle and adductor muscle tissues (as the injection site). The selection of the tissues was based on the following two criteria: (i) the migration and infiltration of hemocyte populations from the hemolymph to exposed or infected tissues, including the site of injection and (ii) the previous evidence of epithelial expression of *Cg-Defm* [1] and *Cg-BPI* [4]. To complete this study, synergic antimicrobial activities of the effectors were investigated.

Results

Expression of oyster antimicrobials after a Vibrio challenge

The expression of genes encoding *Cg-Defhs, Cg-Defm*, *Cg-Prp* and *Cg-BPI* was analyzed by quantitative PCR (qPCR) in hemocytes, gills and mantle tissues collected from oysters 12h after challenge **(Fig. 1A)**. In our experiments, oysters received an intramuscular injection of *V. splendidus*, which is still considered as the only way to obtain reproducible and standardized *Vibrio* infections [8 ,

Complementary results: Evidence of cationic antimicrobials in *C. gigas* gills

Introduction

The innate defense at epithelial linings is both a physical and a chemical barrier to infections. As such, it is fundamental for organisms living in aquatic environments rich in microorganisms. Epithelia participate in immune regulation and generation of biologically active molecules, because these surfaces are in constant contact with a variety of potential pathogens (Ganz, 2002). To avoid infection, unbroken epithelia are a requirement. If a pathogen breaches these barriers, the innate immune system provides an immediate response through the epithelia, and then the immune cells and soluble factors operate in a coordinated way to provide protection (Pruzzo *et al.*, 2005). Therefore, many antimicrobials have been isolated from body surfaces and mucosal linings from aquatic organisms (Park *et al.*, 1996; Birkemo *et al.*, 2003; Bergsson *et al.*, 2005).

In the oyster *C. gigas*, knowledge is increasing on the innate defenses at epithelial linings. One AMP belonging to the defensin family, namely *Cg*-Defm, was found to be continually and specifically expressed in mantle tissues (Gueguen *et al.*, 2006b). Besides, one protein member of the Bactericidal Permeability Increasing protein family, *Cg*-BPI, was evidenced to be continually expressed in the epithelia of various organs of the oysters, including gills, mantle, labial palps or digestive system (Gonzalez *et al.*, 2007b). In addition, a high transcript abundance of the hemocyte defensins *Cg*-Defhs was evidenced here in oyster gill tissues (*cf. Chapter II. Section 3. Role of antimicrobials in C. gigas immune response*).

This prompted us to isolate and identify by biochemical approaches the antimicrobial components present in gills. Most AMPs are cationic, a property that promotes the interaction with the negatively charged microbial membranes (Bulet *et al.*, 2004). Therefore, the isolation method used in this study was designed to selectively enrich gill extracts in cationic molecules. We recovered a mixture of peptides that displayed antimicrobial activity against both Gram-positive and Gram-negative bacteria. By partial N-terminal sequencing and LC MS/MS, we identified several fragments of histones and ribosomal proteins. These findings suggest that a variety of antimicrobial peptides can be present in oyster gill tissues and provide evidence that other antimicrobials like histone-derived non conventional AMPs are present in these tissues.

Experimental procedure (cf. Chapter IV. Materials and Methods III.7.).

Results

Cationic peptide extracts from C. gigas gills display antimicrobial activity

In an attempt to purify cationic antimicrobial peptides from *C. gigas* epithelial tissues, frozen gills were pulverized in liquid nitrogen and the resulting powder was subjected to an acid extraction followed by cation-exchange chromatography. The resulting extract was enriched in cationic peptides and proteins, as evidenced by electrophoresis in non-denaturant Acid Urea-gels (AU-PAGE). Indeed, several bands with distinct electrophoretic behavior were observed after commassie blue staining **(Fig. 14A).** By comparing the electrophoretic profiles of extracts from oysters subjected or not to a bacterial challenge, we evidenced a very similar pattern in both extracts. However, they differed in the intensity of three bands, referred to as bands 0, 1 and 3, which suggested the generation of cationic peptides upon the bacterial challenge.

To detect antimicrobial activity in the extracts, we used a gel overlay technique that imprints peptides electrophoresed by AU-PAGE onto a layer of bacteria (Lehrer *et al.*, 1991). After overnight incubation, inhibition halos were observed, corresponding to different bands in the extracts from both non-challenged and challenged oysters. A total of 5 different bands showed antimicrobial activities against both the Gram-positive *S. aureus* SG511 **(Fig. 14B)** and the Gram-negative *E. coli* SBS363 bacteria. Interestingly, the band 1 which is more intense in challenged oysters, showed antibacterial activity in the extract of challenged oysters only.

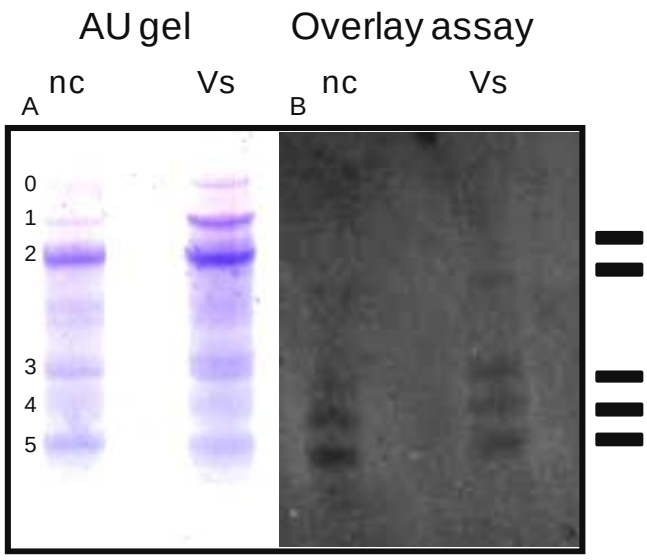

Figure 14. A. Acid-Urea PAGE of cationic extract from gills of non challenged (nc) and challenged (Vs) oysters with *V. splendidus* LGP32. **B.** Overlay antibacterial assay against *S. aureus*.

Identification of cationic peptides from active bands

Every active band from the extract of challenged oysters was excised from AU-PAGE and subjected to Edman degradation. We obtained one single sequence from bands 4 and 5. The 11–residue N-terminal sequence obtained from band 4 (NH_2-AGGKAGKDSGK-COOH) corresponds to a member of the histone family. Indeed, this fragment is highly similar to the histone H2A sequence from *Crassostrea virginica* [Genbank CD649294]. In addition, by BLAST searches in the *C. gigas* EST nucleotide database (http://public-contigbrowser.sigenae.org:9090/Crassostrea_gigas/index.html), several sequences which unknown functions perfectly matched with the corresponding peptide sequence. From the Edman degradation sequence, it was concluded that the isolated peptide derives from the translated sequence by elimination of N-terminal Met **(Fig. 15A)**. The 6–residue N-terminal sequence obtained from band 5 (NH_2-AKSKNH-COOH) was identified as the 60S ribosomal protein L29 (RPL29) by exact match to the highly conserved 60S ribosomal protein L29 from many vertebrates and invertebrates **(Fig. 15B)**. The full-length RPL29 sequence was absent from the *C. gigas* EST database. However, from the alignment with L29 proteins from other species (mammals and insects), it was inferred that similar to Histone 2A, the antimicrobial RPL29 derived from the translated sequence by elimination of the N-terminal Met **(Fig. 15B)**. Bands 2 and 3 contained a mixture of 2-4 peptides as evidenced by Edman sequencing. The band 1, which is more intense in challenged oysters, did not give any signal by Edman degradation. Based on the intensity of the band, it was hypothesized to contain a terminally blocked peptide/protein.

In order to better identify the peptides/proteins present in the active bands, they were excised from AU-gel, subjected to in-gel trypsin digestion and analyzed by LC MS/MS. Peptide identification was obtained by matching MS/MS sequenced fragments against the *C. gigas* EST sequences. This resulted in the detection of several ribosomal and histone proteins **(Table 1)**. Histone H2A was confirmed to be present in band 4 (one fragment from the N-terminal region), together with additional ribosomal proteins. In addition, a fragment similar to H2B from *S. mansoni* [Genbank CAZ35189] was identified, suggesting the presence of different histone fragments in this band. We speculate that the high sensitivity of the MS/MS sequencing revealed the presence of minor products co-migrating with histone 2A, which appeared as pure by Edman degradation.

Surprisingly, MS/MS sequencing applied to band 5 did not reveal any trypsic fragment from RPL29. Instead, we found other ribosomal proteins and the same H2A fragment as in band 4 **(Fig. 15B)**. Due to the absence of the RPL29 sequence in the *C. gigas* database, we could not determine by *in silico* analysis whether the RPL29 trypsic fragments are susceptible to be lost during the LC MS/MS due to poor hydrophobicity.

Noteworthy, in all analyzed bands we identified fragments of ribosomal proteins. Strikingly, fragments from RPL35 were found in active bands 2 to 5 **(Fig. 15C)**. Although we cannot rule out the

possibility of cross contamination at one of the analytical steps, this result strongly suggest that active fragment of various length are generated from RPL35, giving rise to a series of cationic AMPs in gill extracts.

Band 1 was the only active band lacking RPL35 fragment in its trypsic digest. Instead, five other ribosomal proteins were identified, two of which have a Ser and a Pro as a N-terminal residue (RPL28 and RPL8 respectively). Acetylation/glycosylation of Ser confers resistance to Edman degradation (Wellner *et al.,* 1990). Besides, examples of N-terminal Pro of proteins forming DNA-protein complexes have been reported to resist to Edman degradation (Zharkov *et al*., 1997). Such a resistance would be consistent with the lack of signal by Edman degradation. Unfortunately, the full-length sequences of the remaining ribosomal proteins from band 1 are not present in the *C. gigas* database. We therefore cannot speculate on their N-terminal blocking.

Discussion

In this study, we aimed to identify antimicrobial components from epithelial linings that may contribute to the immune defense of *C. gigas.* By using AU-PAGE and overlay antimicrobial assays, we evidenced several peptide bands displaying antimicrobial activity against both Gram-positive and Gram-negative bacteria. The expected antimicrobials, *Cg*-BPI or *Cg*-Defhs failed to be isolated by this method. We assume that their concentrations were below the detection limits. Active peptides were identified by Edman and LC MS/MS sequencing as corresponding to fragments of histones and ribosomal proteins.

There are growing evidences that histones are involved in a multitude of biological functions including DNA stabilization, cellular signaling and innate immunity (Parseghian and Luhrs, 2006). Histone fragments with antimicrobial properties have been identified and isolated from tissues of mammals, fish and toads, as well as in invertebrates like shrimp and mollusks. Three AMPs derived from the N-terminus domain of histone H2A have been purified and characterized from fish and toad, the buforin (Park *et al.*, 1996), the parasin (Park *et al.*, 1998b) and the hipposin (Birkemo *et al.*, 2003). In marine invertebrates, N-terminal H2A fragments with antibacterial activity were also isolated from hemocytes of the shrimp (Patat *et al.*, 2004), scallop (Li *et al.*, 2007) and from the digestive gland of the abalone *Haliotis discus* (De Zoysa *et al.*, 2009). Moreover, the complete H2A protein with antimicrobial activity was found in skin mucus of the rainbow trout (Fernandes *et al.*, 2002). At this stage, we do not know if full length H2A or fragments are the active antimicrobials isolated from *C. gigas*. However, we only obtained sequences from the N-terminal region, which

A. Histone 2A

```
C. gigas H2A MAGGKAGKDSGKAKAKAVSRSARAGLQFPVGRIHRHLKNRTTSHGRVGATAAVYSAAILE
Band 4       ----------------------AGLQFPVGR--------------------------
Band 5       ----------------------AGLQFPVGR--------------------------
N-ter-4      -AGGKAGKDSGK-----------------------------------------------

C. gigas H2A YLTAEVLELAGNASKDLKVKRITPRHLQLAIRGDEELDSLIKATIAGGGVIPHIHKSLIG
Band 4       ------------------------------------------------------------
Band 5       ------------------------------------------------------------
N-ter-4      ------------------------------------------------------------

C. gigas H2A KKGTQKTA
Band 4       --------
Band 5       --------
N-ter-4      --------
```

B. Ribosomal protein L29

```
N-ter Band5     -AKSKNH-------------------------------------------------
L29-M.-mula     MAKSKNHTTHNQSRKWHRNGIKKPRSQRYESLKGVDPKFLRNMRFAKKHNKKGLKKMQAN
L29 M. sexta    MAKSKNHTNHNQNRKAHRNGIKKPTKVRHESTLGMDPKFLRNQRFSKKGNLKPAKQL---

N-ter Band5     ------------------------------------------------------------
L29-M.-mula     NAKAMSARAEAVKALVKPKEVKPKIPKGVSGKLDRLAYIAHPKLGKRARARIAKGLRLCR
L29 M. sexta    ------ARAAERKAVREAKAKK-----------------------------------

N-ter Band5     ----------------------------------
L29-M.-mula     PKAKAKDQTKAQAAAPASIPAQAPKGAQATTKATE
L29 M. sexta    ----------------------------------
```

C. Ribosomal protein L35

```
C. gigas RPL35 XXXDLKQELGTLRVAKVTGGAASKLSKIRVVRKSIARVLTVMHQTQKENLRKYYKSKRLK
Band 2         ------QELGTLR-----------------------------------------------
Band 2         QLDDLKQELGTLR-----------------------------------------------
Band 3         ----------------------------------VLTVMHQTQK-------------
Band 3         ------------------------------------------------------------
Band 4         ------------------------------------------------------------
Band 5         ------------------------------------------------------------
Band 5         ------------------------------------------------------------

C. gigas RPL35 PTDLRKKKTRAMRRALTPFEKSIKSRKQQRRERLYPMRKFAVKQ
Band 2         ------------------------------------------
Band 2         ------------------------------------------
Band 3         ------------------------------------------
Band 3         -------------RALTPFEK-----------------------
Band 4         -------------RALTPFEK-----------------------
Band 5         ------------------------------ERLYPMR------
Band 5         -------------RALTPFEK-----------------------
```

Figure 15. Alignment of aminoacid sequences of fragments obtained by LC MS/MS and Edman degradation from active bands from gills extract. **A.** *C. gigas* histone 2A [Genbank AM856086]. **B.** N-terminal sequence from band 5 obtained by Edman degradation with RPL29 aminoacid sequences from M. mulatta [Genbank XP_001091393] and M. Sexta [Genbank GU084325]. **C.** *C. gigas* ribosomal protein L35 [Genbank ABY27350].

indicates that the active H2A sequence contains the N-terminal region **(Fig. 15).** MS analysis of uncleaved band 4 should help answer this question. Besides, the American oyster, *C. virginica,* produces an AMP derived from the histone H2B which displays antimicrobial activity in gill tissues (Seo *et al.*, 2009). The epithelial surface is under constant risk of abrasion, so damage to the cells may allow histones to become exposed to potential pathogens. Enzymes, such as cathepsin D, also expressed in fish epidermal mucosa, are known to promote the release of histone fragments (Cho *et al.*, 2002). The presence of H2A and H2B fragments with antibacterial activity in gills of different oyster species strengthen the hypothesis of their implication in oyster epithelial defense.

Unlike histones, many fewer reports describe antimicrobial properties of ribosomal proteins or derived fragments. Ribosomal proteins have been identified as antimicrobials in the skin mucus of fish (Bergsson *et al.*, 2005) and in human colon epithelium (Howell *et al.*, 2003; Tollin *et al.*, 2003). In mouse, an heparin/heparan sulfate interacting protein (HIP/RPL29), which is identical to the ribosomal protein L29 and exhibits antimicrobial activity, was identified from lung and small intestine (Meyer-Hoffert *et al.*, 2008). Again, further MS analysis is required to identify the length(s) of the active RPL35-derived fragment(s) found in oyster gill extracts. That those peptide fragments are actual effectors of oyster immunity is supported by a recent study in which the cellular mechanism of autophagy was shown to generate antimicrobial peptides from the processing of a ribosomal protein, RPS30 (Ponpuak *et al*., 2010). This study proposes that cytoplasmic proteins with no evident immunological function have an "afterlife" following their autophagic degradation. Thus, autophagy produces antimicrobial peptides from cytosolic proteins, that are normally engaged in anabolic processes (protein synthesis on ribosomes), and available in principle in all cells. These proteins have an affinity for negatively charged molecules and as such, they are a potential source of cationic antimicrobials.

Although further work will certainly be required to isolate and definitely characterize the active peptides from gill extracts, conventional AMPs (*Cg*-Defs, *Cg*-BPI, *Cg*-Prp) were not found at concentrations sufficient for purification in oyster gills. They could not be detected in paraffin embedded gill sections neither *(cf. Chapter II. Section 3. Role of antimicrobials in C. gigas immune response).* Until now, the only method that evidenced the presence of *Cg*-Defs and *Cg*-Prp in gills was by competitive ELISA (B. Romestand, unpublished data). Altogether, this indicates their poor abundance in oyster gills. On the contrary, histone- and ribosomal protein-derived AMPs appeared both abundant and active against Gram-positive and Gram-negative bacteria. This may endow unconventional AMPs with a protective role in the oyster surface tissues rich in potential invaders from the marine environment.

Although supported by an increasing literature, results from the present study must be interpreted with care. Indeed, the cation-exchange chromatography step used in this approach

favors the isolation of cationic peptides/proteins such as histones and ribosomal proteins. Studies on the antimicrobial activities of purified or synthetic H2A and RPL35 will help validating their role in the antimicrobial activity of cationic extracts and their interest in the control of pathogens. Finally, it will be interest to determine if these antimicrobials derived from histones and ribosomal proteins are present in organs/tissues other than gills and if their generation is continuous/inducible.

Table 1. Peptides identified by Edman sequencing and LC MS/MS from the 5 AU-PAGE bands of the cationic extract from oyster gill tissues presenting antimicrobial properties. The numbers of peptide fragments found for each sequence are indicated at right.

