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Lipopolysaccharides of anaerobic beer spoilage bacteria of the genus *Pectinatus* – lipopolysaccharides of a Gram-positive genus

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Abstract

Bacteria of the genus *Pectinatus* emerged during the seventies as contaminants and spoilage organisms in packaged beer. This genus comprises two species, *Pectinatus cerevisiiphilus* and *Pectinatus frisingensis*; both are strict anaerobes. On the basis of genomic properties the genus is placed among low GC Gram-positive bacteria (phylum *Firmicutes*, class Clostridia, order *Clostridiales*, family *Acidaminococcaceae*). Despite this assignment, *Pectinatus* bacteria possess an outer membrane and lipopolysaccharide (LPS) typical of Gram-negative bacteria. The present review compiles the structural and compositional studies performed on *Pectinatus* LPS. These lipopolysaccharides exhibit extensive heterogeneity, i.e. several macromolecularly and structurally distinct LPS molecules are produced by each strain. Whereas heterogeneity is a common property in lipopolysaccharides, *Pectinatus* LPS have been shown to contain exceptional carbohydrate structures, consisting of a fairly conserved core region that carries a large non-repetitive saccharide that probably replaces the O-specific chain. Such structures represent a novel architectural principle of the LPS molecule.

Keywords: *Pectinatus*; Lipopolysaccharide; Gram-positive; Beer spoilage

Contents

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1. Introduction

Pectinatus strains were initially isolated from spoiled beer in the late seventies and assigned to a new genus and species *Pectinatus cerevisiiphilus* [1]. Later a new species *Pectinatus frisingensis* has been established among the initial isolates [2]; the type strain of *P. frisingensis* is a Finnish isolate first identified as *P. cerevisiiphilus* in 1981 [3]. Based on a recent characterization of a total of 32 *Pectinatus* strains by ribotyping, it was suggested that certain strains that by conventional tests are identified as *P. cerevisiiphilus*, most probably belong to a new species [4]. The emergence of *Pectinatus* almost simultaneously in breweries from several countries including Germany, Finland, United States and Japan has been linked to improvements in filling techniques rendering less oxygen in the beer bottles, and also to the trend towards lighter beers that provide a more favourable growth environment [5]. Minimal access to oxygen is a brewing technology advance intended to improve the important chemical stability of beer. As a consequence, these strict anaerobes are mainly encountered as contaminants in large, modern breweries equipped with effective filling technology.

Most of the described *Pectinatus* strains have been isolated from spoiled beer. Attempts to detect *Pectinatus* have revealed its presence in lubrication oil mixed with beer, in drainage and water pipe systems, in filling hall air, in the filling machine, on the floor of the filling hall, in condensed water on the ceiling, in chain lubricants, and in steeping water of malt before milling (for review, see [6]). It is thus considered obvious that *Pectinatus*, despite its anaerobic nature, can survive in aerosols and be transferred via the air into beer, as reviewed in [7]. According to Hakalehto [8], who showed that *Pectinatus* is generally present in different parts of the brewery, the contaminating strains were permanent inhabitants rather than occasional invaders of the brewery, and find niches in the factory where they can survive. It is therefore perhaps not surprising that no clear-cut source of contamination outside the brewery has been demonstrated.

2. Phylogeny of *Pectinatus*

A number of studies over the last decade utilizing 16S rRNA, 23S rRNA and the ATPase β-subunit gene sequences, the 16S–23S rDNA intergenic spacer regions as well as DNA–DNA hybridization as markers have established the phylogenetic position of *Pectinatus* [2,9–14]. *Pectinatus* species, with *Selenomonas*, *Megasphaera*, *Sporomusa*, *Veillonella* and *Zymophilus*, have been assigned to the *Sporomusa* sub-branch of the *Clostridium subphylum* of Gram-positive bacteria [2,12]. The more recent assignment places all members of the *Sporomusa* sub-branch in the family "*Acidaminococcaceae*" of the class "Clostridia" of the phylum *Firmicutes* [15]. According to the 16S rRNA sequences, the similarity between *P. frisingensis* and *P. cerevisiiphilus* was 95%, the nearest other species (similarities with *Pectinatus* spp.) being three *Selenomonas* species (88–89%), *Clostridium quercicolum* (87–88%), *Sporomusa paucivorans* (86–88%), *Veillonella parvula* (86–87%) and *Megasphaera elsdenii* (86%; [12]). From 16S rRNA analysis, it is quite clear that the genus *Pectinatus* does not belong to the family Bacteroidaceae, as originally suggested [1], because the genus *Bacteroides* and related genera represent a distinct phylum within the phylogenetic tree of bacteria [2,12]. A comprehensive review covering the phylogeny, taxonomy, habitat, isolation, identification, and physiology of the genus *Pectinatus* is available [7].

