O-antigen structural variation: mechanisms and possible roles in animal/plant–microbe interactions

Inge Lerouge, Jos Vanderleyden *

Centre of Microbial and Plant Genetics, Katholieke Universiteit Leuven, Kasteelpark Arenberg 20, B-3001 Heverlee, Belgium

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Abstract

Current data from bacterial pathogens of animals and from bacterial symbionts of plants support some of the more general proposed functions for lipopolysaccharides (LPS) and underline the importance of LPS structural versatility and adaptability. Most of the structural heterogeneity of LPS molecules is found in the O-antigen polysaccharide. In this review, the role and mechanisms of this striking flexibility in molecular structure of the O-antigen in bacterial pathogens and symbionts are illustrated by some recent findings. The variation in O-antigen that gives rise to an enormous structural diversity of O-antigens lies in the sugar composition and the linkages between monosaccharides. The chemical composition and structure of the O-antigen is strain-specific (interstrain LPS heterogeneity) but can also vary within one bacterial strain (intrastrain LPS heterogeneity). Both LPS heterogeneities can be achieved through variations at different levels. First of all, O-polysaccharides can be modified non-stoichiometrically with sugar moieties, such as glucosyl and fucosyl residues. The addition of non-carbohydrate substituents, i.e. acetyl or methyl groups, to the O-antigen can also occur with regularity, but in most cases these modifications are again non-stoichiometric. Understanding LPS structural variation in bacterial pathogens is important because several studies have indicated that the composition or size of the O-antigen might be a reliable indicator of virulence potential and that these important features often differ within the same bacterial strain. In general, O-antigen modifications seem to play an important role at several (at least two) stages of the infection process, including the colonization (adherence) step and the ability to bypass or overcome host defense mechanisms. There are many reports of modifications of O-antigen in bacterial pathogens, resulting either from altered gene expression, from lysogenic conversion or from lateral gene transfer followed by recombination. In most cases, the mechanisms underlying these changes have not been resolved. However, in recent studies some progress in understanding has been made. Changes in O-antigen structure mediated by lateral gene transfer, O-antigen conversion and phase variation, including fucosylation, glucosylation, acetylation and changes in O-antigen size, will be discussed. In addition to the observed LPS heterogeneity in bacterial pathogens, the structure of LPS is also altered in bacterial symbionts in response to signals from the plant during symbiosis. It appears to be part of a molecular communication between bacterium and host plant. Experiments ex planta suggest that the bacterium in the rhizosphere prepares its LPS for its roles in symbiosis by refining the LPS structure in response to seed and root compounds and the lower pH at the root surface. Moreover, modifications in LPS induced by conditions associated with infection are another indication that specific structures are important. Also during the differentiation from bacterium to bacteroid, the LPS of Rhizobium undergoes changes in the composition of the O-antigen, presumably in response to the change of environment. Recent findings suggest that, during symbiotic bacteroid development, reduced oxygen tension induces structural modifications in LPS that cause a switch from predominantly hydrophilic to predominantly hydrophobic molecular forms. However, the genetic mechanisms by which the LPS epitope changes are regulated remain unclear. Finally, the possible roles of O-antigen variations in symbiosis will be discussed. © 2001 Federation of European Microbiological Societies. Published by Elsevier Science B.V. All rights reserved.

Keywords: Lipopolysaccharide; Bacterial cell envelope; Infection; Pathogenicity; Symbiosis

* Corresponding author. Tel.: +32 (16) 321631; Fax: +32 (16) 321966. E-mail address: jozef.vanderleyden@agr.kuleuven.ac.be (J. Vanderleyden).