	N-terminal sequencing	**MALDI TOF LC MS/MS**	**Genbank N°**	**N° peptide fragments** **(between 7 and 13 residues)**
Band 1	No result. Probably bloqued N-ter.	RP L26 *A. irradians* RP L8 *C. gigas* RP L4 *C. gigas* RP L24 *C. gigas* RP L28 *C. gigas*	DV736295 AJ563478 BQ426269 CAD91424 CX069200	3 3 2 2 2
Band 2	_*A*_-(_I_KL)-(DV_K_)-(AGN_K_)-L_A_-K-(Q_E_)-(T_R_)-K-G-S (3-4 peptides mixture)	RP L35 *C. gigas* RP L4 *C. gigas* RP L24 *C. gigas* RP L28 *C. gigas*	CB617368 BQ426269 CAD91424 CX069200	4 3 2 2
Band 3	Peptide mixture Weak signal	RP L35 *C. gigas* RP S18 *C. gigas*	CB61736 AB199895	1 1
Band 4	A-G-G-K-A-G-K-D-S-G-K- Histone H2A	RP L35 *C. gigas* RP L24 *C. gigas* RP L32 *C. gigas* H2A *C. gigas* H2B *S. mansoni* RP L31 *C. gigas*	CB617368 CAD91424 CAD79337 AM856086 CAZ35189 CAD91431	2 2 2 1 1 1
Band 5	AKSKNH 60S RP L29	RP L36 *C. gigas* RP L35 *C. gigas* H2A *C. gigas*	AJ565680 CB617368 AM856086	1 1 1

Chapter 3

General Discussion and Future Prospects

The overall aims of this thesis were to identify and characterize the molecular diversity of the *C. gigas* antimicrobials and to understand the importance of this diversity in oyster immunity. More specifically, I examined i) the molecular diversity of *Cg*-Defensins (*Cg*-Defs) and *Cg*-Proline rich peptide (*Cg*-Prp) AMPs and the Bactericidal Permeability Increasing protein (*Cg*-BPI), as well as the underlying mechanisms that generate their molecular diversity; ii) the biological activities and mechanism of action of three variants of oyster defensins against the Gram-positive bacteria *Staphylococcus aureus* and iii) the role of *Cg*-Defs, *Cg*-Prp and *Cg*-BPI in protecting oysters against pathogenic infection, by the analysis of their transcript expression and localization after a *Vibrio splendidus* challenge and by the *in vitro* analysis of their biological properties. Additionally, a brief survey of the presence of antimicrobials in oyster gill tissues was performed.

I. Diversification of AMPs and the coevolutionary "arm race" between host and pathogens

Over the past years, knowledge has increased on the molecular bases of oyster immunity through the characterization of several antimicrobials from *Crassostrea gigas* (Gueguen *et al.*, 2006b; Gonzalez *et al.*, 2007a; Gonzalez *et al.*, 2007b; Itoh and Takahashi, 2007; Gueguen *et al.*, 2009). In a previous work, Gonzalez *et al.* amplified several variants of *Cg*-Defhs suggesting polymorphism (unpublished data). We speculated that oyster antimicrobials might present a high level of diversity produced by the coevolutionary pressures between host and pathogens. Indeed, the diversification and polymorphism of immune genes has been related with theoretical models of coevolution, like the "Red Queen hypothesis" (Van Valen, 1974) applied on the host/parasite interactions (Combes, 2000). It predicts an "arm race" between hosts and parasites, with a reciprocal evolution and antagonism between the resistance mechanisms of the host and the infectivity/virulence of the parasite. Therefore, molecules that play an essential role in the interaction between host and pathogens should present high levels of diversity and polymorphism, in order to assure the coevolutionary dynamic (*cf.IV.1 Host pathogen interaction and coevolutionary theory*).

There is increasing evidence that all species present a particular arsenal of diversified antimicrobial families (Vanhoye *et al.*, 2003; Conlon *et al.*, 2009; Nicolas and El Amri, 2009) and it is proposed that the diversity of AMPs in a host may play a role in determining the pathogenicity of a microbe in that species (Ganz, 2003). Therefore, great efforts are currently done to identify the exclusive arsenal that each species can develop and to further recognize the bases of the diversity (Clark and Wang, 1997; Lee *et al.*, 2005; Tennessen and Blouin, 2007; Cohuet *et al.*, 2008; Hollox and Armour, 2008).

We evidenced high and variable diversity of *C. gigas* AMPs, generated by a combination of different genetic mechanisms previously described in the diversification of several immune genes (Holmes, 2004; Lazzaro, 2008). As observed for oysters, penaeid shrimp have a diverse AMP family referred as the penaeidins (Gueguen *et al.*, 2006a; Kang *et al.*, 2007). Each penaeidin class (from 2 to 5) is composed by several isoforms and is encoded by unique genes. As found here for oyster AMPs, diversity of penaeidins is generated by gene duplication and allelic polymorphism within each gene family, and isoforms arise from multiple length and single nucleotide polymorphisms (O'Leary and Gross, 2006; Padhi *et al.*, 2007). Evolutionary analyses in penaeidins also indicated that not all codon sites are positively selected; hence selection appears to act differently on different codons and could be implicated in the involvement of these sites in the AMP function (O'Leary and Gross, 2006). Thus, like penaeidins, oyster AMPs appear to be under strong evolutionary pressures to diversify and create new variants by several genetic mechanisms and selection pressures.

In contrast to the evidence of adaptive evolution in shrimp and oyster AMPs, in *Drosophila,* several studies have suggested that, despite remarkable dynamics in genome duplication and deletion of genes encoding AMPs, their molecular evolution is not driven by natural selection (Clark and Wang, 1997; Lazzaro and Clark, 2003; Schlenke and Begun, 2003). Despite the rejection of diversifying selection by evolutionary analyses, the dynamic state of gene duplication in *Drosophila* AMPs suggests the potential generation of new functional genes.

In the insect class, a great number of different AMP families have been described, but most of them are restricted to a few closely related species. For example, *Drosophila* produces eight classes of AMPs, but only three are found in the genomes of bees, mosquitoes and beetles. On the other hand, these insects also produce their own unique set of AMPs (Christophides *et al.*, 2002; Evans *et al.*, 2006; Waterhouse *et al.*, 2007; Zou *et al.*, 2007). Thus, it appears that each insect lineage has developed its own repertoire of antimicrobials, probably due to evolutionary strategies directed to confer ecological niche specialization (Lazzaro, 2008). Niche specialization and, consequently, the adaptation to new pathogens might produce high levels of inter- and intraspecific diversity in insects, as already proposed for amphibians (c*f. IV 5.1.2. Ecology*).

Altogether, these evidences support our hypothesis that the generation of several variants in AMP families may be indicative of neo- or sub-functionalization processes, due to (i) the high exposure of hosts to potential pathogens, as observed for aquatic organisms, and/or (ii) a niche specialization, both phenomena often invoked in the course of evolution of immune genes and in the coevolutionary arm race between hosts and pathogens.

II. Biological meaning of the diversity in oyster antimicrobials

The diversity of oyster AMPs as a result of selective pressures driving evolution motivated us to investigate (i) the antimicrobial activities and mechanism of action of three defensin variants representative of their diversity (*Cg*-Defh1, *Cg*-Defh2 and *Cg*-Defm) and (ii) the potential interplay between the different AMP families. From the results obtained in these studies we propose that the significance of their diversity could be explained at least by their different antimicrobial spectra and the variation in potency between variants, and by the existence of synergistic effects.

II.1. Antimicrobial spectra

We confirmed here the spectra of antimicrobial activities of oyster antimicrobials: oyster defensins were mainly active against Gram-positive bacteria, *Cg*-Prp was barely active alone, and *Cg*-BPI was only active against Gram-negative bacteria. In addition, we showed that the different variants of each AMP family could display different antimicrobial potency. *Cg*-Defh2 was the most potent among oyster defensins, being from 2 to 40-fold more potent than *Cg*-Defh1 and *Cg*-Defm against Gram-positive strains. Regarding *Cg*-Prp variants, although they showed reduced activity against all tested bacteria, the long variants, which displays an extra Arg-Pro motif in the putative active region, was more potent that the short ones.

Such a difference in the antimicrobial spectra of AMP variants was previously reported for other invertebrate AMPs. Like in oyster, the four variants of the mytilin AMP family (mytilin B, C, D, and G1) from the mussel *Mytilus galloprovincialis*, were reported to display different antimicrobial activities (Mitta *et al.*, 1999a). Indeed, whereas mytilins B, C and D isoforms are active against both Gram-positive and Gram-negative bacteria, G1 displays only anti Gram-positive antimicrobial activity. Additionally, mytilins B and C possess differential activities against the fungus *Fusarium oxysporum* and Gram-negative bacteria. In shrimp, the antimicrobial spectra of the synthetic penaeidins from *Litopenaeus setiferus Litset* Pen4-1 and *Litset* Pen3-4 were compared to the spectrum of the recombinant *Litvan* Pen3-1 from *L. vannamei*. Penaeidin class 3 has a broader range of microbial targets and is more potent against certain bacterial species than class 4 (Destoumieux *et al.*, 1999; Cuthbertson *et al.*, 2005), but the antimicrobial activity of class 4 appears to be more specifically directed against certain microbial strains (Cuthbertson *et al.*, 2008). In *Drosophila*, the 6 variants of the drosomycin AMP family display different antifungal activities, which has been correlated with the presence of variable residues among variants (Yang *et al.*, 2006; Tian *et al.*, 2008). Taken together,

these evidences are consistent with the theory that **different variants in an AMP family could be the result of the functional divergence of isoforms to extend the antimicrobial spectra.**

The variation in oyster defensin potency and the evidence of diversifying selection at certain sites suggest that selection pressures on the evolution of AMPs may have occurred to preserve an adaptive phenotype, increase functional divergence, and enhance microbe killing efficiency. Indeed, the most active oyster defensins, *Cg*-Defh2 and *Cg*-Defm, display a charged residue under diversifying selection (Lys16 and Arg16, respectively), instead of Gly16 in *Cg*-Defh1, which is a less active variant (Schmitt *et al.*, 2010a; Schmitt *et al.*, 2010b). Those residues are highly exposed at the surface of oyster defensins. Since the initial mechanism by which AMPs target microbes relies on electrostatic interactions (Yeaman and Yount, 2003; Brogden, 2005), we can hypothesize that such a mutation improves the initial interaction with bacterial membranes. This has also been proposed for a variant of the fungal AMP plectasin, in which an additional lysine on the surface improved its antibacterial activity, probably by promoting a better binding to the membrane of target bacteria (Schneider *et al.*, 2010). In addition, it was demonstrated that amino acid substitutions at sites subjected to diversifying selection increase the antimicrobial activity of β-defensins against bacterial pathogens (Higgs *et al.*, 2007). Besides, the increase in antimicrobial activity of two homologous of β-defensin (BD2) from human and macaque is also caused by amino acid residues subjected to diversifying selection (Antcheva *et al.*, 2004). Altogether, these evidences allow us to propose that the **variation in oyster defensin potency likely depends, at least in part, on charge distribution driven by sites under diversifying selection**.

II.2. Oyster defensins: inhibitors of peptidoglycan biosynthesis

As earlier mentioned in this manuscript, AMPs have long been considered to kill microbes by non-specific pore formation and disruption of the membrane bilayer (*cf. III.3. Mechanisms of action of AMPs*). Nevertheless, we and others have shown that AMPs can also inhibit a broad diversity of cellular processes. While oyster defensins can be membrane-active (Duperthuy *et al.*, 2010), they can also not be membrane-active but be bactericidal by inhibiting the peptidoglycan biosynthesis of *S. aureus* in the absence of membrane damages. This relies on binding to the cell wall precursor lipid II, the membrane bound component involved in peptidoglycan biosynthesis.

The trapping of peptidoglycan precursors is common to many antimicrobials including glycopeptides, lipopeptides, lipodepsipeptides, lantibiotics, and other AMPs (Schneider and Sahl, 2010). However, such a mechanism of action was only very recently identified for one fungal defensin named plectasin (Schneider *et al.*, 2010) and two mammalian defensins representative of the α- defensins, namely HNP-1 (Leeuw, 2010), and β-defensins, namely HBD-3 (Sass *et al.*, 2010).

II.2.1. Trapping of Lipid II: an ancient mechanism kept through evolution?

Oyster defensins show a high degree of structural and sequence similarity with plectasin [GenBank: CAI83768] and with invertebrate defensins from tick [GenBank: BAB41028], mussel [GenBank: AAD45118], dragonfly [GenBank: AAB24032], spider [GenBank: AAW01792] and scorpion [GenBank: AAB27538]. Remarkably, the most similar AMPs to oyster and mussel defensins come from representative species of ancient arthropod taxa (Bulet *et al.*, 1992; Volkoff *et al.*, 2003; Froy and Gurevitz, 2004) and from saprophytic fungi (Mygind *et al.*, 2005). While other possibilities such as convergent evolution can also account for the similarities observed between these AMPs, a common genetic origin for the defensins of fungi, mollusks and ancient arthropods seems highly probable. This theory is supported by the conservation among these AMPs of the Cs$\alpha\beta$ motif and of four residues at the N-terminus, proposed to be involved in the plectasin/lipid II interaction (Schneider *et al.*, 2010). Furthermore, we evidenced that these sites are under negative selection in oyster defensins, and consequently under strong functional constraints (Schmitt *et al.*, 2010a), which could be the result of their potential implication in lipid II-trapping. Nevertheless, the three defensin variants reveal a differential binding for lipid II that correlates with the variation in their antimicrobial potency. Consequently, we cannot rule out the possibility that other unidentified residues could be also implicated in the lipid II-trapping.

Aside from Cs$\alpha\beta$-containing defensins, two other defensin families characterized by distinct cystein arrays have been shown to inhibit peptidoglycan biosynthesis. Indeed, mammalian defensins members of the α- and the β-defensins (HNP-1 and NBD3 respectively) were recently shown to use lipid II-trapping as a mode of action (Leeuw, 2010; Sass *et al.*, 2010). Thus, throughtout evolution, both invertebrate and mammalian defensins have probably evolved by convergent evolution, to present the lipid II-trapping abilities and acquiring membrane-disrupting properties. Not only gene-encoded AMPs like defensins but also AMPs synthesized by multi-enzimatic pathways have developed the ability to both entrap lipid II and disrupt membranes. This has been intensively demonstrated for bacteriocins (AMPs from procaryotes) in the group of H.G. Sahl. The best described example is certainly Nisin, the structure of which is completely distinct from defensins. This renforces our hypothesis of **lipid II-trapping and membrane disruption as essential antimicrobial mechanisms conserved throughout evolution among families of AMPs of the entire living kindom.**

No doubt that the combination of those properties is of major advantage in the fight against bacteria, since membrane permeabilization (i) enables lipid II-trapping in Gram-negative bacteria, and (ii) gives access to the intracellular pool of lipid II present at the inner leaflet of the cytoplasmic membrane in both Gram-negative and Gram-positive strains (see **Fig. 2**, synergistic activities).

II.3. Synergistic activities between oyster antimicrobials

Another important aspect that sustains our hypothesis of the functional diversity of oyster antimicrobials is that their combination produces strong synergistic antimicrobial activities that enlarge their spectra of antimicrobial activities to both Gram-positive and Gram-negative bacteria. Synergistic activities between oyster antimicrobials were first evidenced between *Cg*-Defm and *Cg*-$Prp_{22\text{-}36}$ (Gueguen *et al*., 2009). Here, we showed that strong synergism can be also found between other antimicrobial families, like *Cg*-Defhs and *Cg*-BPI, and between variants of the same AMP family. Additionally, we also observed different potencies of synergism (as measured through FIC indexes) according to the combination of variants. This reinforces our theory on the functional diversity of oyster AMPs. In consequence, it is tempting to speculate that **the production of a large number of structurally similar AMPs is a strategy used by evolution to increase the spectrum of antimicrobial activities through combinations of peptide isoforms.**

For a long time, the biological activities of AMPs have typically been measured for single peptides, but synergistic relationships between AMPs are now well recognized. It is increasingly evidenced that AMPs may interact simultaneously against microbial pathogens as a result of their colocalization (Yeaman and Yount, 2003). Indeed, synergisms occurring between co-expressed AMPs are now documented. In amphibians, several AMP families display synergism. The S dermaseptins variants exhibit synergy when combined, resulting in some cases in a 100-fold increase in antibiotic activity of the mixture over the activity of the peptides when tested alone (Mor *et al*., 1994). As observed for oyster defensins or *Cg*-Prp when mixed with *Cg*-BPI, temporins 1Ta and 1Tb are weakly active toward Gram-negative bacteria, but they show a marked synergism when each of them is mixed with temporin 1Tl (Mangoni and Shai, 2009). Interestingly, the synergistic activity between the amphibian AMPs, magainin-2 and PGLa, takes place by the formation of a complex between both peptides. Additionally, a single amino acid mutation in magainin-2 significantly alters the synergistic activity, suggesting that a precise molecular recognition is involved in the complex formation (Matsuzaki *et al*., 1998; Hara *et al*., 2001).

The different antimicrobial spectra and the synergistic activities between oyster antimicrobials tempt us to propose a model that could explain in part, the underlying mechanisms of their interplay. The mechanisms of action of oyster antimicrobials differ between families. *Cg*-BPI presents membrane-disrupting properties against Gram-negative bacteria while *Cg*-Defs are inhibitors of peptidoglycan biosynthesis. Regarding *Cg*-Prp, although its mechanism of action is still not characterized, it may exert its activity by the interaction with intracellular targets, as known for several proline-rich AMPs (Kragol *et al*., 2001; Otvos, 2002). Thus, we propose that the combination of *Cg*-BPI with *Cg*-Defs or *Cg*-Prp against Gram-negative bacteria, allows a better access to lipid II for

Cg-Defs (both in the periplasmic space and at the inner leaflet of the cytoplasmic membrane), or to the intracellular target for *Cg*-Prp, resulting in killing bacteria **(Fig. 17)**. Consequently, the combined action contributes to reduce the peptide concentrations required to kill bacteria when peptides are acting alone. Still, the molecular mechanisms underlying the synergistic activities between variants of the same AMP family, or between *Cg*-Defs and *Cg*-Prp against Gram-negative bacteria remain to be studied.

