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Review

Molecular Structure of Endotoxins from Gram-negative Marine Bacteria: An Update

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Abstract: Marine bacteria are microrganisms that have adapted, through millions of years, to survival in environments often characterized by one or more extreme physical or chemical parameters, namely pressure, temperature and salinity. The main interest in the research on marine bacteria is due to their ability to produce several biologically active molecules, such as antibiotics, toxins and antitoxins, antitumor and antimicrobial agents. Nonetheless, lipopolysaccharides (LPSs), or their portions, from Gram-negative marine bacteria, have often shown low virulence, and represent potential candidates in the development of drugs to prevent septic shock. Besides, the molecular architecture of such molecules is related to the possibility of thriving in marine habitats, shielding the cell from the disrupting action of natural stress factors. Over the last few years, the depiction of a variety of structures of lipids A, core oligosaccharides and O-specific polysaccharides from LPSs of marine microrganisms has been given. In particular, here we will examine the most recently encountered structures for bacteria belonging to the genera *Shewanella*, *Pseudoalteromonas* and *Alteromonas*, of the γ-*Proteobacteria* phylum, and to the genera *Flavobacterium*, *Cellulophaga*, *Arenibacter* and *Chryseobacterium*, of the *Cytophaga-Flavobacterium-Bacteroides* phylum. Particular attention will be paid to the chemical features expressed by these structures (characteristic monosaccharides, non-glycidic appendages, phosphate groups), to the typifying traits of LPSs from marine bacteria and to

the possible correlation existing between such features and the adaptation, over years, of bacteria to marine environments.

Keywords: endotoxin, lipopolysaccharide, lipid A, O-polysaccharide, marine bacteria

1. Introduction

Gram-negative bacteria are ubiquitous in marine environments. As in the case of other microrganisms from sea habitats, they represent an interesting field of research, being a valuable source of natural substances provided with powerful bioactivity. Among the compounds that marine bacteria are able to synthesize, we can mention a wide range of antibiotics, toxins and antitoxins, antitumor and antimicrobial agents and enzymes with a wide spectrum of action. Furthermore, marine bacteria have recently gained visibility because of the capacity they have shown to contribute, through peculiar metabolic pathways, to the biological decontamination of polluted sites. Head *et al*. [1] recently reviewed the processes underlying hydrocarbon degradation by marine microrganisms belonging to the different classes of Proteobacteria, a promising process that could find application in the bioremediation strategies. For instance, several species belonging to the genus *Shewanella* have been considered for their great biotechnological potential, since they are capable of dissimilatory reduction of a wide range of electron acceptors, including metal oxides [e.g., those of Fe(III) and Mn(IV)] and organic pollutants [2].

From another perspective, marine bacteria can be in many cases classified as extremophiles, i.e. a class of prokaryotes adapted to life in inhospitable and harsh environments [3]. The parameters that define their habitat as “extreme” depend on the specific isolation site, and usually encompass high or low temperatures, elevated pressure and, in some cases, high salinity. For instance, deep-sea microrganisms are usually barophiles and psychrophiles, whereas bacteria living in hydrothermal-vents are obliged barophiles and thermophiles. All marine bacteria have adapted over millions of years in order to thrive these unusual environments, finding a niche for their survival. Microbial adaptation to such habitats has been accomplished by developing a complex series of processes charging the biomolecules (proteins, nucleic acids and membrane lipids) composing the cell, in order to assure the correct physiology and functionality under these conditions. Thus, the research on the marine bacteria glycolipids offers interesting insights in the understanding of the molecular adaptation process that may have occurred. Several of the typifying structural features encountered in marine Gram-negatives can therefore be seen as a response to the evolutionary pressure exerted by their environment. This adaptation provides special significance also for evolutionary phylogeny studies, allowing us an overlook on evolution, since many deep-sea environment have remained substantially unchanged for eons.

The majority of the Gram-negative marine bacteria investigated to date belong to the *Alteromonadaceae* family of the γ-subclass of proteobacteria that encompasses, among others, the genera *Shewanella, Alteromonas, Pseudoalteromonas, Glaceicola* and *Idiomarina*. More recently, studies have started, focusing on species from the *Cytophaga-Flavobacterium-Bacteroides* phylum.

These bacteria are essential components of marine environments and can be encountered in diverse habitats, including coastal and open water areas, deep-sea and hydrothermal vents, bottom sediments as well as marine plants and animals, with which they can establish symbiotic or pathogenic interactions.

In nearly all Gram-negative and in all marine Gram negative bacteria, the outermost layer of the cell envelope is constituted by the outer membrane, an asymmetric bilayer in whose outer leaflet are embedded Lipopolysaccharides (LPSs). These characteristic and vital molecules represent the contact between the bacterial cell and the surrounding environment, therefore it is plausible that many of the functional changes induced by the harsh habitats can target LPS structure.

2. LPS molecular architecture and activity

Lipopolysaccharides are the major constituents of the outer layer of Gram-negative bacteria's outer membrane. They are often referred to also as "endotoxins", given their ability to elicit the immune response in animal and vegetal organisms during the infectious events [4]. The occurrence of LPSs in the blood stream may in fact generate an unregulated activation of the immune system and trigger the biosynthesis of abnormal amounts of cytokines, which may lead to the condition of "septic shock" [5]. Generally, LPSs display a structure organized into three subdomains, covalently linked to each other: a glycolipid region, termed lipid A, the oligosaccharide portion of the core and, finally, a polysaccharide termed O-side chain (O-chain) or O-specific polysaccharide (OPS) [6]. Molecules that exhibit such architecture are also referred to as "Smooth-type LPSs" (S-LPSs), based on the typical regular appearance conferred to the colonies. This definition allows to make a distinction from the so-called "Rough-type LPSs" (R-LPSs), also named Lipooligosaccharides (LOSs), expressed in variable percentages either by native and mutant strains, that lack the polysaccharide portion [7]. LPSs are essential for the correct assembly of the cell membrane, and therefore for correct cellular physiology and replication. The structural variability of these three regions increases shifting from the lipid A to the polysaccharide moiety. This latter is the antigenic determinant of the molecule and, in the case of pathogen strains, is recognised by specific antibodies of the host acquired immune system. Bacterial polysaccharides are generally composed of oligosaccharide repeating units. During the biosynthesis, the so-called 'biological' repeating unit is initially preassembled and subsequently polymerised. Peculiar structural decorations and motives occurring in the OPS may prevent the specific binding of the polysaccharide to the antibodies, therefore masking the bacterial colonization and infection [8].

Marine bacteria OPSs often are of an anionic nature. This has been related to the process of adaptation to the marine environment, since the availability of negatively charged sites on the polysaccharide chains creates a suitable site for the formation of ionic interactions mediated by divalent cations. These bridges strengthen the overall packing of the membrane, thus providing further stability towards external stressors as high pressure. Moreover, the OPSs from marine bacteria LPSs often contain higher (up to 10 carbon atoms) or peculiar (presence of deoxy, amino and carboxyl functions; ester, ether and amide groups) monosaccharides, as well as non-carbohydrate appendages (i.e., phosphate or sulphate groups, polyols, carboxylic and amino acids. Marine bacteria polysaccharides have been previously reviewed by Nazarenko *et al*. [9]).

The OPS is covalently attached to the core oligosaccharide. This region of the LPS shows lower *intra*-species variability, and is characterized by the presence, in the inner region, of typical monosaccharides, namely 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo) and L-*glycero*-D-*manno*-heptose (L,D-Hep) [7]. Besides, in the outer core region, neutral or acidic monosaccharides, as well as 2-deoxy-2-aminosugars, are typically encountered. In marine bacteria, archetypal chemical features of the core region have been encountered as well, like the replacement of Kdo by its 8-deoxy-8-amino analogue (Kdo8N) in *Shewanella* [10-12], the occurrence of the D-*glycero*-D-*manno*-heptose (D,D-Hep) [11,12], the occurrence of monosaccharides connected *via* phosphodiester bonds to the linear backbone of the oligosaccharide, as observed in the core region of the LPS from *Arenibacter certesii* [13] and *Alteromonas addita* [14]. Furthermore, as for the OPSs, core oligosaccharides from marine bacteria often show remarkable negative charge density, inferred by the presence of a great number of phosphate substituents and/or acidic monosaccharides.