Despite the assignment to the order *Clostridiales* of Gram-positive bacteria, cells of *Pectinatus* spp. stain Gram-negative, and possess an outer membrane [1–3,16]. Our early studies also confirmed that they contain lipopolysaccharide (LPS) typical of Gram-negative bacteria [17], the LPS preparations exhibiting normal endotoxic potency in classical biological activity assays [18]. Electron microscopic examinations have revealed that the outer membrane of *Pectinatus* spp., however, exhibits an unusual wrinkled appearance with numerous bulges [1,3,16]. Architecture of the cell envelope in *Pectinatus* is in part reminiscent of Gram-positive bacteria, as indicated by the thick peptidoglycan layer and by invaginations of the cytoplasmic membrane [16]. These features are evident in Fig. 1, showing a cell of *Pectinatus frisingensis* in thin section [16]. In the micrograph the presence of "mesosomes" is indicated; it should be noted in this context that such cytoplasmic membrane-derived internal membranous structures are considered to be artefacts resulting from preparation techniques for electron microscopy [19]. It is, however, of significance that mesosome formation is typical for Gram-positive bacteria [20].

3. Architecture of bacterial cell walls – importance of lipopolysaccharide

As noted above, the cell envelope of *Pectinatus* exhibits characteristics of both Gram-negative and Gram-

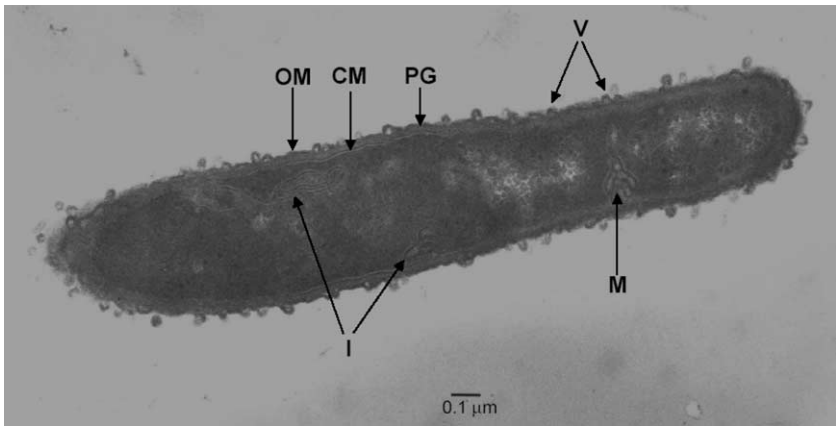


Fig. 1. Electron micrograph of thin-sectioned of *Pectinatus frisingensis*. The outer membrane (OM), the cytoplasmic membrane (CM), the thick peptidoglycan layer (PC) constitute the cell envelope around the cytoplasm (C). Numerous vesicles (V) are seen on the OM surface. Inside the cytoplasm invaginations (I) of the CM and the mesosomes (M), most likely artefacts of preparation for electron microscopy (see text) are seen. Bar = 0.1 μm.

positive bacteria. The presence of LPS is normally considered as a hallmark of Gram-negativity, as LPS is a unique functional constituent of the OM. Accordingly, Gram-positive organisms should be devoid of LPS. Such a dilemma – LPS of a bacterium assigned to the class of Gram-positive organisms – has prompted the studies underlying this review. After our initial studies aiming at clarification of taxonomic position had revealed the presence of typical LPS molecules in *Pectinatus* strains [17], further research was performed to elucidate the composition and structure of this important cell surface component. LPS molecules also play a decisive role in the permeability barrier function of the OM against external molecules, and their structure could provide clues to the ability of *Pectinatus* bacteria to persist in breweries and to grow in beer.