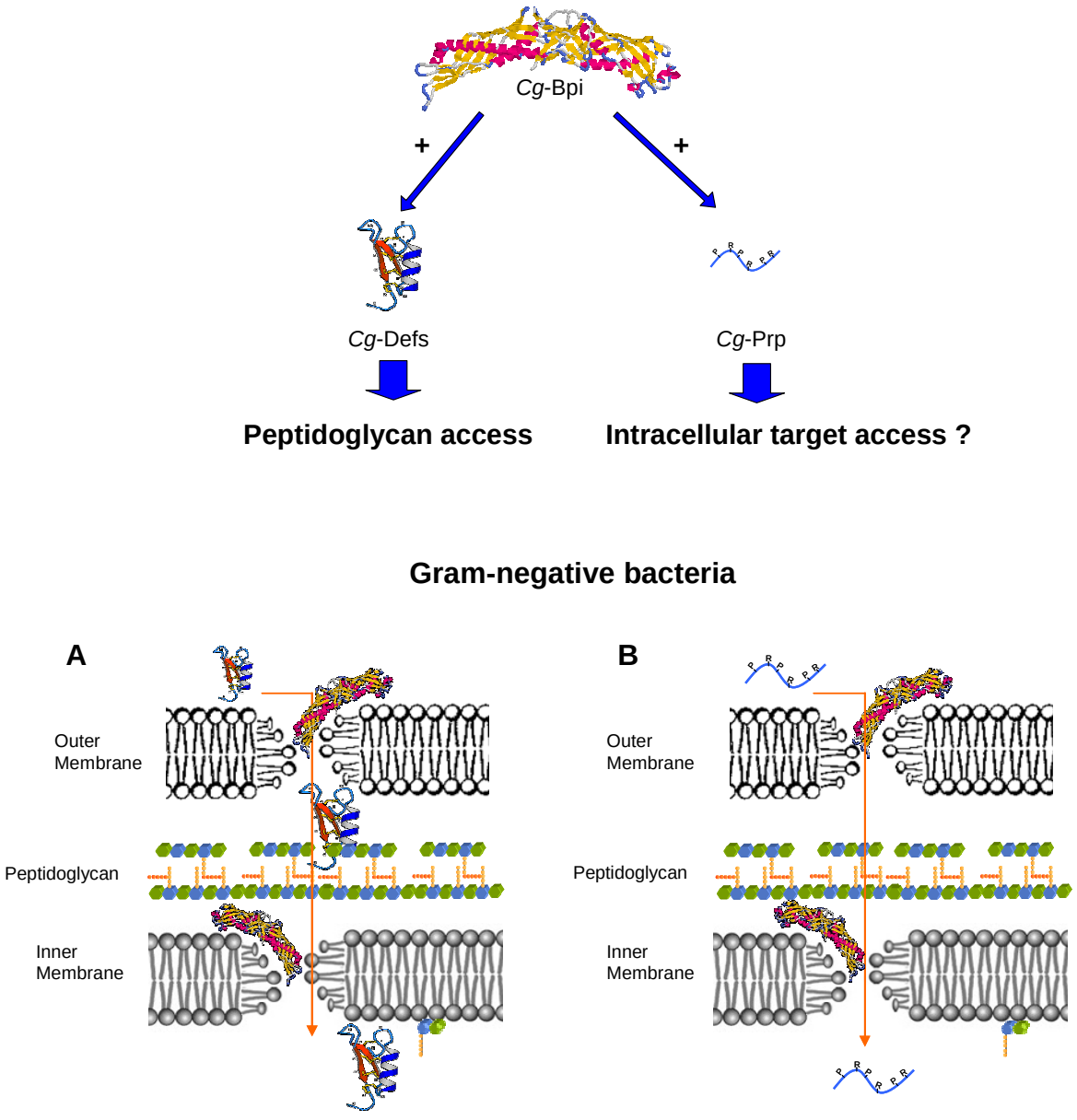

Figure 16. Model of synergism between oyster antimicrobials against Gram-negative bacteria. Proposed mode of action between **A.** *Cg*-BPI and *Cg*-Defs. and **B.** *Cg*-BPI and *Cg*-Prp.

III. Role of antimicrobials in *C. gigas* immune response

Through the study of the transcript expression, immunolocalization and biological activities of *Cg*-Defs, *Cg*-Prp and *Cg*-BPI, the aim of the third part of my thesis was to approach the implication of antimicrobials in the oyster immune response against a pathogenic infection. Results revealed a great diversity of expression profiles between AMPs in hemocyte populations and oyster tissues following challenges. We also evidenced a potential interplay between antimicrobials as a result of their colocalization in hemocytes and of the migration of AMP-expressing hemocytes to AMP-expressing tissues.

III.1. Chemotactic properties of hemocytes: a major element in the oyster antimicrobial response?

Our *Cg-BPI* and *Cg-Defs* gene expression studies confirmed previous results from our group (Gueguen *et al.*, 2006b; Gonzalez *et al.*, 2007a; Gonzalez *et al.*, 2007b). The *Cg-BPI* protein was the only antimicrobial found to be up-regulated in circulating hemocytes following stimulation by heat-killed bacteria and continually expressed in epithelia of several tissues. *Cg-Defhs* and *Cg-Defm* were confirmed to be continually expressed in hemocytes and mantle, respectively, and their expression appeared not to be modulated. However, we also observed here an increase in *Cg-Defhs,* but not in *Cg-BPI* transcript abundance at the site of injection, suggesting differential chemotactic properties among hemocytes populations expressing these effectors.

Strikingly, in our experimental infections (injection of live pathogenic bacteria), the expression profiles obtained for *Cg-Prp* was different from that earlier observed by Gueguen *et al*. using *In Situ* Hybridization on tissue sections of oysters bathed in sea water containing heat-killed bacteria (Gueguen *et al.*, 2009). While the latter challenge appeared to promote *Cg-Prp* expression, in our study, the *Vibrio splendidus* injection challenge resulted in a decrease of *Cg-Prp* transcript abundance in circulating hemocytes, with no increase in gills nor at the site of injection. *Cg-Prp* decrease could be due to the down-regulation of this AMP gene expression by the pathogen, as also shown in other species (Islam *et al.*, 2001; Salzman *et al.*, 2003a), or to the migration of hemocytes expressing *Cg-Prp* toward other tissues not analyzed in our study. Further analyses including *in situ* hybridization will help elucidate this question. Indeed, we failed in detecting *Cg*-Prp on whole oyster body by immunochemical methods, probably due to the low concentration of this peptide. Today, we do not know whether such divergent results could be related to the challenge methods, bath *versus* injection, and/or to a distinct response to killed *versus* live virulent bacteria.

In this study, we highlighted the importance of chemotactic properties of hemocytes in the oyster immune response. As in oysters, continual expression of certain AMPs in hemocytes

presenting different chemotactic properties was also described in the mussel *Mytilus galloprovincialis* and in the shrimp *Litopenaeus vannamei* (Mitta *et al.*, 2000b; Munoz *et al.*, 2002). In mussels, hemocytes are the main site for the production of several AMPs, including defensins, mytilins and myticins. Similarly, subjecting mussels to bacterial challenge did not result in an increase of transcript abundance of defensins or mytilin B but numerous hemocytes expressing mytilins migrated and accumulated around the injection site (Mitta *et al.*, 2000a; Mitta *et al.*, 2000b). Besides, a differential distribution of hemocytes expressing mytilins B and defensins was established (Mitta *et al.*, 1999b; Mitta *et al.*, 2000c; Mitta *et al.*, 2000d). Interestingly, whereas numerous hemocytes expressing defensins infiltrate the digestive tubule epithelia, hemocytes expressing mytilins and myticins are much less or not represented in these epithelia, but they are well represented in gills, where no defensin-expressing hemocytes were observed. In addition, the colocalization of mytilins and defensins in some granular hemocytes was evidenced. In shrimp, penaeidins are expressed in granular hemocytes and their expression and distribution are regulated through dramatic changes in hemocyte populations, both circulating and infiltrating shrimp tissues after a bacterial challenge. Two distinct phases in the immune reactions were evidenced: a migration of hemocytes towards the infection site with a local and massive release of peptides and the appearance into the blood circulation and tissues of a hemocyte population displaying increased penaeidin-transcriptional activity. Therefore, like in these species, oyster antimicrobials are expressed in different hemocyte populations that likely transport the different AMPs to different body areas in response to infection/injury.

III.2. Involvement of antimicrobials in oyster epithelial defense

Additionally to hemocytes, the epithelia of various organs appear to express AMPs. We have evidence of tissue expression of various antimicrobials in oyster (Gueguen *et al.*, 2006b; Gonzalez *et al.*, 2007b). Interestingly, in every tissue we considered, oyster antimicrobials with complementary spectra of activity were detected. It is well known that the first line of defense in bivalves is represented by protective barriers such as mantle or gills, and by mucus (Glinski and Jarosz, 1997). Gill and mantle are involved in major physiological functions, namely food acquisition and gaseous exchange for gills, and shell secretion and sensory role for mantle. Both are exposed to foreign microorganisms. Therefore, the expression and colocalization of antimicrobials in pathogen-exposed tissues/organs argue in favor of a local participation of oyster AMPs in the first line of defense. Such a role of AMPs in the epithelial local responses against infection has been described in both vertebrates and invertebrates. In mammals, epithelial cells of the skin, the respiratory, alimentary, and genitourinary tracts synthesize and release AMPs (Ganz, 2002; Howell *et al.*, 2003; Zasloff, 2006), either synthesized by epithelial cells or released from storage granules in neutrophils, localized in the

vicinity of the epithelium. In insects, AMPs are expressed at basal levels in barrier tissues and are up-regulated systemically in response to infection (Uvell and Engstrom, 2007). In *Drosophila*, different AMPs can be induced in surface epithelia in a tissue-specific manner, and all barrier epithelia express at least one of these AMPs (Tzou *et al.*, 2000). Furthermore, in *Drosophila* and mammals, multiple peptides with overlapping spectra of antimicrobial activity have been described from epithelial tissues (Huttner and Bevins, 1999; Ouellette and Bevins, 2001).

In the frame of the thesis, we evidenced for the first time a significant level of *Cg-Defh* transcripts in gill tissues, which could be a new form of defensin. This prompted us to focus on the purification of cationic AMPs from gills. In many invertebrate species numerous AMPs have been isolated by biochemical approaches (Bulet *et al.*, 1992; Destoumieux *et al.*, 1997; Mitta *et al.*, 1999a; Boulanger *et al.*, 2004; Cytrynska *et al.*, 2007). We identified here a mixture of the non-conventional antimicrobials (histone 2A and ribosomal protein fragments) but could not detect oyster defensins as expected. Therefore, from this first approach of identification of antimicrobials from gill tissues, it appears that oyster can present a wide array of proteins and protein fragments that are derived from compounds with other primary biological functions that might contribute to the epithelial immune network. Epithelial immune responses are tightly linked to injury and subsequent wound repair. The epithelial surface is constantly at risk of abrasion and sloughing, so damage to the cells from such minor injuries might permit histones and ribosomal protein fragments to become exposed to potential pathogens from the surrounding water. However, this does not tell how histones or other intracellular antibacterial factors might interact with infectious agents within the oyster tissues, and this question remains to be elucidated.

III.3. Antimicrobials and oyster endobiont microflora

Oysters live in permanent contact with the marine environment, and are consequently exposed to a high variety of microorganisms. A great number of bacteria found in this milieu belong to the oyster endobiont microflora. With evolution, oysters may have developed an effective immune system that permits to detect and discriminate beneficial microorganisms from pathogenic ones to keep infections under control. The continual expression of AMPs in several barrier epithelia probably contributes to control both the alochtone and endobiont microflora (Zasloff, 2006). In mammals, the constitutive expression of AMPs in exposed tissues regulates the numbers of colonizing microbes by creating an antimicrobial environment that prevents heavy colonization and helps to shape the composition of the colonizing microflora (Salzman *et al.*, 2007; Duerkop *et al.*, 2009). Besides, the endobiont microflora contributes to host homeostasis and defense against pathogens, by competing with pathogens for attachment sites and nutrients, and through elaboration of bacteriocins, a bacterial AMP class (Bevins, 2006). Indeed, in *Anopheles* mosquitoes,

the gut microflora stimulates the epithelium to baseline activity and is thereby primed to respond vigorously when the malaria parasite *Plasmodium faciparum* tries to cross it. If the natural flora is removed, mosquitoes are less competent to deal with *Plasmodia* (Dong *et al.*, 2006b). Therefore, through their constitutive expression of antimicrobials in contact epithelia, oysters probably control their endobiont microbial flora at basal levels beneficial for outcompeting potential pathogens from the environment.

III.4. Hypothesis on the role of AMPs in oyster antimicrobial defense

From the previous and the present studies, oysters respond to infections by an antimicrobial response that differs from those described in other invertebrate species. We have earlier discussed that similar migrations of hemocytes are observed in infected oysters and mussels. However, in the mussel *Mytilus galloprovincialis*, all the AMPs characterized to date are continuously expressed in hemocytes where they are stored. This is in contrast with *Cg-Prp* and *Cg-BPI* (Gonzalez *et al.*, 2007b; Gueguen *et al.*, 2009). Hemocytes containing the AMP mytilin could migrate rapidly toward site of bacterial injection and could exert its microbicidal activity on engulfed bacteria within hemocytes after phagocytosis, but also could massively release their AMP content in the immediate hemocyte environment by exocytosis (Mitta *et al.*, 2000b). Indeed, experimental infections induced a plasma increase of mytilins and defensins concentrations. We have no evidence of such a release of antimicrobials in oyster plasma. However, this can be due to their low concentrations. Whether oyster AMPs are involved in intracellular killing of phagocytosed bacteria remains to be studied. From their results, Mitta *et al*. proposed that in mussels, the AMP-expressing granulocytes could be involved in an early phase response by migrating towards infectious sites and by phagocytizing microorganisms, and at a later stage in the release of AMPs resulting in systemic response (Mitta *et al.*, 2000c)**.**

Systemic responses have been observed in several other species. In *Drosophila*, the expression of genes encoding AMPs is induced after bacterial challenge and AMPs are rapidly synthesized by fat body and immediately secreted into the hemolymph, leading to a systemic response (Hoffmann, 2003). In the chelicerate *Tachypleus tridentatus*, AMPs are produced and stored in hemocytes, and after bacterial challenge, hemocytes degranulate and release AMPs into the plasma (Iwanaga, 2002). As another example, in the *Litopenaeus* shrimp species, penaeidins are produced and stored in granular hemocytes and released into the plasma and tissues in response to *Vibrio* infection by a phenomenon of intracellular degranulation followed by hemocyte lysis (Destoumieux *et al.*, 2000; Munoz *et al.*, 2002; Munoz *et al.*, 2004). Besides, the process of hematopoiesis would be triggered, contributing to a massive hemocyte invasion of the shrimp tissues, thus providing a systemic response to infection.

From our present data, **we have no evidence of an AMP systemic response in oysters.** Indeed, after a bacterial challenge, neither plasma increase of oyster AMPs nor hemocyte proliferation has ever been observed. **Oyster antimicrobial response seems to be highly based on phagocytic processes** (Cheng, 1996; Hine, 1999; Takahashi and Mori, 2000). Therefore, AMPs could have an intracellular role in the oyster antimicrobial response after the recognition and phagocytosis of microorganisms by hemocytes, where diverse AMPs might interplay to increase their antimicrobial spectra by synergistic activities. In addition, they could display antimicrobial activities in a coordinate manner with additional microbicidal reactions that involve lysosomal enzymes and reactive oxygen species (ROS). The expression and colocalization of oyster AMPs like *Cg*-Defm and *Cg*-BPI in exposed epithelial tissues could argue also in favor of a local participation in the antimicrobial response.

Our hypothesis is reinforced by works performed by pathologists. The cellular hemocyte reactions in oyster appear to be the target of *V. splendidus* and *V. aestuarianus* pathogenicity (Schott *et al.*, 2003; Labreuche *et al.*, 2006)(*Duperthuy et al., cf. Annexes*). Both pathogens, which have been associated to the summer mortalities of oysters, evade hemocyte reactions in two different manners. On the one hand, *V. splendidus* invades the oyster hemocytes by the adhesive properties of its major outer membrane protein OmpU. Then, it retains intracellular viability within hemocytes by the inhibition of the phagolysosome formation and an attenuated ROS production, two mechanisms involved in the elimination of intracellular pathogens (*Duperthuy et al., cf. Annexes*). On the other hand, the evasion of *V. aestuarianus* from oyster immune response relies on the avoidance of phagocytosis by the secretion of inhibitory extracellular products (Labreuche *et al.*, 2006). Thus, these two pathogens have developed different strategies that would reduce the probability to encounter oyster AMPs within the hemocytes. In addition, a variety of resistance mechanisms to AMPs are known in several microorganisms (Yeaman and Yount, 2003; Kraus and Peschel, 2006; Peschel and Sahl, 2006) and we have evidence that *Vibrio* spp. display high *in vitro* resistance to oyster AMPs (Duperthuy *et al.*, 2010; Schmitt *et al.*, 2010b) that could also be related to the strategies developed by *Vibrio* spp. to inhibit the antimicrobial response. However, oyster AMPs are active against a large spectra of bacteria that could belong to oyster microflora, suggesting their potential implication in the maintenance of oyster homeostasis through the regulation of its microflora.