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I. Introduction on lipopolysaccharides

The bacterial surface is the first line of defense against antimicrobial molecules and stress caused by changes in the environment surrounding the bacterium. In the case of animal- and plant-microbe interactions, many bacterial cell surface molecules are virulence determinants. Thus, in order to understand the molecular basis for bacteria-host interactions, it is important to characterize the molecular architecture of the bacterial cell surface, and to understand how the bacterium modifies this architecture in response to its different environments, including its environment in the host plant or animal. These environments are mostly heterogeneous and can be very complex. For instance, due to soil types and other non-biotic factors (e.g. water supply and temperature) considerable variation in microenvironments may lead to the need for surface molecules adapted to these conditions.

Among the surface molecules undergoing structural changes during symbiotic as well as pathogenic interactions are lipopolysaccharides (LPS). LPS constitute the major structural component of the outer membrane of Gram-negative bacteria. It is the exposed part of the LPS, also referred to as O-antigen, that contributes to the variation of the Gram-negative bacterial cell wall. LPS are a class of glycoconjugates unique to Gram-negative bacteria and are present in the outer monolayer of the outer membrane along with phospholipids (innerleaflet) and proteins (Fig. 1). The LPS fraction comprises about 10–15% of the total molecules in the outer membrane and is estimated to occupy about 75% of bacterial surface area. LPS provide a permeability barrier to large, negatively charged and/or hydrophobic molecules and contribute to the structural properties of the cell envelope. LPS are complex amphiphilic molecules with a molecular mass of about 10 kDa, which vary widely in chemical composition both between and within bacterial species.

1. Introduction on lipopolysaccharides

1.1. Structure

LPS consist of three different components or regions: lipid A, core oligosaccharide and O-poly saccharide. Lipid A is the lipid component of LPS that contains the hydrophobic, membrane-anchoring region of LPS and is the bioactive component responsible for some of the pathophysiology associated with severe Gram-negative infections in animals and humans. The structure of lipid A is highly conserved among Gram-negative bacteria. Among Enterobacteriaceae lipid A is virtually constant. Escherichia coli lipid A, which shares most features of those characterized from other Gram-negative bacteria, consists of β(1→6)-linked disaccharide of α-glucosamine that is acylated at positions 2, 3, 2', and 3', involving six fatty acid chains (Fig. 2). This acylated disaccharide is also phosphorylated at positions 1 and 4' [1]. Interestingly, lipid A of Rhizobium etli CE3 differs from that of other Gram-negative bacteria. It is devoid of phosphate and it contains an unusual C28 acyl chain, a GalA residue at the 4' position instead of phosphate, and it has an acylated aminoglucosamine unit instead of the proximal glucosamine [2,3].

The core (R) oligosaccharide regions consist of a short chain of sugars, which connects the lipid A anchor to O-antigen and can be divided into inner and outer core regions [4]. The inner core region, which is proximal to lipid A, is composed of the unusual monosaccharides 2-keto-3-deoxyoctonoic acid (Kdo), L-glycero-D-manno-heptose, often together with phosphate or sulfate groups and ethanolamine [5,6]. Kdo is unique and invariably present in LPS and is therefore an indicator in assays for LPS (endotoxin). This residue attaches the core oligosaccharide to lipid A through an acid-labile ketosic linkage, and three to six additional monosaccharides comprise the rest of the inner core region. The minimal LPS required for growth of E. coli consists of lipid A and two Kdo units. The outer
core is attached to the O-antigen and is generally referred to as the hexose region, as it is composed predominantly of neutral and basic hexoses. With minor variations, the core polysaccharide is common to all members of a bacterial genus (e.g. Salmonella), but it is structurally distinct in other genera of Gram-negative bacteria. Salmonella, Shigella and Escherichia have similar but not identical cores.

The O-antigen or O-side chain is attached to the core polysaccharide and extends from the core out into the environment. It consists of repeating oligosaccharide subunits made up of three to five sugars. The individual
chains can vary in length ranging up to 40 repeat units. The O-polysaccharide is much longer than the core oligosaccharide and it contains the hydrophilic domain of the LPS. At least 20 different sugars are known to occur and many of these sugars are characteristically unique dideoxyhexoses such as abequose, colitose, paratose and tyvelose, which are rarely found elsewhere in nature. Variations in sugar content of the O-polysaccharide contribute to the wide variety of antigenic types between species and even strains of Gram-negative bacteria. In this regard, *Sinorhizobium* spp., which show limited variation in O-polysaccharide structure among each other [7], are unusual, as the O-antigens of most Gram-negative bacteria are highly variable, strain-specific surface antigens. In this genus, that role is fulfilled by the K-antigens [8].