As implied by their name, LPS molecules consist of lipid and polysaccharide in covalent linkage. While this very general definition holds true for all LPS studied so far, an enormous diversity of chemical structures is encountered among LPS of different bacterial genera, species, and strains. The lipid part of LPS is termed lipid A and it functions as the lipid anchor in the OM. Lipid A is linked to a heteropolysaccharide chain (the core oligosaccharide) by a special sugar, 3-deoxy-D-*manno*-oct-2-ulosonic acid (Kdo). The core oligosaccharide can usually be divided into functionally and structurally distinct ''inner'' (lipid A-proximal) and ''outer'' core [21]. In many bacteria the core oligosaccharide carries an O-specific chain built of repeating oligosaccharide units in a polymeric structure with a specific set of chain lengths. LPS molecules carrying O-specific chains are often called smooth-type LPS or S-LPS (S, smooth, derives from colony morphology of the respective bacteria), whereas those lacking these chains either inherently or as a result of mutation are called rough-type or R-LPS. As a result of different degrees of polymerization of the O-specific chains, smooth-type lipopolysaccharides typically contain a family of molecules of different O-chain lengths as readily revealed by analysis in sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS–PAGE; [22]).

The structural diversity of LPS among distinct bacterial groups is smallest in the lipid A part and largest in the O-specific chains. A huge number of structural reports exist to date on lipopolysaccharides of different bacterial species; structures are covered in recent reviews by Zähringer et al. [23,24] on lipid A, Holst, [21,25] on the core oligosaccharide and Knirel [26] and Jansson [27] on the O-specific polysaccharides. Although O-specific chains are common in many pathogenic bacteria, helping their survival against the host's defense mechanisms, they are generally not required for growth in the laboratory, and in fact several species of wild-type pathogenic Gram-negative bacteria are known to inherently lack this distal part of LPS [28].

4. Lipopolysaccharides of *Pectinatus* species

4.1. General

Detailed structural studies of *Pectinatus* LPS have been carried out with the type strains *P. cerevisiiphilus* ATCC 29359^T (VTT E-79103) and *P. frisingensis* ATCC 33332^T (VTT E-79100) and with *P. frisingensis* strains VTT E-82164 and VTT E-79104. Whereas the two type strains elaborate mainly, but not exclusively, S-type LPS with polymeric O-specific chains, strains VTT E-82164 and VTT E-79104 represent a different type in that their LPS consists of R-type material only, yielding two prominently separate bands in polyacrylamide gel electrophoresis (PAGE). Silver-stained patterns of the LPS of the above-mentioned strains in sodium deoxycholate

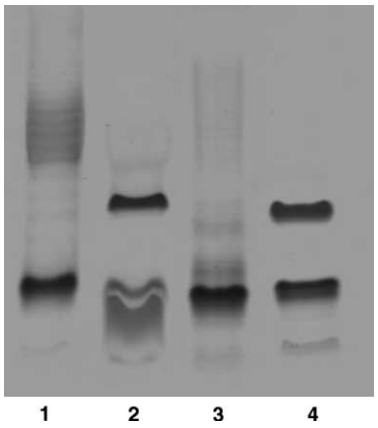


Fig. 2. Deoxycholate-PAGE profiles of *Pectinatus* LPS. Lane 1, *P. frisingensis* type strain VVT E-79100^T; lane 2, *P. frisingensis* VTT E-79104; lane 3, *P. cerevisiiphilus* type strain VTT E-79103^T; lane 4, *P. frisingensis* VTT E-82164.

PAGE (DOC-PAGE) are shown in Fig. 2. Such dual nature of LPS, most obvious in VTT E-82164 and VTT E-79104, is shared by all *Pectinatus* LPS studied so far, including those of the type strains. By applying a specific extraction method (phenol/chloroform/petroleum ether (PCP) method, modification PCP 5:5:8, [29]) the LPS of both type strains could be separated into S-type and R-type LPS in major and minor quantities, respectively [30]. These fractions differed drastically in their hydrophilicity, as indicated by their different precipitability from phenol. There was also evidence of differences in the respective chemical compositions of the R-type and S-type LPS, indicating that the resolution into two LPS types represented a phenomenon distinct from the previously known forms of LPS heterogeneity (for reviews of LPS heterogeneity see [22,31]). It is emphasized in this context that the LPS preparations of *P. cerevisiiphilus* ATCC 29359^T (VTT E-79103) and *P. frisingensis* ATCC 33332^T (VTT E-79100) in Fig. 2 were selectively prepared (acetone-precipitated PCP extracts, for details see [30]) fractions exhibiting no heterogeneity with respect to the core; instead the normal chain length heterogeneity typical of S-type LPS is evident. However, as discussed in detail in [30], the presence of two macromolecularly and compositionally different LPS types in one bacterial strain is an exceptional property. A similar situation probably occurs in *Rhizobium loti*, which displays an analogous case: R-form LPS is harvested after phenol–water extraction from the aqueous phase, whereas S-form LPS with its highly hydrophobic O chain (homopolymer of 6-deoxy-L-talose) is harvested from the phenol phase [32].