IV. Future Prospects

By the characterization of oyster antimicrobial diversity and its potential role in the antimicrobial response, this thesis is a contribution to the knowledge of the molecular bases of oyster immunity.

Pending questions on the immune function of oyster antimicrobials

One major difficulty encountered in this study to better characterize the oyster antimicrobial response was our impossibility to detect and localize antimicrobial peptides/proteins in oyster tissues. Attention must be paid to the immunological detection and ultrastructural localization of the antimicrobials in the whole oyster body and hemocyte populations in response to both stimuli and pathogenic infections. Such data would help clarifying the different migratory behaviors of hemocyte populations. Our results evidenced once again that the characterization of the hemocyte populations or lineages, never elucidated until now in this mollusk species, is of prime importance. Indeed, besides potential differences in hemocyte migratory behavior, we also showed that different hemocyte populations can express different antimicrobials. Such ultrastructural data should help fully elucidate the colocalisation of AMPs in hemocytes and investigate their ability to encounter pathogens intracellularly.

Progress has been made in the antimicrobial response of the oyster by the characterization of various conventional AMP families. Additionally, we evidenced here that non conventional AMPs, such as peptides derived from histones and ribosomal proteins are present in gills, which may participate in the oyster immune protection. Such new results motivate further investigation. Are they produced in other tissues and do they interact with conventional AMPs to eliminate pathogens?

Interplay between antimicrobials and oyster microflora

In numerous species, it is now established that AMPs interact with the endobiont microflora (Dong *et al.*, 2006b; Duerkop *et al.*, 2009). Nevertheless, the organization of the microbial communities that constitute the oyster microflora and the host factors involved in the regulation of this microflora remain to be documented. In this species, which lives in permanent contact with a great variety of microorganisms, it is of prime importance to understand the interaction of AMPs with the microflora and the mechanisms developed for maintaining the oyster homeostasis and for discriminating commensal and pathogenic bacteria.

Role of AMPs in fighting pathogenic *Vibrios*

Several questions remain to be elucidated, in particular the real implication of AMPs in the oyster capacity to fight pathogenic bacteria. This could be clarified by developing functional genomics approach such as the RNA interference method. This technique has been successfully used in oysters for demonstrating the implication of the *vasa*-like gene (*Oyvlg*) in the germ cell development (Fabioux *et al.*, 2009). Regarding AMPs, the role of crustins and ALF in protecting shrimp against *V. penaeicida* and *V. harveyi* infection respectively, was evidenced by RNAi (Tharntada *et al.*, 2008; Shockey *et al.*, 2009). RNAi could also help in understanding the role of AMPs in maintaining oyster homeostasis and its dialogue with the endobiont microflora.

Potential role of AMPs in oyster resistance to summer mortalities

Through this thesis we have now evidence that *Cg*-Defs and *Cg*-Prp are composed by multigenic families presenting a great gene copy number polymorphism, a subject worthy to be further investigated. Copy number variations in immune genes could possibly contribute to immune differences observed between individuals (Ballana *et al.*, 2007). Contrary to what one might guess, a higher number of gene copies is not necessarily related with an enhanced immune protection. As an example, high copy number of human neutrophil peptides 1-3 and β-defensin genes was associated with propensity to severe sepsis and psoriasis respectively (Hollox *et al.*, 2008; Chen *et al.*, 2010). Therefore, as genetic variability exists for susceptibility to oyster summer mortalities (Degremont *et al.*, 2005; Boudry *et al.*, 2007), it could be interesting to determine the copy number variation of AMP genes between oyster populations with different genotypes associated to survival capacities. In this context, we can propose a comparison of individuals belonging to the “resistant (R)” and “susceptible (S)” oyster lines, produced by divergent selection in the MOREST project (Boudry *et al.*, 2007). By studying the variation of AMP gene copy number between these two selected lines we might provide new arguments to clarify the role of AMPs in oyster resistance to diseases.

Finally, we characterized the oyster AMP diversity from a pool of a great number of oyster individuals issued from a large spectrum of origin and challenge conditions, in order to give a good insight into the overall polymorphism. Present evidence obtained by our group suggests the existence of a high inter-individual variability in terms of sequence polymorphism, including the presence and absence of complete groups of AMP variants at the level of transcript and/or genome sequences (unpublished data). Thus, the analysis of AMP diversity at individual level can give news clues to understand their role in resistance to infectious diseases. This argument is supported by the different potency of AMP variants shown here. As a consequence, it is temping to speculate that the presence or absence of specific variants could have an effect on the overall resistance of oysters.

Interestingly, a high variability among individuals is also found in the transcript expression level of AMPs (unpublished data). Therefore, future work must be oriented toward the comprehension of the inter-individual variability that may contribute to successful immune response in oyster.

Chapter 4

Materials and Methods

I. Biological material

I.1. Bacterial strains and culture media

Table 2. List of bacterial strains used in the antimicrobial assays, oyster challenges and study of mechanism of action of oyster defensins.

Strains	Culture media	T(°C)	Origin
Gram-positive			
Staphylococcus aureus SG511-Berlin	LB or HMB	37°	(Sass and Bierbaum, 2009)
Micrococcus lysodeikticus CIP 5345	LB	30°	Institute Pasteur Collection
Staphylococcus aureus CIP 103428	LB	37°	Institute Pasteur Collection
Bacillus megaterium CIP 6620	LB	30°	Institute Pasteur Collection
Mycrobacterium maritypicum CIP 105733T	EPS	30°	Institute Pasteur Collection
Brevibacterium stationis CIP 101282	EPS	30°	Institute Pasteur Collection
Staphylococcus. haemolyticus	LB	37°	IBMC Strasbourg
Gram-negative			
V. splendidus LGP32 CIP 107 715	Zobell	20°	Institute Pasteur Collection
Vibrio aestuarianus CIP 102971 LPi 02/41	Zobell	20°	IFREMER Collection
Vibrio tasmaniensis LMG 20 012	Zobell	20°	IFREMER Collection
Escherichia coli SBS 363	LB	30°	(short-chain LPS)
Vibrio nigripulchritudo CIP103195	Zobell or EPS	25°	Institute Pasteur Collection

Table 3. Bacteria and yeast strains used in general cloning and recombinant expression.

Strains	plasmid	resistance	Characteristics	Culture media	T(°C)	Origin
E. coli BL21 Rosetta (DE3)	pLysS	Cm	DE3 expresses T7 polymerase upon IPTG induction. The pLysS plasmid produces T7 lysozyme to reduce basal level expression of the gene of interest. Thus it is suitable for expression of toxic genes.	LB	37°	Novagen
E.coli TOP10			Chemically competent strain. Use for general cloning.	SOC or LB	37°	Invitrogen
Pichia pastoris			Designed for high-level expression of recombinant protein	BMMY	30°	Invitrogen

Culture Media

Luria-Bertani broth (LB): 10g of tryptone, 5g of yeast extract, and 10g of sodium chloride in 1 liter of distilled water. Adjust the pH to 7.4 using sodium hydroxide. It is a rich broth and is the most common liquid medium used in the cultivation of bacteria.

Mueller-Hinton broth (MHB) (Oxoid): 2g of beef extract powder; 17.5g of acid digest of casein and 1.5g of starch in 1 liter of distilled water (final pH 7.3). It is also a general purpose medium that is used in the cultivation of a wide variety of bacteria.

BMGY broth: 10g of yeast extract and 20g of peptone in 700ml distilled water. After autoclave, add 100ml of 1M potassium phosphate buffer (pH 6), 100ml of 13.4 yeast nitrogen base (without amino acids), 2ml of 0.02% biotine, and 100ml of 10% glycerol.

BMMY broth: the same as BMGY but replacing the 100ml of 10% glycerol for 100ml of 5% methanol.

EPS broth: 15g of bacto peptone and 15g of Sodium chloride in 1 liter of distilled water. Adjust the pH to 7.2 using sodium hydroxide.

SOC medium: (Invitrogen) 2% of tryptone, 0.5% of Yeast Extract, 10mM of sodium chloride, 2.5mM potassium chloride, 10mM of magnesium chloride, 10mM of magnesium sulfate and 20mM of glucose.

Poor Broth (PB): 10g of tryptone and 5g of sodium chloride in 1 liter of distilled water. Adjust the pH 7.5 using sodium hydroxide. This broth is used in the antimicrobial tests by liquid growth inhibition assay. When needed, it is supplemented with 0.5M of sodium chloride (PB + NaCl).

Artificial sea water (ASW): Prepared at 10X. 234g of sodium chloride, 14g of potassium chloride, 12g of magnesium sulfate, 2g of calcium chloride in 1 liter of distilled water.

Zobell broth: 4g of bactopeptone and 1g of yeast extract in 1 liter of ASW 1X. Adjust the pH 7.5 using sodium hydroxide. This broth is used for the growth of marine bacteria.

Tryptic Soy Broth (Difco): 17g of pancreatic digest of casein, 3g of papaic digest of soybean meal, 2.5g of dextrose, 5g of sodium chloride and 2.5g of dipotassium phosphate in 1 liter of distilled water. Final pH 7.3.

Overlay agar: 6% of Tryptic Soy Broth (Difco) and 1% of agarose.

Underlay agar: 10mM of Sodium phosphate buffer p 7.4, 0.03% of Tryptic Soy Broth (Difco) and 1% of agarose.

To obtain culture media agar, 20g of agar was added to 1 liter of specific broth.

All culture agar and media are autoclaved at 120°C for 15 min to sterilize them. In some cases, selective broths were produced by the addition of antibiotics after the broth cool down: Kanamycin (50µg/ml), Chloramphenicol (34µg/ml) or Tetracycline (10µg/ml).

I.2. Oysters

Adult *C. gigas* oysters (~1-2 years old) from three geographic origins of France (Atlantic coast: La Tremblade, Normandie-Bay des Veys and Mediterranean Sea-Thau lagoon) were purchased from local oyster farms. Oysters were kept in sea water tanks at 15°C until bacterial challenges (described below) and RNA extraction. RNAs obtained from these oysters were used for the sequence obtention of the three antimicrobials and the non immune gene *Cg*-actin for molecular diversity analyses.

For the transcript expression analyses of antimicrobials, rapid amplification of cDNA ends (RACE) of *Cg-defs* and purification of cationic peptides from gills tissue, adult oysters (~1-2 years old) were purchased from the local farm "SODIMER" located in the Mediterranean Sea-Thau lagoon, Mèze, France. Oysters were kept in sea water tanks at 15°C at the IFREMER research Center at Palavas les Flots, France, until their use.

I.2.1. Oyster bacterial challenges

Two different types of challenges were performed:

For the obtention of sequences coding for the three antimicrobials and the non immune gene Cg-actin, *C. gigas* adult oysters were exposed to four kinds of bacterial challenges, performed by oyster immersion in sea water supplemented with: (i) alive non virulent *Micrococcus lysodeikticus* CIP 5345 and *Vibrio tasmaniensis* LMG 20 012 (2.5 x 10^8 CFU/ L for each strain), (ii) alive virulent *V. splendidus* LGP32 CIP 107715 (5 x 10^8 CFU/ L), (iii) mix of heat killed virulent *V. splendidus* LGP32 and

V. aestuarianus CIP 102971 LPi 02/41 (2.5 x 10^8 CFU/ L for each strain) and (iv) unchallenged oysters. For each condition, hemolymph was collected at 12 and 24 hours after challenge from the pericardial cavity through the adductor muscle. After hemolymph collection, hemocytes were isolated by centrifugation to discard plasma (700g for 10 min. at 4°C) and resuspended in TRIzol® Reagent (Invitrogen) for further RNA extraction.

For transcript expression analyses, rapid amplification of cDNA ends (RACE) and purification of cationic peptides from gills, a small notch was carved in the dorsal side of the oyster shell, adjacent to the adductor muscle. After filing the shell in the seawater tanks, animals were acclimated for 5 days to let oysters recover from the stress induced by the notch and also to naturally clear the notch from small shell debris. After the acclimatization period, oysters were challenged by the injection in the adductor muscle of 100µl of (i) alive virulent *V. splendidus* LGP32 (1.5 x 10^8 CFU/oyster) or (ii) sea water as a control. Bacteria doses were calculated to stimulate the oyster immune response but not to produce oyster mortality. In parallel, bacterial strains were grown separately overnight at 20°C in Zobell broth. The bacterial cells were collected by centrifugation (1000g, 10min 20°C), washed twice, centrifuged and resuspended in sterile seawater before injection. Bacterial concentration was calculated from the optical density at 600nm (1 unit OD600 corresponds to 2 X 10^8 CFU/ml). After injection, oysters were returned to seawater tanks. Hemolymph from unchallenged or challenged oysters was collected at 12 h and 24 h using a 1ml plastic syringe fitted with a 25-gauge needle via the notch, from the adductor muscle or directly from the pericardial cavity, and immediately centrifuged at 700xg for 15min at 4°C. Hemocyte pellets were resuspended in 1 ml of TRIzol® reagent (Invitrogen) and used for RNA extraction. For RNA extraction of mantle and gills, tissues were harvested by dissection and a pool from each tissue was resuspended in 1ml of TRIzol® reagent. For protein extraction of gills, tissues were harvested by dissection, stored in clean plastic bags and immediately frozen in liquid nitrogen. Bags were kept at -80°C until use. All experimental infections were performed according to the IFREMER animal care guideline and policy.

I.2.2. Hemocyte obtention for immunochemistry

For immunofluorescence assays, hemolymph was collected as described above in ice cold 1:1 anti-aggregant Modified Alsever Solution MAS (9mM EDTA, 27mM Na-citrate, 336mM NaCl, 115mM Glucose, pH 7). Hemocytes were collected by centrifugation (700g, 10min, 4°C) and then fixed in MAS 4% paraformaldehyde for 10min at room temperature. Then, the number of hemocytes was determined using a Malassez counting chamber (0.2mm cell depth). Concentration was adjusted to obtain 2 x 10^5 hemocytes/slide and 200µl was centrifuged on L-polyLysine slides at 1000rpm for 5min at room temperature in a Cyto-TEK® centrifuge.

II. Methods in Molecular Biology

II.1. Primers and plasmids

Table 3. Primers used for 5‘ and 3’ rapid amplification cDNA ends (RACE), PCR amplification from cDNA and gDNA, plasmid sequencing and construction of recombinants *Cg*-Defhs

	5'-3'
RACE amplification	
race(dT) anchor	GACCACGCGTATCGATGTCGAC
oligo(dT) anchor	GACTCGAGTCGACATCGATTTTTTTTTTTTTTTV
Cg-Defh5’ sp1	CACAGTAGCCCGCTCTACA
Cg-Defh5’ sp2	CGGACACCCAAATCCAGC
Cg-Defh5’ sp3	CCATYTCTGCAGAAACCATC
Cg-Defm5’ sp1	CATCTTAACCAGAGCGTGGC
Cg-Defm5’ sp2	GGACTTGCAGTGATTGTTGC
Cg-Defm5’ sp3	CATCAGAAGGACAGCTAGTG
Cg-Defh3’ sp1	GCTGGATTTGGGTGTCCG
PCR amplification from cDNA and gDNA	
Cg-Prp F	CACCATGTTCTCTCGGAGGA
Cg-Prp R	TCTCTTCATCAAAACAAAGTCG
Cg-Defm F	CCACTTTCTGGTTTGCTGAG
Cg-Defm R	TCTTGGTCAGATTCAGWCTGG
Cg-Defh F	CTACCAGTTGTTCATACAGAG
Cg-Defh R	TCTTGGTCAGATTCAGWCTGG
Cg-BPI F	CTACCAGTTGTTCATACAGAG
Cg-BPI R	GGATTTAATATATCCGTCTTCTG
Cg-Actin F	CTTCACAATGGGAGATGAAGA
Cg-Actin R	GTAAACTCCTATCACAGCCAC
Plasmid amplification	
M13 F	GTAAAACGACGGCCAG
M13 R	CAGGAAACAGCTATGAC
T7	TAATACGACTCACTATAGGG
T3	ATTAACCCTCACTAAAGGGA
***rCg*-Defhs construction**	
Cg-Defh F	GCGCGAATTCATGGGATTTGGGTGTCCG
Cg-Defh R	ATATATGTCGACCTTGAAAGATCTTTACTTC

Table 4. Primers used for gene copy number estimation and expression analysis

	5'-3'
Gene copy number estimation	
Cg-prp F	CCACCATGTTCTCTCGGAGG
Cg-prp R	CTTGTAGAACGGGCTAGCAC
Cg-defs F	CTGCAGACATGGCTTTTGCTG
Cg-defs R	GTACATCTTGACCAGAGCGTG
Cg-bpi F	AGATAGAAATAGGAATGGACGG
Cg-bpi R	GTTATAGATCCACGCTGCTCC
Cg-actin F	CAGCTATGTAGGAGACGAG
Cg-actin R	CACGGAGTTCATTGTAGAAGG
qPCR analysis of transcript expression	
Cg-Prp F	CCACCATGTTCTCTCGGAGG
Cg-Prp R	CTTGTAGAACGGGCTAGCAC
Cg-Defs F	GCTGGATTTGGGTGTCC
Cg-Defs R	ACAGTAGCCCGCTCTACAAC
Cg-Defm F	CGGGTAACCAGTTAAAGTGC
Cg-Defm R	ACATCTTAACCAGAGCGTGG
Cg-Defh F	GTTGTAGAGCGGGCTACTGTG
Cg-Defh R	CTTGGTCAGATTCAGACTGG
Cg-BPI F	GATAGAAATAGGAATGGACGG
Cg-BPI R	GTTATAGATCCACGCTGCTCC
Cg-Elf-1 F	GAGCGTGAACGTGGTATCAC
Cg-Elf-1 R	ACAGCACAGTCAGCCTGTGA

Table 5. List of plasmids used in cloning and expression.

plasmids	resistance	Characteristics	Origin
pET28a+	Kanamycin	Expression vector which carry an N-terminal His-Tag configuration. Allow the expression of His-Tag fusion proteins in presence of IPTG.	Novagen
pCR®2.1-TOPO	Kanamycin Ampicillin	Cloning vector for the direct insertion of *Taq* polymerase-amplified PCR products.	Invitrogen
pPICK9	Geneticin ® (G418 sulfate) Ampicillin		Invitrogen

II.2. Extraction and quantification of total RNA

RNA extraction was performed following the TRIzol® Reagent manual (Invitrogen). Samples were homogenized by vigorous vortexing between 10-18h prior to extraction. Following homogenization, insoluble material from the homogenate was removed by centrifugation at 12000xg for 10min at 4°C. The resulting pellet contained extracellular membranes, polysaccharides, and high molecular weight DNA, while the supernatant contained RNA. The supernatant was transfer to a fresh tube and incubated for 5min at room temperature to permit the complete dissociation of nucleoprotein complexes. Then, 0.2ml of chloroform per 1ml of TRIzol® was added. Tubes were shacked by hand for 15s and then incubated at room temperature for 3min. Tubes were centrifuged at 12000xg for 15min at 4°C. Following centrifugation, the mixture separates into a lower pink, phenol-chloroform phase, an interphase, and a colorless upper aqueous phase. RNA remains exclusively in the aqueous phase. Thus, the aqueous phase was transfer to a fresh tube and RNA was precipitated from the aqueous phase by mixing with 0.5ml of isopropyl alcohol. Samples were incubated at room temperature for 10min and centrifuged 12000xg for 10min at 4°C. Then, the supernatant was removed and the RNA pellet was washed once with 1ml of 75% ethanol, and centrifuged at 7500*g* for 5min at 4°C. Ethanol was discarded and the RNA pellet was vacuum-dried for 5min at room temperature. It was important not to let the RNA pellet dry completely as this greatly decreases its solubility. RNA was dissolved in RNase-free water by passing the solution a few times through a pipette tip, and incubating for 10min at 55°C.