Particular sugars in the structure, especially the terminal ones, confer immunological specificity of the O-antigen, in addition to ‘smoothness’ of the strain. These strains have a mucous appearance or colony morphology on agar plates due to the production of ‘smooth’ LPS (S-LPS) or LPS I. Loss of the O-specific region by mutation results in a ‘rough’ or R phenotype with production of LPS II (lipid A and core). For instance *E. coli* K-12 has long been known not to produce an O-antigen due to two mutations in the O-antigen gene cluster which have occurred before any of the current stocks were established [9]. These mutations probably improved adaptation to a laboratory environment but destroyed its ability to survive in its natural environment, which suggests that the core is required for viability, but that outer core and O-antigen are needed only in natural environments. Interestingly, also the LPS produced by species of *Neisseria* and *Haemophilus* are quite distinct from those produced by the Enterobacteriaceae and other Gram-negative bacteria in that they lack O-antigens. Therefore, these molecules are termed lipooligosaccharides (LOS) because the oligosaccharide chain attached to the lipid A moiety is equivalent in size and location to the core region of enterobacterial LPS [10].

### 1.2. Function

LPS are polysaccharide cell surface components that are intimately associated with the Gram-negative bacterial outer membrane, and the correct structure is essential to establish a disease (pathogens) or to produce a beneficial outcome (symbionts) in host-microbe interactions. LPS are important virulence factors of bacteria pathogenic in animals or humans, e.g. *Salmonella* spp. [11]. On the other hand, many reports have shown that also during symbiosis, LPS play crucial roles in the interaction between *Rhizobiiaceae* members and plants [12]. The structural function of LPS in stabilizing the outer membrane of the bacterium is evident. Nevertheless, LPS may also play important additional roles, necessary for effective host-microbe interactions.

### 1.2.1. LPS and pathogenesis

#### 1.2.1.1. Animal pathogenesis

The study of pathogenic bacteria in animals has shown that LPS have an important role in the infection of and survival in the host, and therefore are important virulence factors. Both lipid A and the polysaccharide side chains act as determinants of virulence.

Lipid A is the toxic component of LPS, evidenced by the fact that injection of purified lipid A into an experimental animal will elicit the same inflammatory responses as intact LPS. LPS (and consequently lipid A) are called endotoxin because of the association with the cell wall of bacteria. LPS molecules can dissociate from the surfaces of Gram-negative bacteria. The O-polysaccharide is hydrophilic and may allow diffusion or delivery of the toxic lipid in the hydrophilic (in vivo) environment. Lipid A molecules are detected at picomolar levels by the innate immune system of animals. During severe infections by Gram-negative bacteria, macrophages and endothelial cells are greatly stimulated by the released LPS, resulting in excess production of cytokines and inflammatory mediators. These products resulting from macrophage activation are thought to be responsible for the complications of Gram-negative sepsis. However, the inflammatory response is primarily protective in nature in that host defenses are recruited and stimulated to counter infection. If infection is not held in check, the overwhelming number of bacteria can then stimulate an excessive host response with ensuing damage (for a review of endotoxic activity, see [13]).