Finally, the core oligosaccharide is connected through Kdo (or Kdo8N in some *Shewanella* strains) to lipid A [15,16]. This is the most conserved portion of the molecule, its general structure being composed by a β-1',6-linked disaccharide of 2-amino-2-deoxy-D-glucose (D-glucosamine, D-GlcN). The reducing GlcN unit, α-configured, (GlcN I) is always phosphorylated at *O*-1, while the non-reducing GlcN (GlcN II) is phosphorylated at *O*-4. Both monosaccharides are variously acylated at *N*-2 and *O*-3 by long chain 3-hydroxy-fatty acids, so-called "primary fatty acids". This terminology allows a distinction from the "secondary" fatty acids, ester-linked, when present, to the 3-hydroxyl groups. The hydrophobic portion of the lipid A is embedded in the outer leaflet of the outer membrane. In general, the lipid A fraction isolated from single bacterial strains reveals intrinsic heterogeneity, due either to changes in acylation pattern and in the number of phosphate groups. As a general feature, the polar substituents of the lipid A backbone carry negative groups. It seems clear that these polar heads are important for the molecular organization and functions of the bacterial outer membrane [17].

The number, nature and distribution of the fatty acid chains varies according to the genus, and is responsible of the lipid A bioactivity. Indeed, the lipid A is the actual endotoxic centre of LPSs of pathogen strains, being able to trigger the innate immune response in the infected organism. The innate immune system is the first line of host defence against pathogens, acting in the first stages of the infection [18]. Different microrganisms are recognised *via* a limited number of germline-encoded Pattern-Recognition Receptors (PRRs), that identify microbial components, known as Pathogen Associated Molecular Patterns (PAMPs). The PAMPs are specific and vital for the microrganisms, and they can not be altered through a mimic mechanism by the colonising cell. LPSs in general, and lipid A in particular, can be considered PAMPs for pathogen Gram-negative bacteria. Most of the pathogenic phenomena associated with the infection are induced by the interaction, mediated by several cytosolic and transmembrane proteins, of the lipid A with a specific receptor, termed Toll-like Receptor 4 (TLR-4), acting as a PRR [18].

Different Gram-negative bacteria produce structurally different lipid As, varying in their phosphate and acyl patterns. This structural variability is responsible for three-dimensional changes in the overall lipid A assembly, leading to changes in the induced response of the innate immune system and consequently in the toxicity of lipid A itself. It has been observed that the higher the acylation degree in the lipid A family, the higher a pathogenic effect is caused in the host immune system and that asymmetrical fatty acid distribution on the glucosamine backbone considerably increase the toxicity of

lipid A. In the same way, the absence of one phosphate groups on the disaccharide reduces the pathogenicity associated with the microrganism [19]. As a integral part of the Outer Membrane, structural changes can occur in lipid A in response to environmental stressing conditions, mostly with regards to the fatty acids composition, to counterbalance the alterations effected by physical and chemical agents [20, 21]. Lately, great attention has been paid to marine bacteria lipids A and LPSs, since they have often shown low levels of toxicity [22]. It is known that some lipid A from Gram-negative bacteria may also express antagonistic activity toward the innate immune system, competing with toxic LPSs for the active sites of the PRRs and protein mediators, thus, limiting their immunological reactivity [23]. The features of low acylation and/or phosphorylation degree have been encountered in lipid As from marine Gram-negatives, where they have been seen as an adaptation to the environment. For instance, low acylation, achieved through short chained fatty acids is a distinctive features of psychrophilic microrganisms, where it is performed in order to retain the cell membranes in the liquid-crystalline state [21]. Therefore, potential candidates for the development of antagonist strategies against septic shock may be sought among the lipid As and LPSs from marine Gram-negative bacteria, adding interest in the research about this topic.

The protocol leading to the structural characterization of LPSs and LOSs comprehends a complex series of extraction, purification and degradation steps. These are obviously supported by an extensive succession of chemical analyses, mainly based on chemical derivatization and GC-MS analyses, in order to achieve the complete definition of the monosaccharide and lipid content, and completed by MALDI Mass Spectrometry and high resolution 1D and 2D-NMR spectroscopy, that allow the description of the full structure of the sub-domains composing the LPS structure.

The present work reviews the structures of several marine bacterial OPSs, core oligosaccharides from 2002 on (pre-2002 see Nazarenko *et al.* [9]) whereas the lipid A structures, the majority of which belongs to the genera *Shewanella*, *Pseudoalteromonas* and *Alteromonas*, of the Alteromonadaceae family of the γ-subclass of proteobacteria (*"Gammaproteobacteria"*), are reviewed herein for the first time. Moreover, some examples of LPSs from bacteria belonging to the genera *Flavobacterium, Cellulophaga, Arenibacter* and *Chryseobacterium*, of the *Cytophaga-Flavobacterium-Bacteroides* phylum are reported. All of these bacteria are abundant in the marine environment, inhabiting coastal, deep-sea and high sea areas, hydrothermal vents and bottom sediments, marine plants and animals. The LPSs and LOSs presented have all been extracted from bacterial cells by means of the conventional extraction protocols of Galanos [24] (for LOSs) and/or Westphal and Jann [25] (for LPSs). Purification steps have been realized by means of several Gel Permeation Chromatography or HPLC. State of art NMR and MALDI analyses have been performed on either the native or the partially degraded molecules, which eventually underwent various de-acylation procedures in order to improve their solubility.

3. Structure of O-specific polysaccharides from marine bacteria

The OPS represents the most variable portion of the LPS. A number of structures from marine bacteria OPSs have been reported and reviewed in the past, but this number is continuously increasing as research progresses. Most of bacterial OPSs are antigenic, and show high immunological specificity, being characteristic of single bacterial strains. Therefore, their oligosaccharide epitopes

may be used to develop vaccines. Many OPSs, as well as extracellular polysaccharides, produced by marine bacteria have been reviewed by Nazarenko *et al*. [9]. In order to characterise the repeating unit of the OPSs, the polysaccharide moiety has to be isolated by mild acidic hydrolysis of the linkage between Kdo and lipid A. This makes the OPS water soluble, allowing the exploitation of analyses in such medium. the presence of the core moiety, less abundant, does not affect the structure elucidation process.

The first group of polysaccharides presented has been isolated from the LPSs of bacteria belonging to the genus *Pseudoalteromonas*. This genus contains about 40 validly described species, including the reclassified former *Alteromonas* species [26]. Gram-negative bacteria of the genus *Pseudoalteromonas* are aerobic non-fermentative prokaryotes. They are widespread obligatory marine micro-organisms that require seawater for their growth, and produce a wide range of biologically active compounds [27, 28].

In recent years, extensive studies of polysaccharides produced by *Pseudoalteromonas* spp. revealed that the common features of most of them are an acidic character and the presence of non-sugar substituents. Among the genus *Pseudoalteromonas*, the O-polysaccharides from the LPSs of the species *P. rubra* ATCC 29570^T, *P. carrageenovora* IAM 12662^T, *P. aliena* KMM 3562^T and *P. atlantica* IAM 14165^T were recently described. Interestingly, all of them present in their repeating unit chemical features that increase the total negative charge of the *O*-antigen moiety.