Besides demonstrating the presence of two distinct LPS types in one strain, the initial characterization of the LPS of *Pectinatus* type strains [30] revealed several remarkable properties such as the predominance of odd-numbered fatty acids in lipid A, lack of heptose (a common constituent of the core region), and a high content of 4-amino-4-deoxy-L-arabinopyranose (L-Ara*p*4N). These will be dealt with below in connection with the respective structures.

4.2. Lipid A

The structure of the lipid A component of *P. cerevisiiphilus* and *P. frisingensis* type strains has been resolved and shown to be virtually identical in these species [33]. The lipid A contains the common backbone of a bisphosphorylated β1–6 linked GlcN disaccharide, the phosphate groups residing at positions 1 (glycosidically linked) and 4′ (ester-linked) of the backbone. Both phosphates are substituted by L-Ara*p*4N; in the ester-linked phosphate this substitution is stoichiometric, whereas in the glycosidic phosphate it is minor with an estimated degree of 5% (Fig. 3). The substitution at the

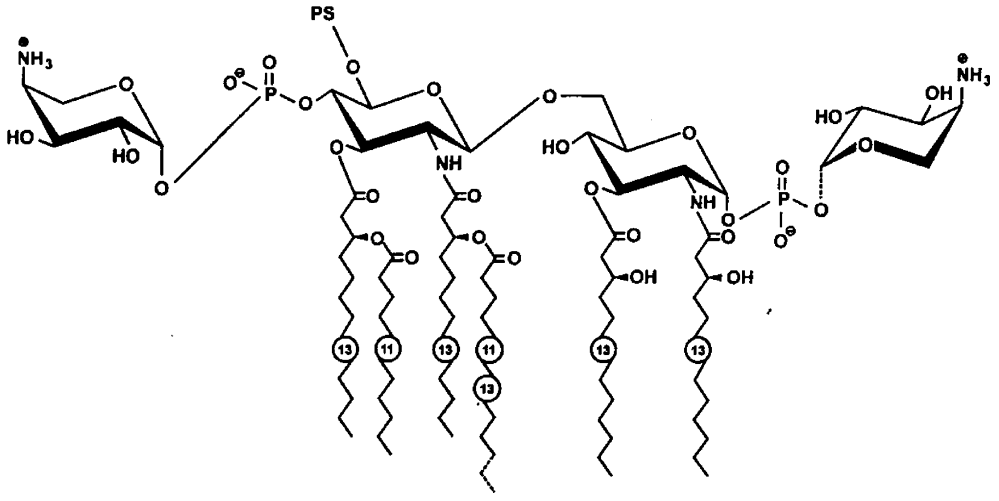


Fig. 3. Structure of the lipid A component of S-form LPS of *P. cerevisiiphilus* and *P. frisingensis* [6]. Numbers in circles refer to the number of carbon atoms in acyl chains. The glycosidic phosphate is assumed to be as the α anomer, and carries L-Ara*p*4N in ≈5% of the molecules.

glycosidic phosphate became evident after the recognition of a similar, albeit more prominent substitution in the lipid A of polymyxin-resistant *Klebsiella pneumoniae* O3 [34]. This minor substitution is represented by the small signal at −2 ppm in the ^{31}P NMR spectrum of de-*O*-acylated LPS of *P. cerevisiiphilus* as recorded at pH 9.7 (Fig. 4). This spectrum contains several signals representing monophosphate monoesters (chemical shift dependent on pH) and monophosphate diesters (small dependence of chemical shift on pH); diphosphates are not present. Based on published literature of ^{31}P NMR signals in LPS [35–37], the signal at 3 ppm was assigned to the glycosidic monoester, and the diester signal at −1.38 ppm (b) was assigned to the ester-linked phosphate of lipid A at position 4′, carrying a stoichiometric substitution by L-Ara*p*4N. The latter was evident, as no significant signal was observed corresponding to a 4′-linked monoester. Furthermore, it can be deduced from this spectrum that in addition to lipid A-associated phosphates, *Pectinatus* LPS contain at least three phosphodiester groups. These are likely to reside in the core oligosaccharide, but hitherto only one has been assigned ([38,39]; see Chapter 4.3).