LPS first bind the serum LPS binding protein. Subsequently, this complex binds to the membrane receptor, CD14, present on certain host cells such as macrophages (Fig. 3). More recently a role for members of the human toll-like receptor (TLR) family in LPS recognition has been proposed, suggesting that the LPS receptor is multimeric, comprised of at least CD14 and one or more TLRs, primarily TLR-4 [14]. The mouse TLR-4 protein consists of an extracellular domain formed by a tandem arrangement of leucine-rich repeats (LRRs) connected by a single transmembrane domain to an intracytoplasmic signaling domain that shares homology with the interleukin 1 receptor (IL-1R) and which is now termed the TOLL-IL-1R (TIR) homology domain [15]. Recognition of LPS by the TLR-4-MD2-CD14 complex (see Fig. 3) generates intracellular signals such as stimulation of transcription of cytokine mRNAs, e.g. tumor necrosis factor and interleukin-1. LPS-induced enzymes, like NO synthase, are also expressed because of transcriptional activation. Excessive production of multiple cytokines and other mediators, including NO and platelet-activating factor, is responsible for the symptoms of endotoxin-induced shock. The cytokines are responsible for most of the systemic effects of endotoxin, including fever, and collectively act on the liver to produce the acute-phase reaction. The characteristic
structural features of *E. coli* lipid A, such as its two phosphate groups, its acyloxyacyl moiety and its glucosamine disaccharide, are needed to trigger the endotoxin response. LPS also directly activate the complement system resulting in the membrane attack complex or MAC. The MAC can integrate into lipid bilayers to form pores that lead to cell lysis. The production of activated complement component C5a induces a localized inflammatory response through the recruitment of host defense cells which then are stimulated to secrete granular contents and produce reactive oxidative intermediates. This inflammatory response can produce localized tissue damage as well as vasodilatation. Finally, the complement system facilitates phagocytosis of the pathogen through its effect as an opsonin by binding to the pathogen.

Although non-toxic, the polysaccharide side chain or O-antigen of LPS is the immunogenic portion of LPS. It presents epitopes for immune responses and has the potential to influence the host-parasite interaction at several levels:

- **Resistance to host complement:**
  The O-antigens are the key targets for the action of host antibody and complement, but when the reaction takes place at the tips of the polysaccharide chains, projected outwards from the overall bacterial cell surface, complement fails to have its lytic effect (MAC killing). Therefore, especially long O-antigens may prevent host complement from depositing on the bacterial cell surface and therefore protect from bacterial cell lysis. If the projecting polysaccharide chains are shortened or removed, antibody reacts with antigens on the general bacterial surface, or very close to it, and complement can lyse the bacteria.

  The importance of the biochemical composition of the O-antigen is demonstrated in the subtle difference between the O-antigen components abequose of *Salmonella typhimurium* and tyvelose of *Salmonella montevideo* resulting in differential activation and deposition of the complement component C3b [16,17] leading to differences in phagocytosis in vitro [17,18] and virulence in the mouse model [19].

- **Resistance to phagocytic engulfment:**
  The first line of defense against phagocytosis is the lack of activation and binding of complement components which could serve as opsonins, as described above. In that context, smooth antigens probably allow resistance to phagocytes. It has been observed in *E. coli* and *S. typhimurium* that rough mutants are more readily engulfed and destroyed by phagocytes than are wild-type strains and that the specific composition of the O-antigen is also important [18]. *Salmonella* transductants and recombinants differing in the O-antigenic side chain of their lipopolysaccharide are taken up at different rates by the murine macrophage-like cell line J774 and these rates correlate well with the known difference in virulence of these strains [18].

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**Fig. 3. Detection of lipid A endotoxin by animal cells and function of the signaling receptor TLR-4 in innate immunity.** The scheme is based mainly on studies of human, mouse and hamster cells [14,207–209]. The TLR-4 receptor may be oligomerized upon binding of lipid A. As indicated by the upper arrow, cell activation can occur without CD14, but requires several orders of magnitude more lipid A. Overproduction of TNF-α and IL-1β during a severe infection can damage small blood vessels, causing fluid leakage and shock. Synthetic lipid A analogs and certain precursors are potent endotoxin antagonists via TLR-4 [209–212]. Abbreviations: TNF, tumor necrosis factor; IL-1, interleukin-1; TLR, toll-like receptor (adapted from [206]).
Molecular mimicry (shielding the organism from host immune defenses):

Resemblance between bacterial antigens and host antigens is referred to as molecular mimicry. In this case, the antigenic determinants of the bacterium are in such close chemical relationship to host ‘self’ components that the immunological cells cannot distinguish between the two and an immune response cannot be raised. So far, one particular example of molecular mimicry through O-antigens has been described in *Helicobacter pylori*.