Next, RNA samples were treated by Deoxyribonuclease I amplification grade (DNase I) (Invitrogen). DNAI treatment is suitable for eliminating DNA during critical RNA purification procedures. DNase I digests single- and double-stranded DNA to oligodeoxyribonucleotides containing a 5' phosphate. 1X DNase I Buffer (200mM Tris-HCl (pH 8.4), 20mM MgCl2, 500mM KCl) and DNAse I (1U/µl) were added to RNA samples and treated 15min at room temperature. DNAse I was inactivated by heating 10min at 65°C. It is important not to exceed the 15min incubation time. Higher temperatures and longer time could lead to Mg++-dependent hydrolysis of the RNA.

A second precipitation was performed to purify RNA. For RNA precipitation from solution, it needs to have a high salt concentration. 1:10 volume of 3M Na-acetate was added to RNA sample and mix briefly. Then 1:1 volume of 100% isopropyl alcohol was added, mixed briefly, incubated at room temperature for 10min and centrifuged at 10000xg for 10min at 4°C. The supernatant was removed carefully and the pellet was washed by adding 5 volumes of 75% cold ethanol. Samples were centrifuged at 10000*g* for 5min at 4°C, and ethanol was discarded. The RNA pellet was vacuum-dried for 5min at room temperature and then resuspended and dissolved in RNase-free water as described above. RNA samples were kept at -80°C until use.

Quantification of total RNA were determined using a *NanoDrop* spectrophotometer (*NanoDrop* Thermo Fisher Scientific) assuming that an OD_{260}=1 the solution has 40µg/ml of RNA. Quality of RNA was verified by 1% agarose gel electrophoresis.

II.3. Extraction and quantification of genomic DNA

For the genomic DNA extractions (gDNA), hemocytes were recovered as described above. Genomic DNA (gDNA) was isolated from oyster hemocytes individually, by incubation in 500µl of buffer (100mM NaCl, 10mM Tris-HCl pH 8, 25mM EDTA pH 8, 0.5% SDS and 0.1mg/ml proteinase K), 4h at 50°C, followed by phenol/chloroform extraction. 500µl of phenol chloroform isoamyl alcohol 25:24:1 was added. Sample was mixed gently for 15s and centrifuged at 12000xg for 30min at 4°C. The aqueous upper phase which contains the DNA was removed, transfer to a fresh tube and an equal amount of chloroform was added to removes phenol. Sample was mixed gently for 15s and centrifuged at 12000xg for 15min at 4°C. The aqueous upper phase (500µl approximately) was recovered and DNA was precipitated with 1.5ml of 100% cold ethanol for 2h at -80°C. Ethanol pulls water from the DNA molecule so that it precipitates and becomes visible as white mucous strands. DNA was spooled with a pipette tip and transfer to tubes containing 75% ethanol. DNA was centrifuged at 4000xg for 3min at 4°C, ethanol was discarded and the DNA pellet was vacuum-dried for 5min at room temperature. The pellet was then resuspended in DNase-free water and treated with RNAse (Invitrogen) 50µg/ml, 30min at 37°C. A second precipitation with Na-acetate was used to purify DNA as describe above. DNA samples were kept at -20°C until use. Quantification of gDNA were determined using a *NanoDrop* spectrophotometer (*NanoDrop* Thermo Fisher Scientific) assuming that an OD_{260}=1 the solution has 50µg/ml of gDNA. Quality of gDNA was verified by 0.8% agarose gel electrophoresis.

II.4. Complementary DNA (cDNA) synthesis

For cDNA synthesis we used the Moloney Murine Leukemia Virus Reverse Transcriptase (M-MLV RT) (Invitrogen). In a 20µl reaction volume it was added 1µl oligo $(dT)_{12\text{-}18}$ (500µg/ml, Invitrogen), 1µg of total RNA, 1µl 10mM dNTP mix (2.5mM of each dATP, dGTP, dCTP and dTTP) and RNase-free water up to 12µl. The mixture was heated 65°C for 5min for denaturation and after brought to 4°C. Then, a mix containing 4µl 5X First-Strand Buffer (250mM Tris-HCl pH 8.3, 375mM KCl, 15mM $MgCl_2$), 2µl 0.1 M DTT and 1µl at 40U/µl of Recombinant Ribonuclease Inhibitor RNaseOUT® (Invitrogen) was added. Mix was incubated at 37°C for 2min. First strand synthesis was carried out with 1µl (200U) of M-MLV RT and samples were incubated 50min at 37°C. Reaction was finally inactivated by heating at 70°C for 15min. cDNAs were kept at -20°C until use.

II.5. Polymerase chain reaction (PCR) amplification

cDNA and gDNA were amplified by PCR. They were all performed by the Taq DNA polymerase (Promega®) following manufacturer protocol. In a 25µl reaction volume was added 1X PCR buffer, 2µM $MgCL_2$ and 0.25U of Taq polymerase supplied by manufacturers, 0.4µM of each primer, 100µM dNTP (25µM each), and 1µl of cDNA or 100ng of gDNA. For cDNA amplification, they were all carried out under the following conditions: 10min at 95°C, followed by 35 cycles of 95°C for 30s, 57°C for 30s, 72°C for 1min and a final elongation step of 72°C for 7min. For gDNA amplification, they were all carried out under the following conditions: 10min at 95°C, followed by 35 cycles of 95°C for 1min, 57°C for 1min, 72°C for 2min and a final elongation step of 72°C for 7min.

All PCR products were analyzed by agarose electrophoresis 1-2%. When it was necessary, PCR products were gel purified using a QIAQuick® gel extraction kit (Qiagen) DNA fragment was excised the from the agarose gel with a clean, sharp scalpel and then it was purified following the manufacturer protocol.

II.6. PCR product cloning

For the direct insertion of *Taq* polymerase-amplified PCR products into a plasmid vector, fresh or purified PCR products were cloned using TOPO TA cloning kit with the pCR 2.1 TOPO® vector (Invitrogen), following the manufacturer protocol. *Taq* polymerase has a nontemplate-dependent terminal transferase activity that adds a single deoxy-adenosine (A) to the 3´ ends of PCR products. The linearized vector supplied in this kit has single, overhanging 3´ deoxy-thymidine (T) residues, allowing PCR inserts to ligate efficiently with the vector. TOPO® cloning reaction was performed in 6µl reaction volume in which 0.5 to 4µl PCR product, 2µl of salt solution (1.2M NaCl; 0.06M $MgCl_2$), water to a total volume of 5µl and 1µl TOPO® vector were added and incubated for 25min at 23°C. Reaction was placed on ice for 5min and then 2µl was added into a vial of One Shot® chemically competent *E. coli* (Invitrogen), mixed gently and incubated on ice for 5min. Cells were heat-shocked for 30s at 42°C without shaking and immediately transferred to ice. 250µl of SOC medium at room temperature was added and the tube was shacked horizontally (200rpm) at 37°C for 1h. 50 and 100µl from each transformation were spread on a pre-warmed selective plate LB agar supplemented with Kanamycin (50µg/ml) and incubated overnight at 37°C. Positive clones were confirmed by PCR as described above, but replacing the cDNA by colonies and amplifying the specific fragment with M13 forward and reverse primers.

II.7. Plasmid purification

Plasmid DNA from positive clones was purified using a silica membrane-based system from the Wizard® SV miniprep DNA purification kit (Promega). 10ml of overnight *E. coli* culture in LB broth supplemented with Kanamycin (50µg/ml) were centrifuged and cell pellet was then treated following the manufacturer protocol. Plasmids were eluted with DNase-free water and kept at -20°C until use.

II.8. Plasmid sequencing

Plasmid sequencing was performed using Big Dye® Terminator sequencing kit on a DNA sequencer model ABI Prism 3130XL (Applied Biosystems). In a 96-well reaction plate, for each reaction was added 400ng of plasmid, 1µl of Big Dye®, 4pmol of M13 primer and water up to 10µl. To incorporate the fluorescent dyes, cycle sequencing were performed under the following conditions: 10min at 95°C, followed by 60 cycles of 95°C for 10s, 50°C for 5s and 60°C for 4min. Reactions were kept at 4°C until purification. The best results are obtained when unincorporated dye terminators are completely removed by an ethanol/EDTA precipitation. For that, 5µl of 125mM EDTA were added to each well follow by adding 60µl of 100% cold ethanol. The plate was sealed with aluminum foil, mixed by vortexing and incubated at room temperature for 5min. Plate was centrifuged at 4200xg for 30min at 4°C. The plate was inverted and centrifuged at 700xg for 30s at 4°C. Then, 60µl of 70% cold ethanol was added to each well and plate was centrifuged at 4200xg for 15min at 4 °C. The plate was inverted and centrifuged at 700xg for 1min at 4°C. After verified the wells were dry, samples were resuspended in 15µl of water, the plate was covered with aluminum foil, protected from light and store at 4°C prior sequencing.

II.9. 5‘ and 3‘ Rapid amplification of cDNA ends (RACE)

5‘ and 3‘ Rapid amplification cDNA ends (RACE) was performed from *C. gigas* hemocytes and mantle tissue RNA to generate the full-length coding sequences (CDS) of *Cg-Defs*. The procedure was performed according to Roche 5’/3‘ Race protocol (Roche). First, we designed three specific reverse primers and one specific forward primer from partial cDNA sequences from hemocytes defensins *Cg-defh1* [GeneBank DQ400101] and *Cg-defh2* [GeneBank DQ400102], and three reverse primers from mantle defensin *Cg-Defm* [GenBank AM050547]**(Table 3)**.

5‘ RACE for hemocytes defensins was carried out using for first strand synthesis 1µg of total RNA, 1.6µM of *Cgdefh5’ SP1* antisense primer, 1mM dNTPs, 40U RnaseOUT® (Invitrogen) and 200units of M-MLV reverse transcriptase in reverse transcriptase buffer (Invitrogen). The cDNA was then treated with 2units of RNaseH (Invitrogen) for 20min at 37°C. ssDNA product was purified with High Pure PCR Product purification kit (Roche), and a poly A end was added with 80U of terminal

transferase (Promega). The 5'UTR was obtained after two PCR reactions, the first reaction with the antisense primer *Cgdefh*5'*SP2* and the oligo d(T)-anchor primer (Roche), and the second one with the antisense primer *Cg-defh*5'*SP3* and the racedT anchor primer (Roche). Both PCR reactions were carried out in a total volume of 25µl under the following conditions: 10min at 95°C, followed by 35 cycles of 95°C for 30s, 53°C for 30s, 72°C for 2min and a final elongation step of 72°C for 5min. 5'RACE for mantle defensin was performed with the three antisense primers *CgDefm5' SP1, CgDefm5' SP2* and *CgDefm5' SP3* and procedure was performed as described above.

3' RACE for hemocytes defensins was performed in the same conditions as 5' RACE but using the oligo d(T)-anchor primer (Roche) for first strand synthesis. Only one round of PCR was performed using the specific sense primer *Cgdefh*3*'SP1* (Table 1) and racedT anchor primer (Roche). PCR reactions, gel purification of PCR products, cloning using TOPO TA with the pCR 2.1 TOPO® vector(Invitrogen), plasmid purification and plasmid sequencing were performed as describe above.

II.10. Gene copy number estimation

For each gene, specific pairs of primers were designed from a conserved region on one single exon. As standard of gene quantification, we used equimolar amounts of pCR 2.1 TOPO® vector plasmid DNA (pDNA) containing the inserts of interest that were pooled and diluted in 40ng of herring sperm DNA. PCR efficiency was calculated by serial dilutions ranging from 10^3 to 10^9 copies per reaction which were tested in duplicate with each primer pair. Primer pair efficiencies were calculated from the given slopes in LightCycler software. The corresponding efficiency (E) of one cycle in the exponential phase was calculated according to the equation: $E = 10^{[-1/slope]}$. Quantitative PCR (qPCR) assays were then performed in duplicate with four serial dilutions of gDNA (between 5 to 40ng per reaction). qPCR was carried out on the LightCycler 480 System (Roche). The reaction mixture consisted in LightCycler 480 master mix (1X), gDNA and 0.5µM of each primer, which was submitted to 900s of denaturation at 95°C, 40 cycles of 10s of denaturation at 95°C, 20s of annealing at 60°C and 20s of extension at 72°C, and fluorescence detection. After an initial 10s denaturation step at 95°C, a melting curve was obtained from a start temperature of 65°C to a final temperature of 95°C, with an increase of 0.06°C/s. The data were analyzed using Light Cycler 480 software version 1.5.0.39 and the 2^{nd} derivative max algorithm.

Gene copy number was calculated by absolute quantification. Standard curves for gDNA and pDNA of each gene were constructed from the mean Cq values using linear regression, from which slope and correlation coefficients were calculated. Using an estimation of the *C. gigas* genome size of about 823 Mb, the number of molecules represented in 20ng of gDNA was then calculated. Quantification was finally achieved by plotting the measured threshold cycle (Ct) on the standard curve obtained with the serial dilutions of pDNA.

II.11. Quantitative PCR (qPCR) analysis of gene expression

Gene expression of antimicrobials after bacterial challenges was determined by qPCR relative quantification, which determines the changes in steady-state mRNA levels of a target gene and expresses it relative to the levels of mRNA levels of a reference gene. Therefore, relative quantification does not require standards with known concentrations and the reference can be any transcript, as long as it is a reference gene. qPCR assays were carried out on the Light-Cycler 480 System (Roche). The 5µl-volume reaction consisted in 1X Light-Cycler 480 master mix, 0.5µM of each primer and 1µl of cDNA diluted at 1/8. qPCR assays were performed in triplicate, and primer pair efficiencies (E) were calculated as described above. qPCR assays were submitted to an initial denaturation step of 900s at 95°C followed by an amplification of the target cDNA (35 cycles of denaturation at 95 C for 10s, annealing at 57°C for 20s and extension time at 72 C for 25s) and fluorescence detection. The melting curves were obtained as described above

To calculate the expression of target genes, we used the method described by Pfaffl (Pfaffl, 2001), using the *C. gigas* Elongation Factor-1 α [GenBank AB122066] as reference gene. The relative expression ratio (R) of the target gene was calculated based on the efficiency (E) and the quantification cycle (Cq) of the target gene expressed in comparison to the reference gene. Cq is defined as the point at which the fluorescence rises appreciably above the background fluorescence.

Thus, changes in relative expression normalized to reference gene were determined using the equation $(R) = (E_{target})^{\Delta Cqtarget\ (control\ -\ sample)}/(E_{ref})^{\Delta Cqref\ (control\ -\ sample)}$, were E_{target} is the amplification efficiency of target gene transcript; E_{ref} is the amplification efficiency of a reference gene transcript and Cq is the quantification cycle of a designated threshold level. ΔCq_{target} is the Cq deviation of control – sample of the target gene transcript; ΔCq_{ref} = Cq deviation of control – sample of reference gene transcript. Efficiencies of tested primers in this study varied between 1.98 and 2.07. Statistical significance was determined using the Student's *t*-test between conditions and differences were considered when $p < 0.05$.

II.12. Agarose gels

To evaluate the presence and quality of total RNA and gDNA extraction, and PCR products, agarose gel electrophoresis were used. Between 0,8% and 2% (w/v) of agarose (depending on analyzed fragment sizes) was dissolved by heat in TAE buffer (40mM Tris, 0.001% acetic acid (v/v), 1mM EDTA, pH 8) and cooled down to about 60°C. Then, agarose solution was put into a gel rack until gel became solid. Samples were mixed with 1X sample buffer (bromophenol blue 0.04% (w/v), xylene cyanol 0.04% (w/v), saccharose 4% (w/v), EDTA 17mM, pH 8) and loaded in the gel. Migration was performed for 30 min-1h at 100V in TAE buffer and DNA ladders of 1kB and 10kB (Eurogentec)

were used as molecular weight markers. Gel was stained by incubation in ethidium bromide solution at 0.01% (v/v) in distilled water for 10min, washed 5min in distilled water and then observed under ultraviolet light.