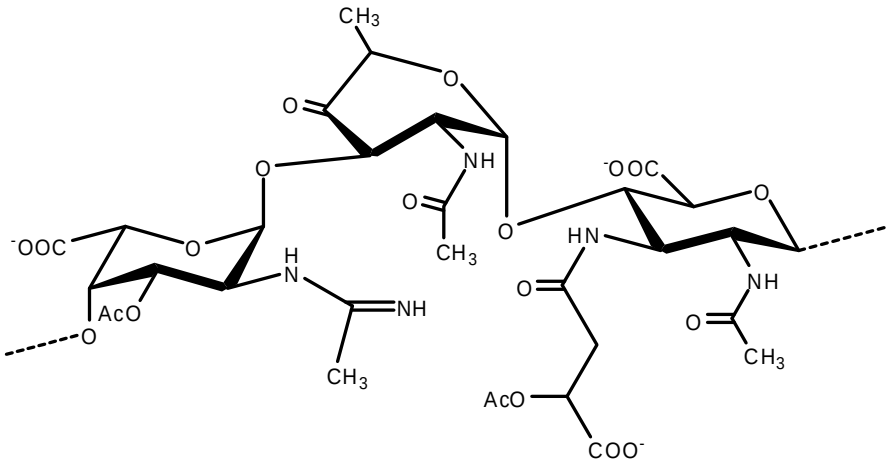
Pseudoalteromonas rubra ATCC 29570^T [29], a red-pigmented bacterium isolated from the Mediterranean Sea off Nice and able to produce an extracellular polyanionic antibiotic that modifies bacterial respiration, assembles an OPS in which two rare constituents are present, namely malic acid (also found in the OPS from *S. algae* BrY [30]), amide linked at *N*-3 of a 2-acetamido-3-acylamido-2,3-dideoxy-D-glucuronic acid (Glc*p*NAc3NAcylA), and a keto-sugar, 2-acetamido-2,6-dideoxy-D-*xylo*-hexos-4-ulose, previously found only in the LPS of the mutant strain *Rhizobium etli* CE166 as the junction point of the OPS to the core [31], and in the capsular polysaccharides from *Streptococcus pneumoniae* type5 [32], *Vibrio ordalii* O:2 [33] and in the OPS of *Flavobacterium columnare* ATCC 43622^T [34]. Moreover, the repeating unit of such OPS also contains a second uronic acid, 2-acetimidoylamino-2-deoxy-L-galacturonic acid, stoichiometrically acetylated at *O*-3 (L-Gal*p*NAm3AcA) The identification of the repeating unit structure has been achieved by means of ^{13}C-NMR and 2D NMR spectroscopy after mild acid hydrolysis, *O*-deacetylation and borohydride reduction of the LPS, and led to the depiction of the following structure:

→4)-α-L-Gal*p*NAm3AcA-(1→3)-α-Sug*p*-(1→4)-β-D-Glc*p*NAc3NAcylA-(1→

Pseudoalteromonas rubra ATCC 29570^T [29]

The marked anionic nature of such polysaccharide is better understood from the molecular structure represented in Figure 1. The OPS from *P. carrageenovora* IAM 12662^T [35] was identified after *O*-deacylation of the LPS, since the standard approach to cleave a lipid A moiety with 1% acetic acid treatment yielded a complex polymer, presumably due to the non-stoichiometric presence of the monosaccharide colitose (3,6-dideoxy-L-*xylo*-hexose, Col), which is partially hydrolysed under these conditions.

Figure 1. Repeating unit of the OPS from the LPS of *Pseudoalteromonas rubra* ATCC 29570^T

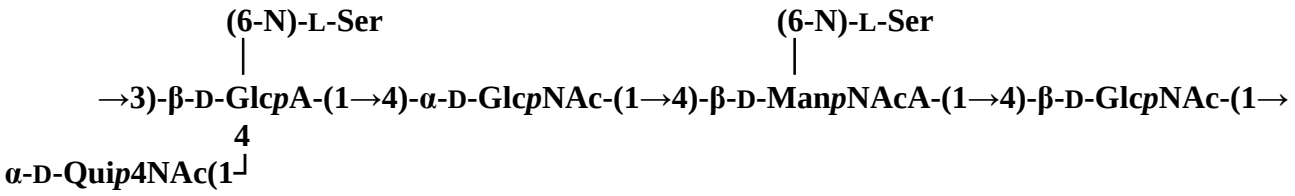


Simple *O*-deacylation of the LPS with anhydrous hydrazine provided a readily soluble polysaccharide, in which the detection of two colitose residues was straightforward. Colitose is either present as the last residue of the side chain or inserted in the hexasaccharide motive, presented below, as also previously found in the OPS from *P. tetraodonis* IAM 14160^T [36]:

→2)-α-Col*p*-(1→4)-β-Glc*p*NAc-(1→4)-β-Glc*p*A-(1→3)-β-Gal*p*NAc-(1→
3
α-Col*p*-(1→2)-β-Gal*p*-(1┘

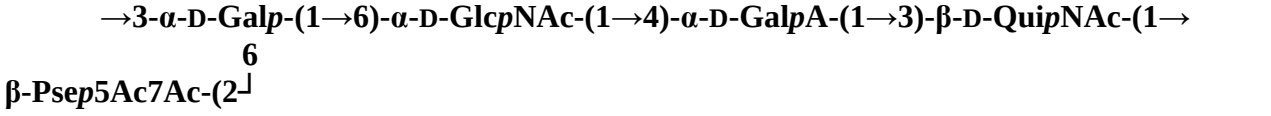
Pseudoalteromonas carrageenovora IAM 12662^T [35]

The acidic OPS from the type strain of *P. aliena* species, SW19^T (=KMM 3562^T=LMG 22059^T), has been subjected to structural investigation [37]. It was found that the polymer contains two *N*-acetyl-D-glucosamine (GlcNAc) residues and one residue each of D-glucuronic acid (GlcA), 2-acetamido-2-deoxy-D-mannnuronic acid (ManNAcA) and 4-acetamido-4,6-dideoxy-D-glucose (*N*-acetyl-D-viosamine, D-Qui4NAc). Besides that, amino acid analysis revealed the presence of L-serine (L-Ser) as constituent of the PS. In this case, a ^{1}H,^{13}C band-selective constant-time heteronuclear multiple-bond connectivity experiment was used to determine amide linkages between serine and uronic acid residues, via $^3J_{H,C}$ correlations between Ser–H_α and C-6 of the uronic acids. As a result, the polysaccharide was shown to be a branched polymer made up of a pentasaccharide repeating unit containing two L-serine residues as amides of both uronic acid residues. This was the first time that two constituents as amides of uronic acids with L-serine have been found as part of a polysaccharide, namely *N*-(2-acetamido-2-deoxy-D-mannuronoyl)-L-serine and *N*-(D-glucuronoyl)-L-serine. Furthermore, this polymer contains another sugar, 4-acetamido-4,6-dideoxy-D-glucose, identified earlier in antigenic polysaccharides from *Escherichia coli* O:7 [38], *Shigella dysenteriae* type 7 [39], *Francisella tularensis* strain 15 [40], *Vibrio anguillarum* V-123 [41] and *Hafnia alvei* 1205 [42].