The nature and location of fatty acids in *Pectinatus* lipid A have been determined by chemical methods and laser desorption mass spectrometry [33]. The major form of *Pectinatus* lipid A contains four residues of the fatty acid (*R*)-13:0(3-OH), two of which are present in ester and two in amide linkage (Fig. 3). One ester-linked and one amide-linked (*R*)-13:0(3-OH) are substituted by 11:0 at the 3-OH group (i.e. forming acyloxyacyl groups). Besides such major form, heterogeneity as regards fatty acid chain lengths, occurs in *Pectinatus* lipid A; less abundant (≈10%) fatty acids replacing those of the major form are represented by (*R*)-11:0(3-OH) and 13:0. Furthermore, in analyses for the cellular fatty acid composition of *P. cerevisiiphilus* and *P. frisingensis* type strains additional 3-hydroxy fatty acids have been found, likely to be minor components of lipid A [40]. In total, six different 3-hydroxy acids, including 13:1(3-OH), which has not been reported in other bacteria or in lipid A [23,24,41] were found in the above analysis of cellular fatty acids.

Apart from its uncommon odd-numbered fatty acids, the lipid A component in the genus *Pectinatus* is thus representative of the asymmetrically hexaacylated bisphosphorylated GlcN disaccharide type commonly encountered in many bacteria (reviewed by Zähringer et al. in [23,24]). The substitution of both phosphates by L-Ara*p*4N has hitherto, however, only been reported in *K. pneumoniae* [34], in cystic fibrosis-associated strains of *Pseudomonas aeruginosa* [42], and in *Proteus mirabilis* [43]. This type of lipid A conforms to the structure required for endotoxic lipid A [28], and *Pectinatus* LPS were indeed shown to possess full endotoxic potency [18].

A prominent substitution of lipid A by L-Ara*p*4N usually renders bacteria resistant to polymyxin B, as discussed by Vaara [44,45]. Surprisingly, *P. cerevisiiphilus* appear to be fully susceptible to polymyxin [33]. It is, however, susceptible also to vancomycin and bacitracin, which are large molecules (1449 and 1411 g mol^{-1}, respectively) that normally are not able to penetrate the OM in Gram-negative bacteria [46]. The conclusion from these data is that the OM of *Pectinatus*, in the conditions utilized, does not act as an effective permeability barrier. Interestingly, Chihib et al. [47] showed that *P. frisingensis* is sensitive to nisin, a large antibacterial peptide (3353 g mol^{-1}) that normally does not affect Gram-negative bacteria [48]; this result is consistent with the above findings of inefficient barrier function of *Pectinatus* OM.

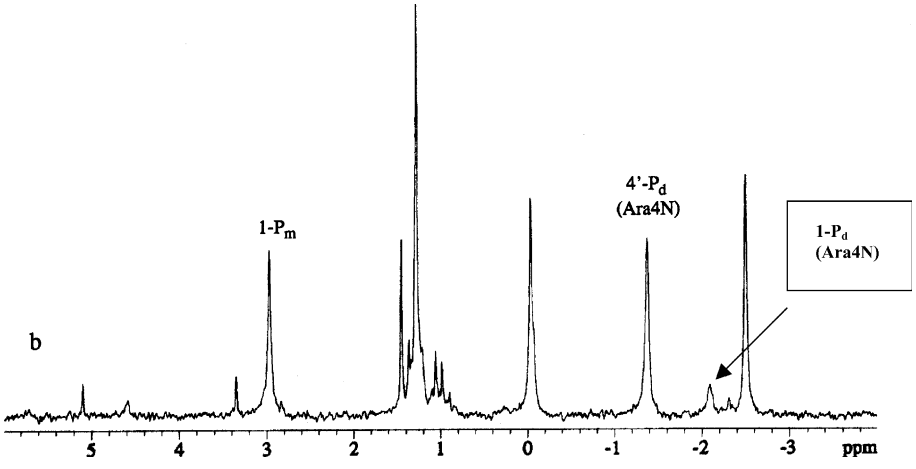


Fig. 4. ^{31}P NMR spectrum of de-O-acylated lipopolysaccharide of *P. cerevisiiphilus* recorded at pH 9.7 [33]. 1-P$_m$, phosphate monoester at the glycosidic position of the lipid A backbone; 1-P$_d$ (Ara4N) at the glycosidic position of the lipid A backbone, 4′-P$_d$ (Ara4N), phosphate diester (Ar4*p*N phosphate) at position 4′ of the lipid A backbone (cf. Fig. 3).