The O-antigen of LPS in most *H. pylori* expresses the Lewis blood group antigens Lewis X (Le^x^), Lewis Y (Le^y^), H type 1, Le^a^, Le^b^, i-antigen, sialyl Le^a^ and blood group A [20]. Only 15% of the *Helicobacter* strains tested lack these blood group antigens. In mammals, Le^x^ and Le^y^ are regarded as tumor-associated markers and some of these antigens and their derivatives can interact with selectins, mediating cell-to-cell adhesion [21]. Moreover, Lewis antigens are also expressed by the human gastric epithelium. Thus, *Helicobacter* seems capable of expressing an LPS serotype similar to that of the host. Data supporting this concept of molecular mimicry were obtained from both studies in human and experimental infection studies. Mimicry may have two diverging roles in pathogenesis. Through molecular mimicry, these Lewis antigen analogs present in the O-antigen may play a role in protecting *H. pylori* from antigen-specific host defenses. Because mimicry may cause ‘invisibility’ of the pathogen to the host, it may aid persistence of infection [22,23]. Secondly, antibodies against the O-antigen, which may be induced during infection, might bind to Lewis antigens in gastric epithelia and cause autoimmune-mediated inflammation [24,25]. In this respect, the case of *H. pylori* is not unique. Autoimmunity due to molecular mimicry between bacteria and host has been documented, as has the involvement of glycoproteins and glycolipids [26]. An example relevant to the study of *H. pylori* is *Campylobacter jejuni*. The molecular structure of *C. jejuni* LPS mimics that of gangliosides and elicits antibodies that react with gangliosides, a process which is believed to be central to the pathogenesis of the autoimmune neurodegenerative Guillain-Barré syndrome [27]. Gangliosides are complex glycolipids found in highest concentrations in the nervous system, particularly in gray matter, where they constitute 6% of the lipids.

Interestingly, both *Neisseria meningitidis* and *Haemophilus influenzae*, which cause bacterial meningitis, are able to covalently attach sialic acid residues to their LOS structures and so mimic the host’s cell surface glycoconjugates [28,29]. By attachment of sialic acid residues to their LOS structures, a bacterium can prevent the formation of C3b complex on its surface, thus avoiding C3b opsonization and subsequent formation of C5b and the MAC on the bacterial cell surface, just as capsules that contain sialic acid can do.

Colonization:

Attachment is a prerequisite for microbial colonization of epithelial surfaces and is mediated by molecules on the bacterial surface, adhesins, that recognize receptor proteins or glycoconjugates on the surface of a eukaryotic cell. The specificity of this interaction and the limited distribution of receptors often result in a restricted range of hosts and tissues utilized for colonization. This phenomenon is known as tropism. Although many diverse biological activities have been associated with LPS, their role in adherence to specific host cells has been recognized only recently [30]. Moreover, the ability of the saccharide moiety of LPS to mediate bacterial adhesion to host tissue, has first been described in *H. pylori* by Edwards and coworkers [31]. Colonization of the gastric mucosa by *H. pylori* NCTC 11637 occurs by tropic adhesion to mucosal epithelial cells. Edwards and coworkers [31] provided the first evidence that Le^a^ structures in the O-antigen polysaccharide of *H. pylori* can promote adhesion of this bacterium to the gastric epithelium. A mutant that no longer expresses the Le^a^ antigen on its LPS failed to show tropic binding to mucosal epithelial cells and adhered to the tissue surface in a haphazard manner.