II.13. Bioinformatics analysis

II.13.1. *In silico* searches

Sequences previously identified for *Cg-prp*, *Cg-defm*, *Cg-defh1*, *Cg-defh2*, *Cg-bpi* and *Cg-actin* were used for the search of homologous sequences among the 29,745 unique sequences contained in the Sigenae *C.gigas* EST database (http://public-contigbrowser.sigenae.org:9090/Crassostrea_gigas/index.html). From all the identified sequences, we kept only sequences with the complete CDS. In addition, to avoid scoring PCR or sequencing artifacts, we have only considered sequences shared between PCR and *in silico* approaches or those observed at least twice in the same approach for global analyses.

II.13.2. Sequence data analysis.

The multiple alignments were generated using the MAFFT alignment program (http://align.bmr.kyushu-u.ac.jp/mafft/online/server/, version 6). Prediction of signal peptide was performed with the SignalP program (http://www.cbs.dtu.dk/services/SignalP/, version 3.0). Construction of phylogenetic trees was performed with PHYML v2.4.4 using the models with the best Akaike information criterion (Phymltest function from the APE package). Measure of sequence diversity as the mean nucleotide diversity (Pi) (intra-group) and the mean nucleotide divergence (inter-groups) values were calculated using the Maximum Composite Likelihood method included in the Molecular Evolutionary Genetics Analysis program (http://www.megasoftware.net/ version 4.0). Polymorphism values and minimum numbers of recombination events (Rm) were calculated with DNAsp program (www.ub.es/dnasp/ version 4.20.2). The ratio of the rate of non-synonymous substitutions (dN) to the rate of synonymous substitutions (dS) for each codon was calculated with Selecton web server (http://selecton.tau.ac.il/index.html), based on M8 evolutionary model which allows for positive selection. Statistical significance of results (p<0.05) was assessed using the likelihood ratio test (LRT) which compares the log likelihood of M8 model to the log likelihood of M8a alternative model, that allows for negative and neutral selection.

III. Methods in Biochemistry

III.1. Characterization of peptides and proteins

III.1.1. Protein quantification

III.1.1.1. Micro BCA® Protein Assay Kit (Pierce)

This colorimetric method utilizes bicinchoninic acid (BCA) as the detection reagent for Cu^{+1}, which is formed when Cu^{+2} is reduced by protein in an alkaline environment. A purple-colored reaction product is formed by the chelation of two molecules of BCA with one cuprous ion (Cu^{+1}). This water-soluble complex exhibits a strong absorbance at 562 nm that is linear with increasing protein concentrations.

A standard curve of seven serial dilutions between 0 and 100µg/ml was constructed with bovine serum albumin (BSA). 1 volume of each standard or diluted target sample was mixed with an equal volume of BCA reaction mix and placed in a microplate. After the incubation for 60min at 60°C, the microplate was read at A_{562} on a Multiscan microplate reader (Labsystems).

III.1.1.2. Protein quantification by spectrometry

NanoDrop spectrophotometer (*NanoDrop* Thermo Fisher Scientific) is used to measure the concentration of proteins, based on the molecular weight and extinction coefficients of the molecules and reading at A_{225} or A_{280}. Nanodrop spectrophotometer employs a sample retention system that micro volumes as small as 2µl can be quantified.

III.2. Electrophoresis

III.2.1. Sodium Dodecyl Sulfate PolyAcrylamide Gel Electrophoresis (SDS PAGE)

Tris-tricine SDS PAGE and Tris-glycine SDS PAGE, based on Tris-glycine and Tris-tricine buffer systems, respectively, are the most commonly used SDS electrophoretic techniques for separating proteins. Tricine–SDS-PAGE are preferentially used for the optimal separation of proteins <30kDa, whereas Tris-glycine SDS PAGE are used for separate proteins >30kDa. Tricine is an organic compound which presents a lower negative charge than glycine. This high ionic strength causes more ion movement and less protein movement, allowing for low molecular weight proteins to be separated in lower percent acrylamide gels. Polyacrylamide gels are characterized by the total percentage concentration (%T) of two monomers (acrylamide and the crosslinker bis-acrylamide) and the percentage concentration of the crosslinker (% C) relative to the total concentration.

In an Acetic Acid-Urea polyacrylamide gel electrophoresis (AU-PAGE), both the molecular size and charge act as bases for protein separation. The pH in a system is commonly about 3.0. Thus all proteins are likely to be positively charged (+) and to travel towards the cathode (-) in an electric field. In such an AU-gel electrophoresis system, two proteins of similar size but different charge may be separated from each other. Since SDS gels may be unable to achieve this end, these two electrophoresis systems usefully complement each other for analysis of small amounts of proteins. In addition, AU-PAGE is a non-denaturant gel electrophoresis, because it runs in the absence of SDS and thus, proteins keep their biological activities.

III.2.2. Tris-tricine SDS PAGE

SDS Tris-tricine gels (100mm wide, 75mm long, 0.75mm thick) were prepared having a resolving and stacking gels. Resolving gel was prepared with 16.5% acrylamide; 38:1.17 (w/w) acrylamide/bis solution, 15.4% glycerol, 1.15 M Tris pH 8.45, 0.12% SDS, 0.06% (v/v) N,N,N',N'-Tetramethyl-ethylenediamine (TEMED), and 0.06% (w/v) ammonium persulfate (APS). Gels were poured and 100µl of isopropanol was added to each gel by gently drooping onto the midth of the gel. Gels were polymerized at room temperature, isopropyl alcohol was removed and gels were washed with distilled water. The stacking gel was prepared with 10% acrylamide; 38/1.17 (w/w) acrylamide/bis solution, 0.75M Tris pH 8.45, 0.075% SDS, 0.08% (v/v) TEMED and 0.08% (w/v) APS and poured above the resolving gel. Combs were put avoiding air bubbles. After complete polymerization, gels were mounted in a Mini PROTEAN® Tetra Electrophoresis Cell (Bio-Rad). Upper chamber was filled with cathode buffer (0.1M Tricine, 0.1M Tris, 0.1%SDS pH 8.25) and lower chamber with anode buffer (0.2 M Tris-base, pH 8.9). Samples were prepared in 1x Tris-tricine sample loading buffer (Biorad), boiled for 3-5min and then loaded. Samples were running at 10mA, 75V for stacking gel and at 20mA, 120V for separating gel. When dye front reached the bottom, the chamber was disconnected, gels were took out and stained with staining buffer containing 40% ethanol (v/v), 7% acetic acid (v/v) and 0.025% Coomassie blue R-250 (p/v). Once gels were colored, they were placed in distaining buffer (40% ethanol (v/v), 7% acetic acid (v/v)) until bands were visible.

III.2.3. Tris-glycine SDS PAGE

SDS Tris-glycine gels (100mm wide, 75mm long, 0.75mm thick) were prepared as described above. Resolving gel was prepared with 12% acrylamide; 30:0.8 (w/w) acrylamide/bis solution, 0.24M Tris HCl pH 8.8, 0.1% SDS, 0.04% (v/v) TEMED, and 0.04% (w/v) APS. The stacking gel was prepared with 5% acrylamide; 30:0.8 (w/w) (w/w) acrylamide/bis solution, 0.12M Tris HCl pH 6.8, 0.1% SDS, 0.08% (v/v) TEMED and 0.06% (w/v) APS and poured above the resolving gel. Both chambers were filled with running buffer (0.1M Glycine, 0.1M Tris, 0.1%SDS). Samples were prepared

in 1x Laemmli loading dye (2% SDS, 10% glycerol, 5% β-mercaptoethanol, 0.002% bromphenol blue, 0.06M Tris HCl, pH 6.8), boiled for 3-5min and then loaded. Running and coloration of gels are performed as described above.

III.2.4. Acid–Urea PAGE

Gels (100mm wide, 75mm long, 0.75mm thick) were prepared having 12.5% acrylamide; 37.5/1 (w/w) acrylamide/bis solution, 4.8M urea, 5% acetic acid (HAc), 0.48% (v/v) N,N,N',N'-Tetramethyl-ethylenediamine (TEMED), and 0.22% (w/v) ammonium persulfate (APS). The gel was polymerized at room temperature. No stacking gel was used. The gel was pre-running with sample solution (3M urea in 5% HAc containing methyl green as a tracking dye)with 5% HAc for 90min at 100 V/20mA with reversed polarity using a Mini PROTEAN® Tetra Electrophoresis Cell (Bio-Rad) until the dye reached the bottom of the gel. Then, the HAc was discarded and gel assembly was rinsed with distilled water. Wells were washed out just before loading samples with 5% HAc using a gauge needle and a small syringe. Samples in 5% HAc were mixed (1:1) with sample solution and electrophoresed with 5% HAc at 100 V/20mA for 90min at room temperature with reversed polarity. After electrophoresis, the gel was stained with staining buffer and appropriately distained in distaining buffer.

III.3. Edman Degradation Sequence analysis

III.3.1. Transfer PVDF membrane

AU gel was performed as described above. In parallel, PSQ-PVDF 0.2µm membrane (Biorad) was cut and activated in 100% methanol for 5min and then immersed in transfer buffer (25mM Tris pH 8.3, 192mM glycine, 20% ethanol, 0.05% SDS) for 10min. After running, AU gel, Whatman papers and filter pads from the minitransblot MTB® module transfer system (Biorad) were also immersed in transfer buffer for 10min. The membrane sandwich was assembled according to the transfer sense (filter pad, Whatman paper, gel, membrane, whatman paper and filter pad) and placed into the transfer assembly. The transfer tank was filled with transfer buffer and transfer migration was reversed by inverting the electrodes. Transfer was performed at 4°C, 160mA, 60V for 25min. After the transfer, membrane was stained 30s with 0.1% (p/v) Comaassie R-250, 1% (v/v) acetic acid and 40% (v/v) methanol and distained with 3 washes of 50% methanol.

III.3.2. Edman degradation

After transfer in PDVF membrane, bands of interest were cut and analyzed by Edman degradation. Edman degradation is a method of sequencing amino acids in a peptide. In this method, the amino-terminal residue is labelled and cleaved from the peptide without disrupting the peptide

bonds between other amino acid residues. Phenyl-isothiocyanate (PTC) is reacted with an uncharged terminal amino group, under mildly alkaline conditions, to form a cyclical phenyl-thiocarbamoyl derivative. Then, under acidic conditions, this derivative of the terminal amino acid is cleaved as a thiazolinone derivative. The thiazolinone amino acid is then selectively extracted into an organic solvent and treated with acid to form the more stable Phenyl-Thio-Hydantoin (PTH)- amino acid derivative that can be identified by using HPLC. This procedure can then be repeated again to identify the next amino acid. Because the Edman degradation proceeds from the N-terminus of the protein, it will not work if the N-terminal amino acid has been chemically modified or if it is concealed within the body of the protein. It also requires the use of either guesswork or a separate procedure to determine the positions of disulfide bridges.

Edman degradation analyses were performed by Marie-Hélène Metz-Boutigue, INSERM Z575, University of Strasbourg, France.

III.4. Matrix-assisted laser desorption ionization mass spectrometry

Mass spectrometry (MS) is an analytical technique for the determination of the elemental composition or the chemical structures of a sample or molecule. The MS principle consists of ionizing chemical compounds to generate charged molecules or molecule fragments and measurement of their mass-to-charge ratios. In a typical MS procedure, a sample is loaded onto the MS instrument, and undergoes vaporization. The components of the sample are ionized by one of a variety of methods (e.g., by impacting them with an electron beam), which results in the formation of charged particles. The positive ions are then accelerated by an electric field and to obtain the mass-to-charge ratio (*m/z*) of the particles, based on the details of motion of the ions as they transit through electromagnetic fields.

In matrix-assisted laser desorption/ionization (MALDI) analysis, the sample or molecule is first co-crystallized with a large molar excess of a matrix compound, usually a UV-absorbing weak organic acid, after which laser radiation of this sample–matrix mixture results in the vaporization of the matrix which carries the molecules with it. One MALDI mass analyzer is the linear time of flight (TOF). TOF analysis is based on accelerating a set of ions to a detector where all of the ions are given the same amount of energy. Because the ions have the same energy, yet a different mass, the ions reach the detector at different times. The smaller ions reach the detector first because of their greater velocity while the larger ions take longer owing to their larger mass (Ragoussis *et al.*, 2006).

Molecular masses of the purified recombinant defensins were verified by MALDI-TOF MS, performed by the Laboratory of Physical Measurements, University of Montpellier 2, France.

III.5. MALDI-TOF Liquid chromatography coupled to tandem MS

Liquid chromatography-mass spectrometry (LC-MS) is an analytical chemistry technique that combines the physical separation capabilities of liquid chromatography (LC) with the mass analysis capabilities of MS. LC-MS is a powerful technique used for many applications which has very high sensitivity and specificity, generally oriented towards the specific detection and potential identification of chemicals in complex mixtures. LC-MS approach to proteomics generally involves protease digestion and denaturation (usually trypsin as a protease, urea to denature tertiary structure and iodoacetamide to cap cysteine residues) followed by LC-MS/MS (tandem MS) to derive sequence of individual peptides (Wysocki *et al.*, 2005). This "shotgun" technology does not require the initial separation of individual proteins and therefore can be applied to complex mixtures. Typically, after the enzymatic digestion of the peptide mixture, is then separated by LC, ionized and sent to a mass spectrometer to measure the mass/charge ratio of each peptide. Peptides of interest are selected for further fragmentation in a collision cell to produce tandem (MS/MS) mass spectra. A MS/MS spectrum consists of a sequence of peaks, each characterizing the mass/charge ratio and intensity of an ion. Computer software is then used to identify the peptide sequence associated with each MS/MS spectrum.

Protein bands were excised and subjected to in-gel trypsin digestion: 0.15 µg of modified trypsin (Promega, sequencing grade) in 25 mM NH_4HCO_3 was added to the dehydrated gel spots for an overnight incubation at 37°C. Peptides were then extracted from gel pieces in three 15 min sequential extraction steps with 30 µl of 50% acetonitrile, 30 µl of 5% formic acid and finally 30µl of 100% acetonitrile. The pooled supernatants were then dried under vacuum. These operations were done automatically on a robot (EVO150, Tecan). Peptides extracts were analyzed by LC-MS/MS on a Ultimate 3000 HPLC (Dionex) coupled with a LTQ-ORBITRAP discovery (Thermo Fisher Scientific) mass spectrometer. The MS/MS sequenced peptides were analyzed by the Mascot search engine for protein identification, against the 29,745 unique EST sequences available for *C. gigas* (http://public-contigbrowser.sigenae.org:9090/Crassostrea_gigas/index.html). Results were validated using the le IRMa (Mascot Results Interpretation) program. Analyses were performed by Ms. Silvie Kieffer-Jaquinod, from the Platform of proteomic and protein analysis "Etude de la Dynamique des Protéomes EDyP Service", INSERM U880, University of Joseph Fourier, Grenoble, France.

III.6. Production of recombinant and synthetic antimicrobial peptides and protein

III.6.1. Production and purification of *Cg*-BPI

The recombinant construction of the bactericidal permeability increasing protein was produced in the recombinant system of *Pichia pastoris* using the pPICK9 vector system (Invitrogen) following manufacturer protocol (Gonzalez *et al.*, 2007b). Expression and purification from culture supernatants was optimized as described below. The yeast cells were grown as a pre-culture in BMGY medium at 220rpm, 30°C for 48h. Pre-culture was used to inoculate 1/100 of BMGY medium and then incubated at 220rpm, 30°C for 72h. Yeast cells were harvested by centrifugation at 3000rpm, 15min at 4°C. Expression of *Cg*-BPI was induced by the resuspension of yeast cells in1/10 BMMY medium and incubated at 220rpm, 30°C for 72 h, adding 0.5% methanol every 24 h. After induction, culture was centrifuged at 3000rpm for 30min at 4°C and supernatant was recovered. Cg-BPI was purified by a step gradient precipitation with 20% and 50% ammonium sulfate. 50% precipitate was resuspended in 10mM Potassium phosphate buffer (pH 6) and subjected to dialysis in Cellu-Sep® cellulose tubular membrane (cut-off 6000-8000)(Interchim) against 25mM Potassium phosphate buffer (pH 6), 150mM sodium chloride, overnight at 4°C. Then, sample was centrifuged at 12000xg for 30min at 4°C, and subjected to a cation exchange chromatography by carboxymethyl weak cation exchange resin (Macro-Prep® CM support, Bio-Rad). The cation exchange resin was washed twice with deionized water and equilibrated with 25mM sodium phosphate buffer pH 7.3. Sample was diluted four times in 25mM potassium phosphate buffer pH 6 and resin was then added in a 1:50 ratio. Sample was incubated overnight at 4°C with gentle agitation. The resin was packed in a 1ml column and washed with 10 resin volumes of 25mM sodium phosphate buffer pH 7.3 at 5ml/min. Elution was performed with 25mM sodium phosphate buffer pH 7.3 supplemented with 200mM NaCl, in 250µl fractions. Protein concentration was determined using the microBCA method and protein profile was controlled by acid-urea gel electrophoresis. The corresponding fractions containing *Cg*-BPI were kept at 4°C until use.

III.6.2. Chemical synthesis of *Cg*-Prp variants

Chemical synthesis of four variants of *Cg*-Prp: *Cg*-Prp22-36 and *Cg*-Prp22-36 P33Q long variants; *Cg*-Prp22-34 and *Cg*-Prp22-34 K28E short variants was performed by Fmoc chemistry and purchased from GENEPEP (Montpellier, France).