The cellular fatty acid composition of the genus *Pectinatus* resembles closely those of anaerobic cocci of the genus *Veillonella* [49]. Furthermore, similarities are evident with the fatty acid composition of the lipid A of *Selenomonas sputigena* [50]. Both *Selenomonas* and *Veillonella* are assigned to the same family as *Pectinatus* [2,15]; similarities in their fatty acid composition are in accordance with this assignment. The cellular fatty acid composition of *Pectinatus* bears, however, no similarity to those of *Megasphaera cerevisiae* and *M. elsdenii* (members of the family *Acidaminococcaceae*; [15]), the former also being a beer spoilage bacterium [7,40].

4.3. Core oligosaccharide and distal oligosaccharide

Structural analysis of LPS core oligosaccharides in *Pectinatus* was initially hampered by the multiple heterogeneity of the LPS preparations obtained from the type strains, as noted above. The interesting LPS of *P. frisingensis* VTT E-82164 with its two separate bands in PAGE (Fig. 2) was, however, better amenable to structural analysis, and the structures of the LPS carbohydrate backbone of this species has been thoroughly elucidated [39]. In agreement with its electrophoretic pattern, this LPS was shown to contain two major structures differing by a decasaccharide fragment. The larger major structure of the carbohydrate backbone consists of 24 monosaccharides (including the lipid A backbone), whereas the shorter is composed of 14 saccharide residues; in addition, minor components lacking a terminal glucose in the smaller (lipid A-proximal) fraction were identified. The shorter variant obviously represents the core-lipid A entity of LPS, and the additional (distal) part can be seen as a replacement for the O-specific polysaccharide. Furthermore, the carbohydrate structure of the LPS of another non-smooth strain *P. frisingensis* VTT E-79104, exhibiting predominance of the upper band in PAGE (see Fig. 2), has been recently elucidated [51]. An analogously dual architecture of LPS is present in *P. frisingensis* VTT E-79104; the largest carbohydrate structure is somewhat smaller than that of *P. frisingensis* VTT E-82164, i.e. comprising 20 monosaccharides. Strain VTT E-79104 also contains a heptose residue (in the D-*glycero*-D-*galacto* configuration) in the outer region of the heteropolysaccharide. These structures are compiled in Fig. 5. The structural analyses revealed that the position of the disaccharide component consisting of GlcN and Kdo, previously reported for LPS of *P. cerevisiiphilus* and *P. frisingensis* type strains [38] is in the inner part of the core oligosaccharide, and confirmed the presence of the putative phosphate-linked Ara4N at position 6 of the GlcN residue.

These results also provided a clue to the core structures of type strains elaborating smooth-type LPS: As depicted in Fig. 5, the *P. frisingensis* type