Pseudoalteromonas aliena KMM 3562^T [37]

The antigenic polysaccharide from *Pseudoalteromonas atlantica* strain IAM 14165 [43] is an example of a polysaccharide containing, within its oligosaccharide repeating unit, a higher monosaccharide with a nine carbon atom skeleton. On the basis of structural characterization data, a pentasaccharide repeating unit was found, containing along with D-Gal, D-GlcNAc, D-QuiNAc and D-GalA, 5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-non-2-ulosonic acid, one of the stereoisomers of pseudaminic acid [43]. This sugar has been identified earlier as the component of the antigenic PSs from *Shigella boydii* serogroup 7 [44], some serogroups of *Pseudomonas aeruginosa* [45,46], *Vibrio cholerae* serogroup 2 [47] and *Escherichia coli* O136 [48]. The identified structure is presented below:



Pseudoalteromonas atlantica IAM 14165 [43]

The genus *Shewanella* comprises more than 20 validly described species, including both free-living and symbiotic forms, and members of this family have been isolated from various marine sources, including water, sediments, fish, algae and marine animals. These bacteria are responsible of the spoilage of the protein-rich foods and two strains, *S. putrefaciens* and *S. algae*, are known as opportunistic pathogens of humans and marine animals and recognised as the causative agents of soft tissues bacteraemia and sepsis. Members of this genus have also been studied for their involvement in a variety of anaerobic processes including the dissimilar reduction of manganese and iron oxides, uranium, thiosulphate and elemental sulphur among others. Because of their metabolic versatility and wide distribution in a variety of aquatic habitats, *Shewanella* and related microrganisms play a crucial role in the cycling of organic carbon and other nutrients [2]. Several *O*-polysaccharides from *Shewanella* have been isolated and characterised. Also in this case, as for *Pseudoalteromonas*, acidic polysaccharides have been identified, often containing peculiar monosaccharides or acidic non carbohydrate substituents, i.e. malic acid in the *O*-polysaccharide from *S. algae* BrY [30] or the peculiar monosaccharide Shewanellose, a novel *C*-branched sugar [(2-acetamido-2,6-dideoxy-4-*C*-(3'carboxamide-2',2'-dihydroxypropyl)-D-galactose, She] first found in the *O*-polysaccharide from *S. putrefaciens* A6, together with a derivative of the 8-epilegionamminic acid [49]. In recent years, the structures of two new *O*-polysaccharides isolated from *Shewanella* LPS have been added to the vast literature describing such kind of molecules (for a review, see Nazarenko *et al*. [9]).

The recently described species *S. fidelis* was isolated from sediment samples from the South China Sea [50]. The *O*-specific polysaccharide from the type strain KMM 3582^T has a tetrasaccharide

repeating unit consisting of two D-GalNAc, one D-GlcA and one D-GalA residues. The latter is amidated with N^{ε}-[(*S*)-1-carboxyethyl]-N^{α}-(D-galacturonoyl)-L-lysine (alaninolysine, AlaLys) [51]. The occurrence of amides of alaninolysine with GalA and GlcA has been reported previously only for the *O*-specific polysaccharides from a number of members of *Enterobacteriaceae* family: *Providencia rustigianii* O14 [52], *Providencia alcalifaciens* O23 [53], *Proteus myxofaciens* [54] and *Proteus mirabilis* O13 [55]. However, this is the first time that this amide compound was found in the *O*-antigenic polysaccharide of a bacterium from a different taxon. The presence of such characteristic functional groups might be an indication of a common ancestor for bacteria of these genera as members of the γ-subclass of *Proteobacteria* [56].

→3)-β-D-Gal*p*NAc-(1→4)-β-D-Glc*p*A-(1→3)-β-D-Gal*p*NAc-(1→2)-α-D-Gal*p*A-(1→
6
***2S,8S*-AlaLys ┘**

Shewanella fidelis KMM 3582^{T} [51]

An *O*-specific polysaccharide was also isolated from the LPS of the type strain of the new species *S. japonica*, which has a linear structure and contains an amino-deoxysugars, *N*-acetyl-L-fucosamine, 4-acetamido-4,6-dideoxy-D-galactose (4-aminofucose, tomosamine, Fuc4NAc) and *N*-acetyl-D-bacillosamine (QuiNAc4NAc), together with D-GalA [57]. Bacillosamine has previously been found in the capsular polysaccharide from another marine bacteria, *Pseudoalteromonas sp*. KMM 155 [58] and in the *O*-antigens, among the others, of *Pseudomonas aeruginosa* [59], *Pseudomonas aurantiaca* IMB 31 [60], *Pseudomonas reactans* [61], *Vibrio cholerae* O3 [62], O5 [63], and O8 [64], *Pseudoalteromonas haloplanktis* KMM 223 [65], ATCC 14393 [66], KMM 634 [67,68]. Fuc4NAc has been found before in the cyclic enterobacterial common antigen from *Yersinia pestis* [69] and in the repeating unit of the *O*-chain polysaccharide of *Pseudomonas fluorescens* biovar A strain IMV 1152 [70] and of *Pseudomonas* sp. OX1 [71].

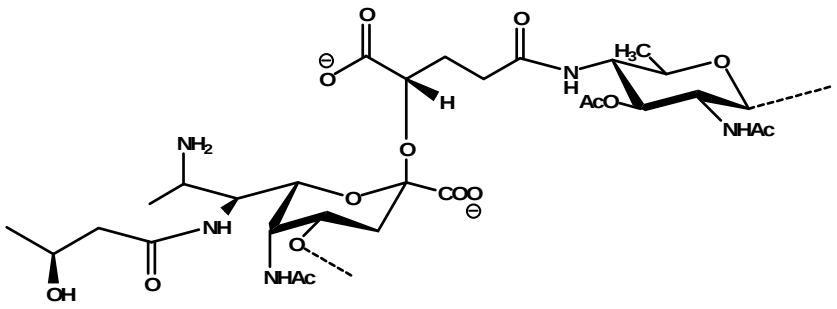
→3)-α-D-Fuc*p*4NAc-(1→4)-α-D-Gal*p*A-(1→3)-α-L-Fuc*p*NAc-(1→3)-β-D-Qui*p*NAc4NAc-(1→

Shewanella japonica KMM 3299^{T} [57]

In order to present a complete picture of the enormous structural variability that may be encountered in marine bacteria LPSs, we also report here the structure of the OPS from the fish pathogen *Flexibacter maritimus* [72]. This microrganism belongs to the *Cytophaga-Flavobacterium-Bacteroides* group. These are a large, heterogeneous group of filamentous, gliding, Gram-negative bacteria infecting a wide variety of fish species and usually forming filamentous biofilms [73], primarily on the tissues associated with the oral cavity. *F. maritimus* has been associated with a disease (Flexibacteriosis) in a number of fish species [74]. The OPS from this bacterium has been characterized by means of extensive spectroscopical analyses performed on the polysaccharide and on its methanolysis products, revealing the occurrence of a higher monosaccharide, 5-acetamido-7-[(*S*)-3-hydroxybutyramido]-8-amino-3,5,7,8,9-pentadeoxynonulopyranosonic acid, together with 2-acetamido-3-*O*-acetyl-4-[(*S*)-2-hydroxyglutar-5-ylamido]-2,4,6-trideoxy-β-glucose (QuiNAc3Ac4NAcyl).

The repeating unit includes a linkage via the (*S*)-2-hydroxyglutaric acid residue, that was reported for the first time in a bacterial polysaccharide. The repeating unit is represented in Figure 2:

Figure 2. Structure of the repeating unit of the OPS from the LPS of *Flexibacter maritimus* [72].



A similar feature, namely the occurrence of *O*-glycosylated amide linked (*R*)-malic acid, was reported to be a component of the OPS from another fish pathogen, *Flavobacterium psychrophilum*, belonging to the same phylum of *F. maritimus* [75]. The structure found in that case is reported below, and again shows the occurrence of both amino-sugars and non glycidic components.