The key to identifying the receptor recognized by the Le^a^ O-antigen may lie in the role played by Le^a^ as a component of human cells. The identification of Le^a^ as an adhesin suggests that molecular mimicry may enable *H. pylori* to exploit cell surface receptors that act as natural ligands for Le^a^ structures expressed on eukaryotic cells. Le^a^ is an oncofetal antigen, frequently expressed on human cancer cells. The Lewis blood group antigens are also present in the gastric mucosa of normal individuals [32] which may aid initial colonization of *H. pylori* in the gastric mucosa. Besides the gastric mucosa, it is also expressed on a variety of other cell types including neutrophils and monocytes, where it is often referred to as CD15 [33]. In this context, CD15/Le^a^ has been implicated as a ligand for P-selectin found on platelets and E-selectin found on the endothelium [33–35]. The Le^a^ structure is also a component of the α and β chains of the integrin, lymphocyte function-associated molecule 1, which is a ligand for intercellular adhesion molecule 1 [33]. Thus, Le^a^ plays an important role in the primary and secondary adhesion of lymphocytes to activated platelets or the endothelium of vessels through selectin and subsequent integrin binding. As such the ability of *H. pylori* to mimic eukaryotic cell structures may play an essential role in colonization of gastric tissue.

Because Le^a^ structures are present on both the bacterial cell and the host epithelial cell, Le^a^-Le^a^ homotypic interactions have also been implicated in the adhesion of
More specific roles in virulence:

- Finally, O-antigen components can have more specific roles in pathogenesis. Sandlin et al. [38] postulated that the core and O-antigen components of LPS of *Shigella flexneri* 2a are critical elements in the correct unipolar localization of IcsA, the protein essential for intracellular motility and intercellular spread. Mutations in two genes, *galU* and *rfe*, which directly affect synthesis of the LPS of *S. flexneri* 2a, result in the inability to spread from cell to cell. More strikingly, the LPS mutants showed aberrant surface localization of IcsA. Unlike the unipolar localization of IcsA seen in the parent strain, the *galU* mutant expressed the protein in a diffuse circumferential fashion. The *rfe* mutant had an intermediate phenotype in that it displays some localization of IcsA at one pole while also showing diffuse localization around the bacterium [38]. These observations indicate a more precise role for LPS in *Shigella* pathogenesis.

Interestingly, data from Del Portillo et al. [39] suggest that intracellular bacterial pathogens might signal the host cells from intracellular locations by releasing bioactive bacterial components such as LPS. *S. typhimurium* releases LPS once it is located in the intracellular environment of cultured epithelial cells. LPS is liberated from vacuolar compartments, where intracellular bacte-

*H. pylori* to gastric epithelial cells. However, a Lea-mediated adhesion of *H. pylori* to another cell type (parietal cell canaliculi), which also expresses the Leb antigen, was not observed. This argues against the involvement of Leb-Lea homotypic interactions [31]. On the other hand, it has been proved that the Leb antigen expressed by the gastric mucosa in most humans, contrary to the Lea antigen expressed by the gastric mucosa, does have a role in adhesion of *H. pylori* and is recognized by the Bab (blood group antigen binding) adhesin present on the bacterial outer membrane [36]. In individuals with blood group O and who are of positive secretor status, the Leb blood group antigen is the predominant blood group-related antigen expressed on surface mucous cells, providing ample opportunity for Bab-mediated adhesion of *H. pylori* to occur. However, in individuals of non-secretor status, where the predominant blood group antigen is Lea, adhesion mediated by Bab would be inefficient. More significantly, this Bab adhesin cannot promote adhesion to gastric tissue from individuals of blood group A and B phenotypes where the Leb determinant is substituted with a terminal GalNAc1-3 residue or Galα1-3 respectively. In such individuals, Leb would be anticipated to play a critical role in establishing colonization of the gastric mucosa. The report of adhesin receptor interactions in Pasteurellaceae further supports the finding that LPS can act as an adhesin in bacterial pathogenesis to facilitate the invasion of animal cells [37].