III.6.3. Production and purification of *Cg*-Defhs

III.6.3.1. Recombinant construction of *Cg*-Defhs

Recombinant *Cg*-Defh1 and *Cg*-Defh2 peptides were obtained according to the method described for recombinant *Cg*-Defm production with some modifications (Gueguen 2006). Recombinant *Cg*-Defh1 and *Cg*-Defh1 were expressed in *E. coli* as an N-terminal His6-tagged fusion protein using the pET-28a system (Novagen, Madison, WI). By PCR amplification, a Met-coding trideoxynucleotide was incorporated 5' of the cDNA of *Cg-defh1* and *Cg-defh2*, and cloned in-frame with the N-terminal His6 in the EcoRI/SalI sites of pET-28a.

To obtain the construct pET-28a/Cg-Defh1 and pET-28a/Cg-Defh2, their coding sequences were amplified from two pCR 2.1 TOPO® plasmid vectors, containing the coding sequence of each hemocyte defensin (primers in **Table 3**). The underlined codon in the forward primer denotes the Met codon to incorporate a CNBr cleavage site immediately upstream of the N-terminus of the designed peptide. In a 25µl reaction volume were added 1X PCR buffer, 2µM $MgCL_2$, 0.25U Taq polymerase, 0.4µM of each primer, 100µM dNTP and 50ng of plasmid. Amplification was carried out under the following conditions: 10min at 95°C, followed by 35 cycles of 95°C for 30s, 50°C for 30s, 72°C for 30s and a final elongation step of 72ºC for 10min. PCR fragment was purified by gel extraction using the QIAEX II Gel Extraction Kit (Qiagen). Ligation was performed with 10ng of digested pET-28a and 30ng of digested PCR product with EcoRI and SalI, for 4h at room temperature and inactivated at 65°C for 10min.

III.6.3.2. Obtention of competent cells and transformation

Competent cells are bacterial cells that have been chemically treated to allow incorporation of foreign DNA/plasmids. Thus, to express recombinant hemocyte defensins, *E. coli* Rosetta (DE3) pLysS strain (Novagen) was chosen for this effect. Cells were grown at 37°C to DO_{600} 0.2 in LB medium, kept on ice for 10min and centrifuged at 3000g for 10min at 4°C. The pellet was gently resuspended on ice in 1/4 volume of ice cold 0.1M $CaCl_2$, treated for 20min on ice and centrifuged at 3000g for 10min at 4°C. Cells were resuspended in 1/20 volume of 0.1M $CaCl_2$ and suspension was kept on ice for at least 2h.

Transformation was performed by adding 2µl of ligate vector to the competent *E. coli* cell suspension, mixed gently and kept in ice for 10min. After that, the cells were heat-shocked for 30s at 42°C without shaking and tubes were transferred immediately to ice for 2min. 250µl of SOC medium were added at room temperature and tubes were shacked horizontally (200rpm) at 37°C for 1hour. 50µl from each transformation were spread on a prewarmed selective plate LB supplemented with 50µg/ml kanamycin. Plates were incubated at 37°C overnight. Positive clones were confirmed by

PCR, amplifying the specific fragment with T7 and T3 primers and further sequencing as described above.

III.6.3.3. Expression of recombinant *Cg*-Defhs

Recombinant *Cg*-Defh1 and Cg-Defh2 were expressed in *E. coli* Rosetta (DE3) pLysS cells (Novagen) transformed with the pET-28a/*Cg*-Defh construct. The cells were grown at 37°C to DO_{600} 0.9 in LB medium supplemented with 50µg/ml kanamycin. Expression of fusion proteins was induced with 0.5mM IsoPropyl-β-D-1-ThioGalactopyranoside (IPTG). After growth for 3h at 37 °C, bacterial cells were harvested by centrifugation at 6000xg for 20min at 4°C, resuspended in 6M guanidine HCl, 50mM Sodium phosphate, 300mM NaCl, 5mM imidazole pH 8.5 and stored at -20 °C. The cells were lysed by the resuspension of bacteria pellets in this solution followed by sonication at 50% amplitude, 3 X 45s using a Vibracell (Bioblock Scientific). The lysate was clarified by centrifugation at 12000xg for 30min at 4 °C prior to protein purification.

III.6.3.4. Purification and folding of recombinant *Cg*-Defhs

Cg-Defhs fusion proteins were purified by affinity chromatography by incubating cell lysates with TALON® metal affinity resin (Clontech) at a ratio of 25:1 (v/v) in 6M guanidine HCl, 50mM Sodium phosphate, 300mM NaCl, 5mM imidazole pH 8.5 for 4h at 4 °C with gently agitation. Then, resin was washed twice by decantation in 6M guanidine HCl, 50mM Sodium phosphate, 300mM NaCl pH 8.5, and fusion proteins were eluted by decantation with two column volumes of 6M guanidine HCl, 50mM Sodium phosphate and 1M imidazole pH 6.4. Elution was desalted using a reverse phase *Sep-pak*® C-18 cartridge (Waters) which was previously activated with 3 column volumes of methanol and equilibrated with 5 column volumes of deionized water/TriFluoroAcetic acid (TFA) 0.05%. After the sample load, it was washed with 10 column volumes of deionized water/TFA 0.05% and then the peptide mixture was eluted with a step gradient of 5% and 80% of Acetonitrile (ACN)/TFA 0.05%. The 80% ACN/TFA 0.05% fraction containing the peptide mixture was then frozen and lyophilized. The methionine residue introduced at the peptide N-terminus was then subjected to CNBr cleavage by dissolving the lyophilized His6 fusion proteins in 50% formic acid, adding CNBr dissolved in 50% formic acid to a final concentration of 10 mg/ml and incubating the mixture for 8h in the dark at room temperature. The cleavage reaction was terminated by adding 10 volumes of deionized water, followed by freezing and lyophilization. Then, the cleaved fusion peptide mixture was directly folded at pH 8.1 in a refolding solution containing 0.1M $NaHCO_3$, 3mM reduced glutathione and 0.3mM oxidized glutathione in the presence of 2M urea and 25% *N*,*N*-dimethylformamide, at room temperature for 72h. The peptide mixture was then prepurified by a reverse phase *Sep-Pak*® C18

chromatography and purified by a reverse phase high performance liquid chromatography (RP-HPLC) as described below.

III.6.3.5. Reverse phase *Sep-Pak*® C18

Sep-Pak® C18 12 cc cartridges were activated with 3 column volumes of Methanol and washed with 5 column volumes of deionized water/TFA 0.05%, pH 2. The refolded peptide mixture was acidified with 3 volumes of deionized water/TFA 0.05%, and then was loaded in a *Sep-Pak*® cartridge. Column was washed with 10 volumes of deionized water/TFA 0.05% and the peptide was eluted with 3 column volumes of a step gradient of 20%, 40% and 70% of ACN/TFA 0.05%. The 40% elution containing the refolded peptide was frozen, lyophilized and resuspended in sterile deionized water to further purification by RP-HPLC.

III.6.3.6. Reverse Phase High Performance Liquid Chromatography

40% ACN/TFA 0.05% *Sep-pak*® fraction was resolved by RP-HPLC with a 0–60% ACN/ TFA 0.05%. gradient developed over 30min at a flow rate of 1ml/min on a UP5ODB-25QS column (Interchrom modulo-cart uptisphere 5 C18, 250 x 4.6 mm). Peptide purity was controlled by Tris-tricine PAGE, and the peptide concentration was determined using a *NanoDrop* spectrophotometer *NanoDrop* (Thermo Fisher Scientific) at 280 nm based on the extinction coefficients of the molecules. Molecular masses of the purified peptides were determined using Matrix-Assisted Laser Desorption Ionization mode Mass Spectrometry (MALDI-TOF MS).

III.7. Purification of cationic peptides from gills

Frozen gill tissues was pulverized in liquid nitrogen and powder were transferred to polypropylene tubes and resuspended in 16 volumes of cold 5% acetic acid (w/v) supplemented with protease inhibitor cocktail (1µM pepstatin A ,25µM leupeptin and 6.1µM aprotinin (Sigma). The tissue was then homogenized on wet ice by sonication at 50% amplitude, 3 X 45s using a Vibracell (Bioblock Scientific). After homogenization, the extract was agitated for 3h at 4°C and then centrifuged at 12000xg for 30min at 4°C. The supernatant was recovered and placed on wet ice, and pH was increased by adding 10M NaOH gradually to avoid protein precipitation. Then, extract was centrifuged at 2500xg for 30min at 4°C, and a carboxymethyl weak cation exchange resin (Macro-Prep® CM support, Bio-Rad) was used to enrich the supernatant in cationic peptides. The cation exchange resin was washed twice with deionized water and equilibrated with acetate ammonium buffer (25mM acetate ammonium pH 6.8). Then, resin was added in a ratio 1:100 resin:extract, and then incubated overnight at 4°C with gentle agitation. The sample was centrifuged at 1000xg, washed twice with 40 resin volumes of 25mM acetate ammonium pH 6.8 and the cationic peptides

bound to the resin were eluted with 2serial elutions using 5 resin volumes of 1%TFA in deionized water. The elutions were pooled, lyophilized, resuspended in deionized sterile water and kept at -20°C. Protein concentration was determined using the microBCA method and protein profile was controlled by acid-urea gel electrophoresis.

1%TFA elution was resolved by RP-HPLC with a 0–60% acetonitrile gradient developed over 60min at a flow rate of 0.3 ml/min on a BEH 130 column (Waters C18, 3.5µm, 100 x 2.1 mm). Peptide purity was controlled by Acid Urea PAGE.

III.8. Surface Plasmon Resonance

When a beam of light passes from material with a high refractive index (e.g. glass) into material with a low refractive index (e.g. water) some light is reflected from the interface. When the angle at which the light strikes the interface (the angle of incidence or θ) is greater than the critical angle (θ_c), the light is completely reflected (total internal reflection). If the surface of the glass is coated with a thin film of a noble metal (e.g. gold), this reflection is not total; some of the light is "lost" into the metallic film. There then exists a second angle greater than the critical angle at which this loss is greatest and at which the intensity of reflected light reaches a minimum. This angle is called the surface plasmon resonance angle (θ_{spr}). It is a consequence of the oscillation of mobile electrons or "plasma" at the surface of the metal film. These oscillating plasma waves are called surface plasmons. When the wave vector of the incident light matches the wavelength of the surface plasmons, the electrons "resonate", hence the term surface plasmon resonance.

Surface plasmon resonance based instruments use an optical method to measure the refractive index near a sensor surface. In the BIAcore, this surface forms the floor of a small flow cell, through which an aqueous running buffer passes under continuous flow. In order to detect an interaction, one molecule (the ligand) is immobilised onto the sensor surface. Its binding partner (the analyte) is injected in aqueous sample buffer through the flow cell, also under continuous flow. As the analyte binds to the ligand, the accumulation of protein on the surface results in an increase in the refractive index, and the BIAcore uses a photo-detector array to measure very small changes in θ_{spr}. This change in refractive index is measured in real time, and the result plotted as response or resonance units (RU) versus time (a sensorgram). Importantly, a background response will also be generated if there is a difference in the refractive indices of the running and sample buffers. This background response must be subtracted from the sensorgram to obtain the actual binding response. The background response is recorded by injecting the analyte through a control or reference flow cell, which has no ligand or an irrelevant ligand immobilized to the sensor surface (van der Merwe and Barclay, 1996).

III.8.1. Preparation of liposomes.

Liposomes (lipid vesicles) are formed when thin lipid films or lipid cakes are hydrated and stacks of liquid crystalline bilayers become fluid and swell. The hydrated lipid sheets detach during agitation and self-close to form large multilamellar vesicles (LMV) which prevents interaction of water with the hydrocarbon core of the bilayer at the edges. Once these particles have formed, reducing the size of the particle requires energy input in the form of sonic energy (sonication) or mechanical energy (extrusion). The general elements of the procedure involve preparation of the lipid for hydration, hydration with agitation, and sizing to a homogeneous distribution of vesicles.

10mg of DOPC lipid (18:1 1,2-dioleoyl-*sn*-glycero-3-phosphocholine, Avanti Polar Lipids ®) was dissolved in chloroform and mixed with 0.8% of lipid II in chloroform/methanol 1:1 to assure a homogeneous mixture of lipids. Once the lipids were thoroughly mixed in the organic solvent, the solvent was removed by 2 hours of rotary evaporation on a vacuum pump to yield a lipid film yielding a thin lipid film on the sides of a round bottom flask. Hydration of the dry lipid film was accomplished by adding 2ml of phosphate saline buffer (PBS) to the container of dry lipid and agitating, without making bubbles. The temperature of the hydrating medium should be above the gel-liquid crystal transition temperature (Tc) of the lipid with the highest Tc before adding to the dry lipid. After addition of PBS, the lipid suspension was maintained above the Tc during the hydration period by spinning the round bottom flask in a warm water bath maintained at 30°C for 1 hour. Then, hydrated large multilamellar vesicles (LMV) were frozen at -20°C and after freezing completely, air was removed with argon. Extemporary, LMV were downsized by sonication for 20min in wet ice by a probe tip sonicator. Disruption of LMV suspensions using sonication produces Small Unilamellar Vesicles (SUV), with diameters in the range of 15-50nm. By sonication, the lipid suspension should clarify to yield a slightly hazy transparent solution. After sonication, particles into the lipid suspension were removed by centrifugation at 12000xg, 10 min at 4°C prior to use.

IV. Methods in Microbiology

IV.1. Antimicrobial assays

IV.1.1. Liquid growth inhibition assay

Minimum inhibitory concentrations (MICs) of peptides were determined in triplicate by the liquid growth inhibition assay based on the procedure described by Hetru & Bulet (Hetru and Bulet, 1997). MIC values are expressed as the lowest concentration tested that causes 100% of growth inhibition (µM). Poor broth was used for standard bacteria, and Zobell medium at a third strength or saline poor broth was used for marine bacteria. Tests were performed as follow: exponential phase bacteria culture (Optical Density at 600 nm (OD_{600}) between 0.2-0.4) was diluted in the corresponding media to obtain a final OD_{600} of 0.001 (about $1x10^6$ CFU/ml). 10µl of peptide was incubated with 100µl of this bacterial suspension in a 96-well plate an incubated at the corresponding temperature for 18h at 200rpm. Two sterility controls were performed: (i) 10µl of solution the peptide was resuspended and (ii) culture media used to the bacterial dilution. After incubation time, growth was monitored spectrophotometrically at 620nm on a Multiscan microplate reader (Labsystems).

IV.1.2. Checkerboard microtiter assay

Synergy between 2 antibacterial molecules was tested using the checkerboard microtiter assay (Rabel *et al.*, 2004). This assay uses the same method of the liquid growth inhibition assay described above. The MIC of each peptide must be well verified, because in this assay the tested peptide concentration begins at half the MIC in order to detect synergistic effects. Thus, a fixed concentration of half the MIC of peptide 1 was distributed in several wells and 2-fold serial dilutions of peptide 2 were tested against the fixed concentration of peptide 1. The same procedure was tested with a fixed concentration of peptide 2 and a variable concentration of peptide 1. An additional control was incorporated, corresponding to the MIC of each peptide in each assay. Volumes, bacterial dilution, incubation and growth monitoring is performed as described above. Results were expressed as the fractional inhibitory concentration (FIC) index according to the following formula: $FIC = (A)/MIC_A + (B)/MIC_B$ where MIC_A and MIC_B are the MICs of peptides A and B tested alone and (A) and (B) are the MICs of the two peptides tested in combination. An FIC index ≤ 0.5 indicates strong synergy (representing the equivalent of a fourfold decrease in the MIC of each compound tested), between 0.5-1.0 indicates synergy, ≥ 1 indicates that the antimicrobial activity of

the two compounds is additive (a twofold decrease in the MIC of each compound tested), =2 indicates no effect and ≥ 2 indicates antagonism.

IV.1.3. Gel overlay antibacterial assay

Gel overlay assay was performed according to the protocol of Lehrer with minor modifications (Lehrer *et al.*, 1991). Briefly, *S. aureus* SG511 or *E. coli* SBS363 cells were grown overnight in LB broth and centrifuged at 1000xg for 10min at 4°C. The pellet was washed twice using ice cold 10mM $NaPO_4$ buffer pH 7.2. The bacteria pellet was resuspended in ice cold buffer and bacterial concentration was calculated from the optical density at 600nm. The underlay agar was kept molten at 42°C and bacteria were added to the gel solution to have the final concentration of 4 x 10^6 CFU in 10ml of gel solution. The mixture was immediately poured into a Petri dish to form a uniform layer of ~1mm deep.

Gill extracts were subjected to non-denaturing AU-PAGE in duplicate. After electrophoresis, the gel was cut into two identical halves. One half was washed 3 times with ice cold 10mM $NaPO_4$ buffer pH 7.2, 5min each to decrease the acetic acid and urea content. Then the PAGE gel was placed on the top of the underlay gel. The Petri dish was incubated for 3h at 37°C to allow the peptides to diffuse into the underlay gel from the AU gel. The gel was removed, replaced by 10ml of overlay agar and incubated overnight at 30°C. Clear bacteria-free regions were seen where the growth of bacteria was suppressed by protein bands with antibacterial activity. The second half of the gel, an exact replica of the first half, was stained with R-250 Coomassie blue to verify the protein bands with antibacterial activity.

IV.1.4. Antagonization assays

Different peptidoglycan precursors, namely undecaprenyl phosphate (C55P), UDP-N-acetylmuramyl-pentapeptide (UDP-MurNAc-pp), Lipid II, or UDP-N-acetyl glucosamine (UDP-GlcNAc) were tested for antagonization of the oyster defensin antimicrobial activity. Tests were performed against *S. aureus* SG511 strain in half-concentrated MH broth. Serial dilutions of oyster defensins were performed from 0.25 to 8 X MIC were incubated in a 96-well plate with the peptidoglycan precursors in a 1:1, 1:2 or 1:5 molar ratio. *S.aureus* SG511 was then added to the microtiter plate as for a conventional MIC determination. After the incubation for 18h at 37°C, the lowest peptide/peptidoglycan precursor molar ratio that antagonized the antimicrobial activity of the highest defensin concentration was determined.