→4)-α-L-Fuc*p*NAc-(1→3)-α-D-Qui*p*2NAc4NR-(1→2)-α-L-Rha*p*-(1→

R = (3*S*,5*S*)-$CH_3CH(OH)CH_2CH(OH)CH_2CO$

Flavobacterium psychrophilum [75]

Finally, the OPS from another member of the family of Flavobacteriaceae, *Cellulophaga fucicola*, was identified, after mild acid hydrolysis with acetate buffer, as constituted by a trisaccharide repeating unit containing a nonulosonic acid residue, pseudaminic acid (5,7-diacetamido-3,5,7,9-tetradeoxy-L-*glycero*-L-*manno*-non-2-ulosonic acid, Pse*p*) [76] . The structure found is the following:

→4)-β-D-Gal*p*-(1→4)-β-D-Glc*p*-(1→4)-β-Pse*p*-(2→

Cellulophaga fucicola [76]

4. Structure of core oligosaccharides from marine bacteria

The structure elucidation of the core region of bacterial LPS is attained after complete deacylation of the LOS. This is normally achieved by alkaline treatment with hydrazine followed by hydrolysis with aqueous KOH, that allows the recovering of the oligosaccharide backbone comprehensive of the lipid A glucosamine disaccharide. This treatment is responsible for the loss of base-labile groups, that are conventionally characterised through a complementary approach that cleaves the linkage between Kdo and lipid A under extremely mild condition (1% acetic acid). In this way, water soluble oligosaccharides can be analysed in aqueous medium. Alternatively, it is possible to simply *O*-deacylate the LOS and carry out spectroscopic analyses in denaturing solvent system (SDS-d_{12} in

D_2O). This latter approach allows one to preserve either acid- and base-labile groups, that can be in this way identified. The chemical and spectroscopical analyses are completed and integrated by means MALDI MS, that allows the identification of the exact molecular weight of these structure, and at the same time allows to determine the occurrence of different glycoforms, varying in the glycosylation and/or phosphorylation pattern.

Exploiting this approach, the core oligosaccharides (OSs) from two new core oligosaccharides from *Pseudoalteromonas* have been recently described, from *P. carrageenovora* IAM 12662^T [35] and *P. issachenkonii* KMM 3549^T [77]. The OS from *P. carrageenovora*, whose OPS has been described in the previous section, has been elucidated by means of compositional analysis, matrix-assisted laser desorption/ionization mass spectrometry and complete ^{1}H-, ^{13}C- and ^{31}P-NMR spectroscopy. It is composed by a mixture of three glycoforms, differing for the length of the sugar chain and the phosphorylation pattern [35], and is characterized by a strong accumulation of phosphate groups in the lipid A-core portion, creating a region with a high charge density, as depicted below.

α-Hep*p*3*P*-(1→5)-α-Kdo*p*4*P*-(2→6)-β-Glc*p*N4*P*-(1→6)-α-Glc*p*N1*P* **(1)**

β-Gal*p*-(1→4)-α-Hep*p*3*P*-(1→5)-α-Kdo*p*4*P*-(2→6)-β-Glc*p*N4*P*-(1→6)-α-Glc*p*N1*P* **(2)**

β-Gal*p*-(1→4)-α-Hep*p*3*P*-(1→5)-α-Kdo*p*4*P*-(2→6)-β-Glc*p*N4*P*-(1→6)-α-Glc*p*N1*P* **(3)**
4
α-Glc*p*-(1┘

Pseudoalteromonas carrageenovora IAM 12662^T [35]

A closely related chemical architecture has been found also in the core region of the LPS from *P. issachenkonii* KMM 3549^T [77], isolated from the thallus of the brown alga *Fucus evanescens*, collected in the Kraternaya Bight of the Kurile Islands in the Pacific Ocean [78]. This halophilic microrganism has bacteriolytic, proteolytic and haemolytic activity and degrades algal polysaccharides, producing a number of glycosyl hydrolases (fucoidanases, laminaranases, alginases, agarases, pullulanases, β-glucosidases, β-galactosidases, β-*N*-acetylglucosaminidases and β-xylosidases). The core oligosaccharide structure has been depicted after alkaline de-acylation, and on the basis of the MALDI MS and NMR data, it has been described as composed by a mixture of three glycoforms differing in the glycosylation and phosphorylation profile.

β-Gal-(1→7)-α-Hep3*R*-(1→5)-α-Kdo4*P*-(2→6)-β-GlcN4*P*-(1→6)-α-GlcN1*P* **(1)**

a R = P
b R = H

β-Gal-(1→7)-α-Hep3*R*-(1→5)-α-Kdo4*P*-(2→6)-β-GlcN4*P*-(1→6)-α-GlcN1P **(2)**
4
β-Gal-(1┘

a R = P
b R = H

β-Gal-(1→7)-α-Hep-(1→5)-α-Kdo4*P*-(2→6)-β-GlcN4*P*-(1→6)-α-GlcN1*P* **(3)**
4
α-Glc-(1→4)-β-Gal-(1┘

Pseudoalteromonas issachenkonii KMM 3549^T [77]

Interestingly, glycoform **3** was present only in its tri-phosphorylated form, where the three phosphate residues on lipid A and Kdo were plainly assigned, thus, with no phosphate residue at *O*-3 of the heptose unit. It should be noted, that in two core glycoforms of the LOS from *P. issachenkonii* (**2** and **3**), a 4,7-di-substituted heptose is present and this is, at our knowledge, the first time that in a core region a heptose with such a substitution pattern is found. It is noteworthy that the only one more core structure of LPS from *Pseudoalteromonas* has been reported to date, *P. haloplanktis* TAC 125 [79]. As in the case of *P. carrageenovora* and *P.issachenkonii*, it possesses the carbohydrate skeleton

β-Gal-(1→4)-α-Hep3P-(1→5)-α-Kdo4P-(2→6)-β-GlcN4P-(1→6)-β-GlcN1P,

that, up to now, can be considered a unique structural feature of the *Pseudoalteromonas* genus.

From the structural investigation on the core oligosaccharides of LPSs from *Shewanella*, interesting information have emerged. For instance, the oligosaccharide from *S. oneidensis* MR-1 constitutes the first case of identification of a monosaccharide other than Kdo, namely 8-amino-3,8-dideoxy-D-*manno*-octulosonic acid (Kdo8N) [10], as junction of the OS with lipid A. This is not the only odd feature of such OS, since a residue of 2-acetamido-2-deoxy-D-galactose in an open-chain form (GalNAc*o*), linked as cyclic acetal to *O*-4 and *O*-6 of D-galactopyranose has also been detected by means of NMR spectroscopy [10]. An open-chain acetal linkage was previously found only in the core part of some *Proteus* LPSs [80]. In addition, the structure contains a phosphodiester linkage between the α-D-galactopyranose and D-*glycero*-D-*manno*-heptose (DD-Hep) residues:

α-DDHep-(1→5)-α-Kdo8N4R-(2→6)-β-GlcN4P-(1→6)-α-GlcN1P
3
α-Gal-(1→6)-α-Gal-(1→3)-α-Gal-(1-P┘
4,6
↑
1
GalNAc*o* **R = P or EtNPP**

Shewanella oneidensis MR-1 [10]

The occurrence of Kdo8N replacing Kdo has been also detected in the oligosaccharides from *S. algae* BrY [11] and from the three strains KMM 3601, KMM 3605 and KMM 3772 of *S. pacifica* [12], which revealed identical core structures, and constitute, together with the OS structure from *S. putrefaciens* CN32 [81], the only known oligosaccharide structures for this genus. Although this latter does not include in its architecture the Kdo8N, it is still possible to suggest that this sugar residue may be considered as a taxonomic marker for the genus. The core structure from *S. algae* BrY is a blend of diverse glycoforms, but all variants are only mere truncations of the following maximal structure.

α-Hep-(1→
2
α-Glc-(1→2)-α-Hep-(1→6)-α-DDHep-(1→5)-α-Kdo8N4*P*-(2→6)-β-GlcN4*P*-(1→6)-α-GlcN1P
7
α-L-Rha-(1→3)-β-GlcN*R*-(1→

R=Ac or H

Shewanella algae BrY [11]

The combined chemical, MS and NMR approach has led to the complete elucidation of the novel oligosaccharide structure from *S. pacifica* KMM 3601, KMM 3605 and KMM 3772 [12]. As in the two examples presented above, in *S. pacifica* LOS the Kdo residue is replaced by Kdo8N. Moreover, as in all *Shewanella* LPSs, this Kdo8N residue bears an unusual heptose residue (D,D-Hep) at the C-5 position, and this carries a non-carbohydrate substituents linked by a phosphodiester linkage, as reported for *S. oneidensis* MR-1 LPS.