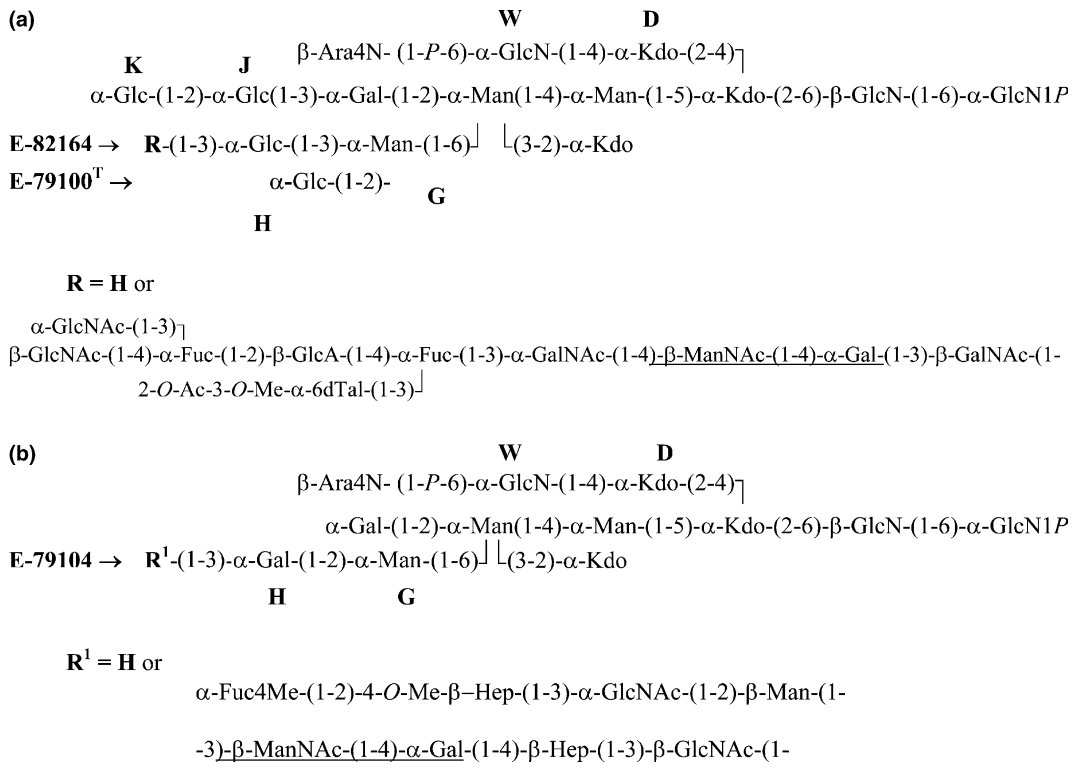


Fig. 5. Proposed structures of the carbohydrate backbones of *P. frisingensis* LPS. (a) Strains VTT E-82164 and VTT E-79100^T; (b) strain VTT E-79104. α-Glc residue K is present partially in (a). The structure of the disaccharide fragment W–D was previously reported [38]. Conserved monosaccharide residues in the distal oligosaccharide region are underlined. Hep, D-*glycero*-D-*galacto*-heptose.

strain was shown to contain a core oligosaccharide that was highly similar to the short variant of *P. frisingensis* VTT E-82164, differing only in one glycosidic linkage, the linkage between Glc and Man (residues H and G in Fig. 5, respectively) being 1–2 in the type strain.

The inner core contains the disaccharide α-Kdo-(2-4)-α-Kdo-linked to position 6′ of the lipid A backbone. Whereas this structure is ubiquitous in LPS of different bacterial groups, the presence of mannose units (instead of the common heptose) in the inner part of the core oligosaccharide is less common, having been reported, e.g. in *Legionella pneumophila* and in different species of *Rhizobium* [21]. The outer part of the saccharide moiety is rich in amino sugars, including three different aminohexoses GalN, GlcN and ManN, all in *N*-acetylated form. An interesting feature is the presence in the distal oligosaccharide of the structure consisting of β-D-GlcA, α-L-Fuc, and 3-O-Me-α-6dTal (see Fig. 5), which is also found in *Rhizobium etli* LPS [52].

Despite being compositionally, and most likely also biosynthetically, clearly different from the core oligosaccharide, the distal carbohydrate portions of *P. frisingensis* VTT E-82164 and VTT E-79104 LPS exhibit no repetitious structures comparable to those encountered in O-specific chains. To date, structures of this dual core type have not been previously described, and such architecture of the LPS molecule represents a most unique feature of *Pectinatus* LPS. The distal oligosaccharide presumably replaces the O chains present in the type strains (see below). The dual oligosaccharide (core + distal oligosaccharide) architecture actually seems to be a common feature among *Pectinatus* isolates, as preliminary screening by SDS- and DOC-PAGE has indicated that most strains are of this type, lacking polymeric O chains (I.M. Helander, unpublished data).

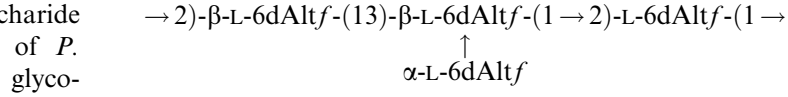
4.4. The O-specific chains

LPS of *P. cerevisiiphilus* and *P. frisingensis* type strains exhibit in SDS–PAGE ladder-like banding patterns indicative of the presence of O chains. The chain length distributions appear characteristically limited, yielding a series of fairly slow-migrating bands with no intermediary material (Fig. 2).

The structures of the repeating units of these strains have been analyzed [53]. In *P. cerevisiiphilus* ATCC 29359^T the structure of the repeating unit is the disaccharide

→ 2)-β-D-Fuc*f*-(1 → 2)-α-D-Glc*p*-(1 →

Structure of the O-specific repeating unit of *P. frisingensis* ATCC 33332^T was shown [53] to consist of a single monosaccharide unit, the rare 6-deoxy-L-altrofuranose in a branched tetrasaccharide structure



Of the two identified 6-deoxyhexoses, 6-deoxy-L-altrose is a component that rarely occurs in Nature. It is found in pyranose form in the O chains of some serovars of *Yersinia enterocolitica*, forming homopolymers [54]. In serovar O:5b of *Y. pseudotuberculosis* 6-deoxy-L-altrose occurs as a constituent in furanose form [55], but homopolymeric O chains of 6-deoxy-L-altrofuranose have not been reported elsewhere. The D-fucose of *P. cerevisiiphilus* also is rather uncommon, having mainly been found in O chains of plant-associated bacterial species. In general, it can be extracted from the reviews of Jansson [26] and Knirel [27] that O chains with a high degree of hydrophobicity are common in plant-associated bacteria.