IV.2. Determination of bacterial membrane potential using TPP+

S. aureus SG511 was grown in half-concentrated MH broth to an OD_{600} of 0.5-0.6. To monitor the membrane potential, 1 µCi/ml of [^{3}H]TetraPhenylPhosphonium bromide (TPP+; 26 Ci/mMol) was added (the lipophilic TPP+ diffuses across the bacterial membrane in response to a trans-negative membrane potential). The culture was then treated with each defensin at 10X MIC (1.2, 2.5 and 5µM *Cg*-Defh2, *Cg*-Defm and *Cg*-Defh1 respectively), and sample aliquots of 100µl were filtered through cellulose acetate filters (pore size: 0.2µm) and washed twice with 5ml of 50mM potassium phosphate buffer. The filters were dried and placed into scintillation fluid, and the radioactivity was measured with a liquid scintillation counter. Non specific TTP+ binding was determined by measuring the TPP+ incorporation in cells treated with butanol. The pore-forming lantibiotic nisin (3.6µM corresponds to 10X the MIC) was used as control. For calculation of the membrane potential ($\Delta\psi$), the TPP^+ concentrations were applied in the Nernst equation ($\Delta\psi$) = (2.3 X *R* X *T/F*) X log(TPP^+ inside/TPP^+outside), where *T* is absolute temperature, *R* is the universal gas constant and *F* is the Faraday constant.

IV.3. Purification of cytoplasmic peptidoglycan precursor pool

The final soluble cell wall precursor UDP-MurNAc-pp accumulates if cells were treated with substances that interact with membrane-bound steps of peptidoglycan biosynthesis, like the antibiotic vancomycin (Kohlrausch and Holtje, 1991). Therefore, *S. aureus* SG511 was grown in 5ml half-concentrated MH broth and incubated overnight at 37°C (160rpm). Next, 50ml of half-concentrated MH broth were inoculated with 100µl of the overnight pre-culture and grown to and OD_{600} of 0.5. Then, 5ml of the culture were filled in a sterile tube (control) and the remaining culture was supplemented with 130µg/ml of chloramphenicol and incubated for 15min at 37°C. Cloramphenicol prevents that cellular autolysis occurs and disrupts feedback mechanisms that normally block accumulation of UDP-MurNAc-pentapeptide (Dai and Ishiguro, 1988). After 15min of incubation, culture was divided and each defensin and vancomycin as positive control was separately added at 10X MIC and incubated for another 30min. The cells were centrifuged at 13000xg for 5min at 4°C, resuspended in 250µl deionized sterile water and the suspensions were added to 500µl boiling deionized water. After boiling for 15min, the suspensions were cooled down and the cell extracts were adjusted to pH 2 with H_3PO_4. Insoluble components were removed by centrifugation at 12000xg for 5min at room temperature and the supernatants were filtered through cellulose acetate filters (pore size: 0.2µm) and analyzed by RP-HPLC in 50mM sodium phosphate buffer pH 5.2, developed in isocratic mode over 30min at a flow rate 1ml/min on a Nucleosil 100-C18 column (Schambeck SFD GmbH, Bad Honnef, Germany). UDP-linked cell wall precursors were analyzed and

corresponding fractions were confirmed using Matrix-Assisted Laser Desorption Ionization Mass Spectrometry (MALDI-TOF MS), in negative mode with 6-aza-2-thiothymine dissolved in 50% (v/v) ethanol/20 mM ammonium citrate as matrix.

V. Methods in Immunochemistry

V.1 Polyclonal antibodies

Antibodies against *Cg*-Defs were raised in New-Zeland rabbits by Eurogentec. Antibodies against *Cg*-BPI were raised in Balb/C mice. Briefly, mice (4 weeks old) received three subcutaneous injections (50µg each) of recombinant *Cg*-BPI at day 0 with complete Freund's adjuvant, and at days 15 and 30 with incomplete Freund's adjuvant. Mouse tumor cells ($5x10^6$ cells in 500µl RPMI 1640 medium, Gibco) were injected in the mouse peritoneal cavity at day 30. Ten days after cell injection, ascitic fluid was withdrawn and clarified by centrifugation (800xg, 15 min, 4°C). Immunoglobulins G (IgG) contained in each immune sera were purified on a Hitrap protein G Sepharose column (Pharmacia). Reactivity and specificity of purified IgG were tested by ELISA against r*Cg*-BPI.

V.2 Immunofluorescence assays

Immunofluorescence analyses were carried out using polyclonal antibodies specific for *Cg*-Prp, *Cg*-Defs and *Cg*-BPI. Fixed hemocytes on L-polyLysine slides were permeabilized for 10min at room temperature with 25mM Tris, 0.2% gelatin, 0.5% Triton X100 and 50mM ammonium chloride, washed with PBS and blocked 2 hrs at room temperature with PBS/5% nonfat milk. After three washes for 3min at room temperature, slides were incubated 2h at 37°C with both anti-*Cg*-Def (20µg/ml) and anti-*Cg*-BPI (20µg/ml) antibodies in PBS/0.5% nonfat milk. As control, non relevant antibodies were used. After three washes in PBS, a second incubation was performed for 1h at room temperature with anti-mouse Alexa Fluor® 488-conjugated (Invitrogen) and anti-rabbit Cy5-conjugated IgG (Jackson) diluted at 1:100 in PBS/0.5% nonfat milk. Slides were washed three times in PBS and mounted in ProLong Gold® Antifade Reagent (Invitrogen). Slides were observed by confocal microscopy Leica TCS 4D.

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Annexes

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Oyster hemocytes express a proline-rich peptide displaying synergistic antimicrobial activity with a defensin

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ABSTRACT

A cDNA sequence that encodes a 61-amino acid polypeptide precursor with homologies to proline-rich antimicrobial peptides (AMPs) was identified in the oyster *Crassostrea gigas*. After release of a hydrophobic signal peptide, the resulting 37-amino acid peptide, *Cg*-Prp, is composed of an acidic region and a cationic proline-rich region. To evaluate the biological properties of *Cg*-Prp, multiple proline-rich peptides corresponding to putative processing of the full-length *Cg*-Prp were synthesized. A limited antimicrobial activity was observed for two of them, which also showed strong synergistic antimicrobial activity with *Cg*-Def, a defensin from *C. gigas*. To our knowledge, this is the first evidence of synergy between a defensin and another AMP in an invertebrate. By *in situ* hybridization, the expression of *Cg-prp* was found to be restricted to hemocytes and induced following bacterial challenge. *Cg-prp* transcripts were also detected in hemocytes infiltrating mantle, where *Cg*-Def is expressed. Additionally, by immunocytochemistry, we showed that *Cg*-Prp or one of its variants is present in some hemocytes together with defensins. In conclusion, we described here the first proline-rich AMP from mollusk. From our study, it is likely to provide a first line of defense against bacterial invasion by acting through synergy with defensins.

1. Introduction

Marine invertebrates including bivalve mollusks have evolved in the continuous presence of microorganisms. Thus, the oysters, such as *Crassostrea gigas*, harbor a diverse microflora both on their surface and inside the body cavities and hemolymph. As filter feeders, they are exposed to a constant challenge by pathogenic and opportunistic bacteria naturally present in their environment. Therefore, the capacity to overcome infections is essential for oyster survival.

Although lacking an adaptive immune system, invertebrates fight microbes with effective mechanisms that include the generation of gene-encoded antimicrobial peptides (AMPs). AMPs are important components of the innate immune system, which have been conserved during evolution (Brogden, 2005; Yang et al., 2002). They form a key line of host defense against pathogens in plants and animals (Bachère et al., 2004; Boman, 2003; Bulet et al., 2004). In animals, AMPs are particularly abundant in tissues that are likely to come in contact with microorganisms, such as at mucosal surfaces (Zasloff, 2006a,b) and within immune cells (Bals, 2000; Zhao et al., 2007). AMPs can be classified into three major groups: (i) linear peptides that can form amphipatic α-helices, (ii) peptides containing cysteine residues engaged in internal disulphide bonds, and (iii) peptides with one or two amino acids over-represented (Pro, Arg, Gly or His) (Brogden, 2005). AMPs from those different groups are often encountered within one organism, as shown in *Drosophila* (Lemaitre and Hoffmann, 2007). Despite their great diversity in terms of size, primary structure and amino acid composition, most AMPs are characterized by a high content in cationic and hydrophobic amino acids (Bulet et al., 2004; Jenssen et al., 2006). The resulting amphipathic structure is considered to be required for the interaction of the peptide with the membrane of the sensitive microorganisms, leading commonly to the disruption of the membrane integrity. Membrane permeabilization is proposed to be one major mechanism by which AMPs kill their target cells, which include bacteria, fungi, parasites and enveloped viruses (Brogden, 2005).

Proline-rich AMPs have a dissimilar mode of action and act without membrane permeabilization. These peptides are characterized by repeated proline-containing motifs and are active predominantly against Gram-negative bacteria. Although relying on unresolved mechanisms, translocation of proline-rich AMPs

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Use of OmpU for attachment and invasion of host immune cells through opsonisation by the extracellular superoxide dismutase in the *Crassostrea gigas* / *Vibrio splendidus* interaction

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OmpU porins are increasingly recognized as key determinants of pathogenic host *Vibrio* interactions. Although mechanisms remain incompletely understood, various species including the human pathogen *V. cholerae* require OmpU for host colonization and virulence in natural and experimental infections. We have shown previously that OmpU is essential for virulence in *V. splendidus* LGP32, an oyster pathogen associated to the massive summer mortalities of *Crassotrea gigas* oysters. Here, we showed that *V. splendidus* LGP32 invades the oyster immune cells, the hemocytes, through subversion of host cell actin cytoskeleton. In this process, OmpU serves as an adhesin required for β integrin recognition and host cell invasion. Besides, the major protein of oyster plasma, the extracellular superoxide dismutase *Cg*-EcSOD, is used as an opsonin mediating the OmpU-promoted phagocytosis through its RGD sequence. Finally, the endocytosed bacteria were found to survive intracellularly, evading the host defense by preventing acidic vacuole formation and limiting ROS production. We conclude that (i) OmpU is a major determinant of host-cell invasion in *Vibrio* species, used by *V. splendidus* LGP32 to attach and invade oyster hemocytes through opsonisation by the oyster plasma *Cg*-EcSOD, and (ii) that *V. splendidus* is a facultative intracellular pathogen that manipulates host defense mechanisms to enter and survive in host immune cells.

Adhesin | Invasin | opsonin | phagocytosis | oxidative burst | innate immunity

Vibrio splendidus LGP32 is an oyster pathogen associated to the massive mortality events that have affected the oyster production over the past years. However, up to now, little is known on the pathology of *LGP32* (1). A metalloprotease has been characterized in LGP32, which contributes to the toxicity of the *Vibrio* extracellular products a metalloproptease has been associated to toxicity (2,3). In addition, we have recently shown that the outer membrane protein (OMP) OmpU is a major determinant of LGP32 virulence (4).

OmpU is a major porin from *Vibrio* species. Aside from its role in environment adaptation through osmoregulation (5,6) and bile resistance (7-9), several recent studies show that OmpU mediates host-*Vibrio* interactions. In pathogenic interactions, OmpU was shown to mediate resistance of *V. cholerae* to antimicrobial peptides (AMPs) (10), or adherence of *V. vulnificus* to host cells (11). Besides, it was identified as critical for pathogen/symbiont recognition in the squid (12). Similarly, from our previous study, OmpU is essential for virulence, and participates to the resistance of LGP32 to oyster AMPs and displays adhesive properties evocative of a role in recognition by host cells (4). However to date, little is known on the molecular basis of the OmpU-mediated host-*Vibrio* interactions. The best characterized mechanism is probably that identified in *V. vulnificus*, which uses OmpU to bind fibronectin, as a process for invasion of HEp-2 cells (11).

More generally, as bacterial surface components, OMPs are used for pathogen recognition and trigger activation of macrophages. Consequently, various pathogens use OMPs as adhesins (adhesion proteins). For instance, in *Salmonella typhimurium*, OmpC mediates adherence to macrophages (13). Similarly, in *Escherichia coli*, OmpA is essential for invasion and virulence (14), while in *Klebsiella pneumoniae* it binds to and activates macrophages (15). As a result, both facultative and obligate intracellular pathogens use OMPs to invade host cells. Thus, human endothelial cells are invaded by *Neisseria meningitis* through OpC, an invasin (invasion protein) which binds to human serum factors (fibronectin and vitronectin). This coating is used for recognition by specific host cell receptors, the RGD-recognizing αvβ3 integrins, and subsequent invasion of host cells (16,17).

As oyster immune cells, hemocytes are equipped with a series of immune receptors and effectors involved in pathogen recognition and control. The best characterized immune receptor of oysters is certainly a β-integrin, named β_{CGH}, the expression of which differs according to the phagocytic ability of hemocyte populations (18). It was proposed that the oyster β-integrin could serve as a receptor for the major plasma protein referred to as extracellular superoxide dismutase, *Cg*-EcSOD, which contains a RGD cell-binding domain (19). A broad diversity of immune effectors was also characterized in oyster hemocytes ranging from reactive oxygen species (ROS) (20), to antimicrobial peptides and proteins (21-25).

Among oyster pathogens, some target hemocytes for invading their host. For instance, the protistan parasite *Perkinsus marinus* enters hemocytes of *Crassostrea virginica* avoiding the induction of the oxidative burst, as a mechanism of immune evasion (26,27). Inside hemocytes, it proliferates and spreads throughout the host. The mechanism of hemocyte entry relies on recognition by a galectin referred to as *Cv*-Gal, which promotes the phagocytosis of *P. marinus* (28).

In this article, we have studied the role of OmpU in the interaction of LGP32 with oyster hemocytes. Green fluorescent protein (GFP)-expressing variants of the wild-type and Δ*ompU* LGP32 were constructed. By a series of *in vitro* hemocyte invasion assays and experimental infections, we showed that LGP32 is a facultative intracellular pathogen that invades the oyster immune cells by using OmpU as an adhesin. In this process, the oyster major plasma protein *Cg*-EcSOD is used as an opsonin recognized by through its RGD sequence by hemocyte β-integrins. *In vitro* and *in vivo* experiments showed that OmpU-recognition subverts the host cell actin cytoskeleton, inducing the expression of host cell trafficking genes and resulting in actin and clathrin polymerisation. Consistent with its intracellular survival, LGP32 appeared to escape from host cellular defenses by avoiding vacuole formation, and by limiting ROS production.

Diversité moléculaire des effecteurs antimicrobiens chez l'huître creuse *Crassostrea gigas*: mise en évidence et rôle dans la réponse antimicrobienne.

Ce travail a contribué à la compréhension des bases moléculaires de l'immunité de l'huître creuse par la caractérisation la diversité de trois effecteurs antimicrobiens de *C. gigas* et par l'appréhension du rôle de cette diversité dans les mécanismes de défense. Des analyses phylogénétiques de deux peptides antimicrobiens (AMPs), *Cg*-Défensines (*Cg*-Defs) et *Cg*-Proline rich peptide (*Cg*-Prp), et d'une protéine de type Bactericidal Permeability Increasing protein, *Cg*-BPI, nous a permis montrer la grande diversité pour les 2 AMPs, qui est générée par plusieurs mécanismes génétiques et par des pressions de sélection directionnelles, suggèrant une diversité fonctionnelle des variants. L'importance biologique de cette diversité a été étudiée pour trois variants de *Cg*-Defs. Une forte activité antimicrobienne a été mise en évidence contre les bactéries à Gram positive, mais celle-ci diffère selon les variants. De plus, nous avons démontré que le mécanisme d'action des *Cg*-Defs contre *S. aureus* repose sur l'inhibition de la biosynthèse du peptidoglycane par le piegeage de son précurseur, le lipide II. Finalement, l'expression des transcrits et la localisation de ces effecteurs en réponse à une infection par un *Vibrio* pathogène ont montré un réseau complexe des profils d'expression des différents antimicrobiens, au niveau des populations hémocytaires et des tissus d'huître, suggérant une interaction entre les antimicrobiens du fait de leur colocalization. La combinaison entre les familles ou entre les variants d'une même famille produit de fortes activités synergiques qui élargissent les spectres d'activité. Ainsi, la diversité produit par la coévolution entre hôte et pathogènes pourrait améliorer l'activité des AMPs d'huître, lui conférant une plus grande protection contre les pathogènes de son environnement.

Molecular diversity of antimicrobials in the oyster *Crassostrea gigas* and role in the antimicrobial response.

This work contributed to the knowledge of the molecular bases of oyster immunity by the characterization of the diversity of three antimicrobials of *C. gigas* and the understanding of the role played by their diversity in the oyster antimicrobial response. Phylogenetic analyses of two antimicrobial peptides (AMPs), *Cg*-Defensins (*Cg*-Defs) and *Cg*-Proline rich peptide (*Cg*-Prp), and one Bactericidal Permeability Increasing protein, *Cg*-BPI, led us to the identification of a high diversity for both AMPs. Further analyses showed that this diversity is generated by gene duplication, allelic recombination and directional selection pressures, suggesting their functional diversification. The biological meaning of AMP diversity was investigated for the three major variants of *Cg*-Defs, which revealed a strong but variable potency against Gram-positive bacteria. We evidenced that oyster defensins kill *S. aureus* through binding to the cell wall precursor lipid II, resulting in the inhibition of peptidoglycan biosynthesis. Finally, transcript expression and localization of oyster antimicrobials after a pathogenic infection evidenced a complex network in their expression profiles in hemocyte populations and oyster tissues, suggesting a potential interplay between antimicrobials as a result of their colocalization. Indeed, the combination of oyster antimicrobials produced strong synergistic activities that enlarged their antimicrobial spectra. Thus, the diversity of oyster antimicrobials may provide significant means in acquiring functional divergence, probably concerned in the evolutionary arms race between hosts and their pathogens.From our data, it would provide oysters with a higher protection against the potential pathogens from their environment.