The replacement of Kdo by a derivative in the inner core of LPS is rather rare and, to the best of our knowledge, the only other occurrence is the presence of the 3-hydroxy-derivative of Kdo, D-*glycero*-D-*talo*-octulosonic acid (Ko), which non-stoichiometrically replaces Kdo in *Acinetobacter haemolyticus* LPS [82], whilst Kdo itself is only present as a branching residue of the oligosaccharide. A feature of the LPS from *S. pacifica* is the presence of glyceric acid, which is attached *via* phosphodiester linkage to the D,D-Hep, contributing to the increasing of the total negative charge of the inner-core region. Although this is a key molecule of the primary metabolism of Gram-negative bacteria, it has never been detected in the core of LPS molecules.

Glc-(1→2)-Hep-(1→6)-Hep7PGroA-(1→5)-Kdo8N4R-(2→6)-GlcN4P-(1→6)-GlcN1P
2
Hep-(1→

R = P or PPEtN

Shewanella pacifica KMM 3772 [12]

The revision of the genus *Alteromonas,* first established by Baumann and co-workers [83], resulted in its partition into two genera: *Pseudoalteromonas* and *Alteromonas* [84]. The newly defined *Alteromonas* genus comprises few validly described species, namely *A. macleodii, A. marina, A. stellipolaris, A. litorea* and *A. addita*. The structure investigation of the LPS structures from bacteria belonging to this genus has only recently begun, and, up to now, only two structures have been given, from *A. macleodii* ATCC 27126^T [85] and from *A. addita* KMM 3600^T [14]. Interestingly, in both cases the bacteria have been found to produce only a R-LPS, provided with an extremely short oligosaccharide chain with a high negative charge density. The structure of the glycidic portion of the LPS therefore coincides, in such cases, with the description of the OS. Within the core oligosaccharide from *A. macleodii* ATCC 27126^T, the negative charge is inferred, among the others, by the occurrence of Kdo residue that is present as β-configured residue. This feature is rather rare in polysaccharides in

general, being sometimes detected in polysaccharide capsules [86], and is very rare in LPSs. In the OPS from *Serratia marcescens*, β-Kdo is present as a single residue at the end of the chain, functioning as a monosaccharide cap in the regulation of the length of the molecule [87].

β-Kdo-(2→7)-α-Hep-(1→5)-α-KdoP-(2→6)-β-GlcN4P-(1→6)-α-GlcN1P

Alteromonas macleodii ATCC 27126^T [85]

The newest species added to the genus, *A. addita*, was first isolated from sea water samples collected at various depth in the Pacific Ocean region of Chazma Bay (Sea of Japan), during a study on free-living microbial colonies in radionuclides contaminated environments [88]. The core oligosaccharide of the LOS from this strain was characterized by means of ^{1}H-, ^{13}C- and ^{31}P-NMR spectroscopy and MALDI MS, after complete de-acylation and selective mild *O*-deacylation [14]. This latter approach allowed the detection of the base labile substitution of a Glc residue linked *via* phosphodiester bond to a heptose moiety:

α-Glc-(1-P-3)-α-Hep-(1→5)-α-Kdo4P-(2→6)-β-GlcN4P-(1→6)-α-GlcN1P

Alteromonas addita KMM 3600^T [14]

The same feature of a phosphodiester bond connecting two monosaccharides units was previously reported in the core oligosaccharide of another marine bacterium, *Arenibacter certesii* KMM 3941^T [13]. The recently described genus *Arenibacter* was established to accommodate Gram-negative, strictly aerobic, heterotrophic, dark-orange pigmented, non-motile marine bacteria belonging to the *Cytophaga–Flavobacterium–Bacteroides* phylum [89]. Also in this case, a combined approach based on spectroscopical and MS analyses was performed on the product of either complete and selective *O*-deacylation. The structural analysis showed the replacement of the β-GlcN (GlcN II) moiety of the lipid A backbone with a unit of 2,3-diamino-2,3-dideoxy-glucose (DAG). DAG has been previously identified as component of the lipid A backbone in other LPSs [90,91]. The DAG-Glc*p*N β-(1→6)-linked disaccharide was found in lipid A from *Campylobacter jejuni* and *Rhodospirillum salinarum* [90], whereas the DAG-DAG β-(1→6)-linked disaccharide is present in the lipid A from the LPS of *Aquifex pyrophilus* [91], *Bordetella pertussis*, and *Legionella pneumophila*, and, as a minor component, in lipid A from *C. jejuni* [90]. The presence of the DAG moiety in the lipid A backbone of *A. certesii* contributes to assure the bacterium a major resistance to environment, given the superior resistance of the amide linkages to the hydrolysis, which can be seen as an adaptation of the bacterium to external hostile environment.

α-D-Rha-(1→6)-α-D-Man-(1→6)-α-D-Man-(1→5)-α-D-Kdo-(2→6)-β-DAG-(1→6)-α-D-GlcN1P
3 4 4
R┘ α-D-GalA-(1-P┘ P┘

R = H or α-D-Rha

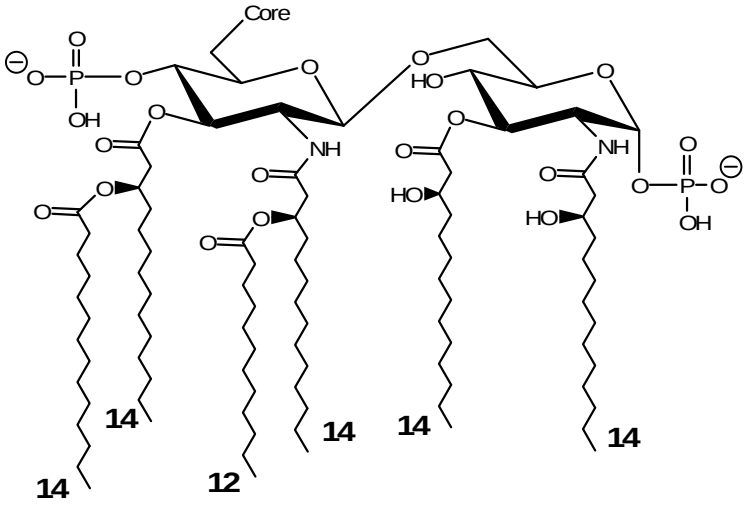
Arenibacter certesii KMM 3941^T [13]

The occurrence of the GalA moiety contributes also in this case to the increase of the total charge density of the molecule. It is deemed that these negatively charged groups allow the establishment of ionic bridges between LPS molecules due to electrostatic interactions with environmentally available bivalent cations (Ca^{2+}, Mg^{2+}), and that these contribute to the rigidity and stability of the Gram-negative cell wall [92]. Thus, the high number of negative charges in these short oligosaccharides from *Pseudoalteromonas*, *Shewanella*, *Alteromonas* and *Arenibacter* could be important for maintaining the integrity of the outer membrane exposed to a peculiar external surrounding.

5. Structure of lipids A from marine bacteria

In order to perform the structure elucidation of lipid A, the glycolipid moiety has to be released from the LPS or LOS. This is generally achieved through the same mild treatment used for the isolation of OPSs, which exploits the susceptibility to acid conditions of the glycosidic linkage of Kdo to β-GlcN. The lipid A is then characterized by means of selective chemical analyses, NMR spectroscopy and, mostly, mass spectrometry, that allows the depiction of the intrinsic heterogeneity of this portion. Selective deacylation with NH_4OH, which cleaves the acyloxy-acylester groups but leaves unaffected the acyloxy-acylamide substituents before MS analysis provides useful insight to the description of the acylation pattern of lipid A [93,94]. The information about the lipid A structure may also derive from the MALDI MS analysis on the intact LOS fraction. In fact, due to the energy associated to the laser source, a phenomenon of β-elimination [95] may be observed, that yields either oligosaccharide ion (termed B-type ions, according to the Domon and Costello nomenclature [96]) and lipid A ions.