The established structures of the O chains of S-form LPS of *P. cerevisiiphilus* and *P. frisingensis* provide an explanation to the finding that S-form lipopolysaccharides (LPS-Ac, [30]) were extractable by the PCP 5:5:8 method and precipitable from the processed phenol solution only by acetone. Such behavior can be explained by the high degree of hydrophobicity of the O chains due to their high content of 6-deoxysugar. Furthermore, it is interesting that due to the nature of the constituent saccharides the O chains of both *Pectinatus* species are relatively labile to acid, whereas the lipid A-polysaccharide linkage instead appears resistant to acid hydrolysis [33], i.e. properties that are usually the opposite in LPS molecules.

5. General discussion and future prospects

A characteristic feature of *Pectinatus* LPS is their heterogenous nature with respect to carbohydrate structures, as detailed above. Most notably, the ''dual oligosaccharide'' type of LPS architecture has not been encountered among other bacterial genera, making it of interest to see whether such an arrangement of the LPS saccharide backbone is shared by other genera assigned to the *Acidaminococcaceae* family of the order *Clostridiales* of Gram-positive bacteria. These genera, i.e. *Selenomonas*, *Megasphaera*, *Sporomusa*, *Veillonella* and *Zymophilus*, have only sporadically been subjected to analyses of LPS composition/structure, yielding no data of the core structures. However, the cellular fatty acid composition of the genus *Pectinatus* resembles closely that of anaerobic cocci of the genus *Veillonella* [49]. Furthermore, similarities are evident with the fatty acid composition of the lipid A of *Selenomonas sputigena* [50]. Future research of the LPS and other OM components would reveal whether a previously unidentified cell envelope architecture is indeed present in these bacteria that represent intermediary organisms between

Gram-positive and Gram-negative types. Considering the novelty of the architecture of the distinct heteropolysaccharide regions of *P. frisingensis* VTT E-79104 and VTT E-82164, the mode of biosynthesis leading to the formation of the distal oligosaccharide and the genes responsible for the generation of this structure would be major aspects to be elucidated. Such biosynthesis studies should constitute a main line of further research of *Pectinatus* and other genera with potentially similar architecture of the cell envelope.

Although the two type strains of *P. cerevisiiphilus* and *P. frisingensis* elaborate polymeric O-specific chains, other strains and most likely the majority of *Pectinatus* isolates, possess the non-repetivive LPS as found in *P. frisingensis* VTT E-79104 and VTT E-82164. This indicates that O-specific chains are not essential to the survival of these bacteria in beer and in breweries, but the bacteria can instead survive and proliferate with the truncated LPS forms, having the distal oligosaccharide part in place of the O-specific chain. Survival and growth of contaminating bacteria at different stages of the brewing process are dependent on specific properties of the beer environment. These include low temperature, relatively low pH, presence of bacteriostatic hop bitter substances, low supply of nutrients, low oxygen availability, high carbon dioxide and alcohol concentration [56]. The finding that the OM of *Pectinatus* does not function as an efficient permeability barrier against external substances [33], together with the imperfect morphological appearance of the OM [16]; Fig. 1 shows that it is certainly not a protective OM that makes *Pectinatus* tolerate and favor the basically hostile beer environment. The leaky character of *Pectinatus* OM may result from the LPS structure, as the concentration of free amino groups (GlcN, L-Ara*p*4N) in the inner core could contribute to this effect by reducing the sites available for stabilizing divalent cations (for review, see [57]). The significance of two distinct LPS types for OM stability clearly warrants further investigation. The possibility that conditions in which permeability assays have been performed may, however, be highly unphysiological for *Pectinatus*. The permeability properties of *Pectinatus* OM in conditions of the real habitat of these bacteria remain thus largely unknown.

The original source of *Pectinatus*, i.e. its habitat outside the brewery, remains equally unknown. Some clues can, however, be drawn to plant-associated bacteria. Accordingly, in various aspects of the LPS, similarities emerge to *Rhizobium* (discrete extractability of S vs. R-LPS in *R. loti* [32], presence in *Pectinatus* VTT E-82164 distal oligosaccharide of a part structure in common with *R. etli* [52]). The tendency that O-chains rich in deoxysugars are often found in plant-associated bacteria might indicate *Pectinatus* as a saprophytic or pathogenic bacterium of plants, being carried to breweries with any of the various plant materials utilized, such as cereals, rice, and hops.

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