Figure 3. Structure of *Escherichia coli* lipid A at physiological pH. Bold numbers indicate the length of fatty acid chains in the most abundant form.



The structural study of marine bacteria lipid A is still at an early stage compared to that of oligo- and poly-saccharides. Nevertheless, in the past few years, a number of structures has been described. In order to give a synoptic description of lipid A, the fatty acid substitution profile referring to the main form of the studied species is reported in Table 1. Unless specifically stated, all the molecules share the conventional glucosamine disaccharide backbone and show phosphorylation at the positions C1 and C4', as exemplified in Figure 3, where the lipid A from *E. coli* is shown.

Table 1. Acylation pattern of the main lipid A species from Gram-negative marine bacteria.

Bacterium	Nature and linkage of acyl substituents bound to				
	GlcN II[a]		GlcN I		
	3'	2'	3	2	Ref.
P. haloplanktis ATCC 14393^T	10:0(3-OH)[b]	12:0(3-O-12:0)[c]	10:0(3-OH)[b]	12:0(3-OH)[c]	97
P. haloplanktis TAC 125	12:0(3-OH)	12:0(3-O-12:0)	12:0(3-OH)	12:0(3-OH)	98
P. issachenkonii KMM 3549^T	10:0(3-OH)[d]	12:0(3-O-12:0)		12:0(3-OH)	77
P. carrageenovora IAM 12662^T	10:0(3-OH)[d]	12:0(3-O-12:0)		12:0(3-OH)	-
A. macleodii ATCC 27126^T	12:0(3-OH)[d]	12:0(3-O-12:0)[c]		12:0(3-OH)	85
A. addita KMM 3600^T	12:0(3-OH)	14:0(3-O-12:0)	10:0(3-OH)	14:0(3-OH)	14
S. pacifica KMM 3772	13:0(3-O-13:0)	13:0(3-O-13:0)	13:0(3-OH)	13:0(3-OH)	12
Marinomonas vaga ATCC 27119^T		10:0[3-O-10:0(3-OH)]	10:0(3-OH)	10:0(3-O-10:0)	99
A. certesii KMM 3941^T	15:0(3-OH)[c]	15:0(3-O-15:0)[c]	15:0(3-OH)	15:0(3-OH)	-
C. scophtalmum CIP 104199^T			*iso*-15:0(3-OH)	*iso*-17:0(3-OH)	100

[a] DAG in *Arenibacter certesii* KMM 3941^T
[b] alternative substitution with 11:0(3-OH); 12:0(3-OH); *iso*-11:0(3-OH) or *iso*-12:0(3-OH).
[c] interchangeable
[d] alternative substitution at position C3

The lipid A portion of the LPS is the most conservative region of the molecule, and exhibits a low *intra*-genus variability. Therefore, only slight differences are encountered, among the genus, in the lipids A from the bacteria herein presented. For instance, three lipid As have been described from the LPSs of bacteria belonging to the genus *Pseudoalteromonas*, namely from *P. haloplanktis* ATCC 14393^T [97] and TAC 125 [98] and from *P. issachenkonii* KMM 3549^T [77].

A structure of the lipid A from *P. haloplanktis* ATCC 14393^T has been proposed on the basis of chemical analyses and NMR spectroscopy, pointing out to two possible penta-acyl distributions, with a variable localization of the secondary amide linked fatty acid, namely a dodecanoic acid moiety (12:0), on GlcN I or GlcN II. Moreover, primary fatty acid substitution at C-3 and C-3' is non-stoichiometrically given by (*R*)-3-hydroxy-dodecanoic acid [12:0(3-OH)], (*R*)-3-hydroxy-undecanoic acid [11:0(3-OH)], by their *iso*-branched isomers [*iso*-12:0(3-OH) and *iso*-11:0(3-OH), respectively], or (*R*)-3-hydroxy-decanoic acid [10:0(3-OH)] [97].

In the case of the strain TAC 125 [98], the structure depiction has been achieved through the use of Electrospray Ionization (ESI) tandem mass spectrometry (MS/MS). Also in this case, the most abundant species is the penta-acyl lipid A with 12:0(3-OH) linked both as ester and amide to 2',3' (distal glucosamine) and 2,3 positions (proximal glucosamine) of the sugar backbone. Secondary substitution was realized at the OH group of the 12:0(3-OH) at position 3' by a 12:0. In addition to the penta-acyl component, a minor tetra-acyl form, lacking the acyl residue at position 3, was also detected. The slight differences among the lipid A fractions from these two strains of the same species have been attributed to a difference in the culture condition employed, since it is known that growth conditions can modify the molecular architecture of lipid A.

The lipid A structure from *P. issachenkonii* KMM 3549^T has been described directly from the MS data obtained for the intact LOS sample [77]. As the opposite of *P. haloplanktis*, in *P. issachenkonii* the tetra-acyl lipid A is the most abundant species, carrying three C12:0(3-OH) and a C12:0 residues. Minor penta-acyl [bearing an additional C10:0(3-OH)] and tri-acyl [lacking a 12:0(3-OH)] forms were also present. The identical composition and acylation profile was found in the lipid A from the LOS of *P. carrageenovora* IAM 12662^T (unpublished data). Also in these two cases, the prevalence of the less acylated species compared to *P. haloplanktis* may be originated by the different environmental conditions from which this microrganism was first isolated.

Together with the structure elucidation of the core oligosaccharide, the molecular characterization of the lipids A from *Alteromonas macleodii* ATCC 27126^T [85] and *Alteromonas addita* KMM 3600^T [14] has been performed. MALDI MS analyses of the intact and NH_4OH-treated lipid A from *A. macleodii* ATCC 27126^T allowed the description of a blend in which the tetra-acyl species constituted the major form, together with minor amounts of tri- and penta-acyl species. In particular, the penta-acyl lipid A carried two C12:0(3-OH) residues in an ester linkage and two C12:0(3-OH) in amide linkages as primary fatty acids and one C12:0 residue as a secondary fatty acid at C2'. One or two ester-linked 12:0(3-OH) lack in the tetra- and tri-acyl forms [85].

Using the same approach, the lipid A from *A. addita* KMM 3600^T has been determined [14], and it presented a predominance of the penta-acyl species, with the same [3+2] distribution of the fatty acids on the glucosamine disaccharide. In this case, the major species exhibited two units of (*R*)-3-hydroxy-tetradecanoic acid [14:0(3-OH)] as amide substituents. This residue was eventually replaced, on the GlcN I, by a (*R*)-3-hydroxy-tridecanoic acid [13:0(3-OH)]. Secondary acylation at GlcN II was realized by a 14:0 or a 12:0 residue, whereas the ester linked fatty acid at C3 position was a 10:0(3-OH).

The acylation pattern for the Lipid A from *Shewanella pacifica* KMM 3772 has been completely described exploiting the MS data associated to the lipid A fraction and to either the intact and *O*-deacylated LOS [12]. In both cases, ions produced by the in source β-elimination of lipid A were in

fact visible. The main lipid A species from *S. pacifica* KMM 3772 is a hexa-acyl form in which both amide- and ester-linked primary fatty acids are (*R*)-3-hydroxy-tridecanoic acid [13:0(3-OH)]. The primary substituents at the GlcN II both bear secondary tridecanoic acid (13:0) residues. Such residues are missing in the minor penta- and tetra-acyl species.

The structure of the lipid A from the LPS of another γ-proteobacterium, *Marinomonas vaga* (formerly *Alteromonas vaga*) ATCC 27119^T, has been described [98]. This microrganism was isolated from the waters off the coast of the Hawaiian archipelago and has a psychrophilic and moderately halophilic character. Its lipid A shows stoichiometric lack of the phosphate ester group at C4'. Moreover, also in this case, the main form is represented by a penta-acyl species, with a [3+2] distribution of fatty acids, and acylation is principally performed by the short chained 10:0(3-OH). It has been reported for the first time in this occasion that the 3-hydroxy moiety is also present as the secondary substituent at the amide linked fatty acid of GlcN II [98].

The lipid A from *Arenibacter certesii* KMM 3941^T, from the *Cytophaga-Flavobacterium-Bacteroides* phylum, is characterised by the prevalence of a penta-acyl form (Silipo *et al*., unpublished results). As discussed above in the core oligosaccharide section, this glycolipid possesses a glycidic backbone constituted by a DAG-Glc*p*N β-(1→6) linked disaccharide. Phosphorylation occurs, also in this case, at the conventional C1 and C4' positions. Singularly, substitution is performed, in the most abundant penta-acyl species, only by fatty acids with an odd number of carbon atoms, namely four units of (*R*)-3-hydroxy-pentadecanoic acid [15:0(3-OH)], and one secondary pentadecanoic acid (15:0). From the MS analyses on the partially degraded lipid A, it has been possible to locate the 15:0 residue in ester linkage on one of the two primary amide substituents on the DAG unit, although it is not yet well understood whether it is the one at position C2' or C3'.

To the same phylum of *A. certesii* KMM 3941^T belongs the bacterium *Chryseobacterium scophtalmum* CIP 104199^T, whose LOS possessed an extremely unusual lipid A that was isolated and characterized [100]. The peculiarity of this molecule consists in the occurrence of a monosaccharidic lipid A moiety, in which a single glucosamine unit, phosphorylated at C1, carries one (*R*)-3-hydroxy-15-methylhexadecanoic [*iso*-17:0(3-OH)] and one (*R*)-3-hydroxy-13-methyltetradecanoic [*iso*-15:0(3-OH)] residues, as primary amide and ester substituents, respectively. This structure closely resembles the so-called lipid X, the biosynthetic precursor of lipid A in *E. coli*. This event probably relates with the absence, in *C. scophtalmum* membranes, of phosphatidylglycerol (PG), one of the three main phospholipids of Gram-negative bacteria. It is been in fact observed that the accumulation of lipid X in cells of mutant bacteria correlates with the deficiency of PG [101-103]. The absence of this phospholipid has been also detected in other bacteria of the same genus, i.e. *C. indoltheticum* and *C. defluvii* sp. [104]. Apparently, there is a certain relationship between lipid A synthesis and the presence of PG in bacterial cells: when PG is absent, lipid As with incomplete monosaccharide structure are synthesized. However, some substantial differences between the *E. coli* mutant strain and *C. scophtalmum* should be mentioned. Primarily, despite of the absence of PG and lipid A (in its classical structural variant), *C. scophtalmum* retains its viability, while the mutant *E. coli* cells grow only at nonpermissive temperatures (42°C) and, already after 3 h, a cessation of their growth is observed [101]. In addition, lipid A from *C. scophtalmum* can be isolated only after acidic hydrolysis of bacterial cells, indicating the existence of a strong, probably chemical, bond with other components of the outer membrane.

Besides the lipid A structures presented above, preliminary studies have been performed on the chemical characterization of the fatty acid compositions (but not their distribution) of the glycolipid moieties from other *Pseudoalteromonas* species [105]. In all the cases examined, the fatty acid composition resembles that from the microrganisms here described, with a strong prevalence of shorter chained fatty acids compared to the wide-spread enterobacteria.

6. Concluding remarks

Marine bacteria are habitual colonizers of all watery environments, and, either as free living colonies or symbiotic forms, they are present in sea habitats worldwide. It is widely accepted that the molecular architecture of the LPSs from Gram-negative marine bacteria strictly relates to the possibility of thriving in such environments. These molecules represent the outermost barrier of the microbial cell towards the surrounding environment, being therefore involved in the cellular resistance against the stressors typifying marine environments. Moreover, LPS endotoxins play a fundamental role in a number of essential processes, like the adhesion to the host tissues as well as to sediments, and the recognition either in the event of symbiotic interaction or animal and human pathogenicity. In this latter case, the most conservative portions of the LPSs structure act as PAMPs, being recognised by specialised receptors of the host immune system, thus eliciting the immune response. In the present paper, the structures of the LPSs from a number of marine Gram-negative bacteria is reviewed. This work provides an overlook on the growing body of information about oligo- and polysaccharides from marine Gram-negative bacteria, and also evaluates, for the first time, a collection of structures of lipids A. Many features encountered during our investigation can be ascribed to the environmental demand imposed by stress factors like elevated pressures, high salinity or low temperatures, to which they might represent an adaptive response. For instance, the majority of the O-specific polysaccharides structures here reported is endowed with anionic groups, variously supplied by acidic (often uncommon) monosaccharides, amino-acid or non-glycidic appendages. This is a general tendency observed in the smooth marine strains, and was already identified in the OPSs that were earlier characterised. A comparison between the structures presented suggests the idea that bacteria acquainted with marine environments provide the outer membrane with molecules with uncommon negative charge density, adapted during evolution in order to provide optimal protection of the cell. In the case of rough strains, producing only lipooligosaccharides (e.g. *A. addita* and *A. macleodii*), the enhancement of the cation binding capacity of the membrane may have been achieved through the constitutive shortening of the oligosaccharide chains and the addition of anionic decorations of both glycidic and non-glycidic nature (i.e. phosphate groups). Such structural features increase the capability of binding naturally available cations (Ca^{+2} and Mg^{+2}), through which the molecules overcome the electrostatic repulsion, otherwise arising among them. In this way, charged glycolipids can organize themselves in a rigid net of cross-linkages, providing higher resistance to physical stressors. This effect is in our opinion closely related to barophily, thus to mechanical pressures acting on the cell. Halophily may reflect in the enhancement of water retention in the immediate surrounding of the cell, as a reaction to the osmotic pressure generating between the cytoplasm and the external sea environment. The salt content in the different cell compartments may be modulated through the creation of a microenvironment around the cell with an increased water attraction capability due to

charged groups, confirming the trend already outlined for glycolipids within the cytoplasmic membrane [106].

The present work also reports for the first time a collection of lipid A structures from marine bacteria. These are the first few examples for which the complete acylation pattern has been described, and some interesting conclusions can be made. For instance, a preference for shorter acyl chains [10:0(3-OH); 12:0; 12:0(3-OH); 13:0(3-OH)] with respect to the lipids A from *Enterobacteriaceae* is evident. The shortening of lipid A fatty acids has been observed in mesophilic bacteria as a short term response to cold stress [20,107] and is in agreement with the fact that marine bacteria presented in this paper express a certain psychrophilic character or at least do not live at the "canonical" 37° C. Evolutionary adaptation in obliged psycrophiles may have led to similar stratagems in membrane glycolipids, bringing to the preference for structures more suitable to counterbalance the freezing effects of cold on long chain fatty acids. Shorter lipids have less Van der Waals interactions between the chains, preserving the physiological fluidity of the Outer Membrane. Moreover, the majority of the lipids A characterized from marine bacteria produces mainly penta- and tetra-acyl species, i.e. species with less fatty acids than endotoxically active molecules. Some species, like *M. vaga*, also possess a single phosphate group on the disaccharide backbone, another feature that is related to a lowering in toxicity. These two features make marine lipid As potential endotoxin antagonists candidates [108-110]. The rather low innate immune system elicitation showed by *Marinomonas vaga* [99], *Alteromonas macleodii* and *A. addita* (unpublished results) lipids A fits well with the above suggestion. Further investigations are needed to confirm this theory.

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