1.2.1.2. Plant pathogenesis. Recent evidence supports the idea that plants may have evolved systems of innate immunity, perhaps analogous to the Toll-like receptor system (TLR) for lipid A in animals [40-42]. *Arabidopsis thaliana* contains a gene encoding a homolog of serum lipopolysaccharide binding protein, which is required for lipid A transport and delivery (Raetz, homepage: http://ives.biochem.duke.edu/Raetz/Lipid%20A1.html). Several cytoplasmic plant disease resistance genes (R genes) with homology in certain modules to members of the TLR family have also been cloned and shown to mediate host defense against specific viral and fungal plant pathogens [43]. This class of disease resistance proteins mediates the hypersensitive response in plants that includes metabolic alterations and localized cell death at the site of pathogen invasion [44]. Examples of R proteins that contain the Toll-interleukin 1 receptor (TIR) homology domain are the N protein from tobacco, the L6 protein from *fx*ax and the RPP5 protein from *Arabidopsis* [43]. The presence of the TIR domain in these plant R proteins suggests a role for this domain in signalling but not in ligand binding (see Fig. 4).
This role is fulfilled by the leucine-rich repeats (LRRs) found in R proteins. These LRRs are highly diverse and appear to be involved in the recognition of a wide array of microbial components.

Interestingly, human Nod1 and Nod2 were recently identified as mammalian counterparts of plant disease resistance gene products based on structural homology. It is proposed that they may function as cytosolic receptors for components derived from invading pathogenic bacteria [45]. Moreover, the authors suggest that Nod1 may act as an intracellular receptor for LPS, in addition to the TLR-4 complex that has been demonstrated to serve as a surface receptor for LPS. Similar to the LRRs in plant disease resistance proteins that are critical for pathogen-specific responses, the LRRs of Nod1 are essential for Nod1 to respond to LPS.

The roles of LPS in the bacterial pathogenesis of plants are as diverse as those observed in the bacterial pathogenesis of animals. These include effects which appear to favor the pathogen and those of benefit to the host. LPS mutants, which have been described in all the major genera of Gram-negative phytopathogens, have been found to have reduced virulence [46]. LPS certainly contribute to the resistance of plant pathogens to plant-derived antimicrobial compounds (barrier function) which presumably promote the ability to infect plants. Conversely, LPS can trigger defense-related responses in plants. The infiltration of heat-killed Ralstonia solanacearum bacteria into tobacco leaves delayed or prevented the appearance of disease symptoms or the hypersensitive response (HR) when the leaves were subsequently inoculated with live bacteria in compatible or incompatible interactions. From this finding it was concluded that bacterial LPS can locally prevent HR normally induced by avirulent bacteria. This activity was shown to reside in the LPS of R. solanacearum, specifically in the lipid A-core structure [47]. This effect was later termed ‘localized induced resistance’ by Sequeira and co-workers [48], who considered that the plant response to LPS reflects an enhanced antimicrobial environment within the plant which prevented bacterial growth and hence HR. LPS also potentiates the phenolic metabolism after bacterial infection. Interestingly, two antimicrobial phenolic conjugates, coumaroyl tyramine and feruloyl tyramine, accumulated more rapidly after inoculation in peppers pretreated with LPS [46,49]. Additionally, LPS from Pseudomonas fluorescens has been implicated in the establishment of induced systemic resistance in radish, an activity associated with the O-antigen component [50]. Moreover, Reitz et al. [51] showed that the LPS of R. etli G12 act as the inducing agent of systemic resistance to infection by the potato cyst nematode Globodera pallida in potato roots. This type of induced disease resistance is often referred to as rhizobacteria-mediated induced systemic resistance (ISR; reviewed by [52]). Rhizobacteria-mediated ISR has been demonstrated in many plant species, e.g. bean, carnation, cucumber, radish, tobacco, tomato and the model plant A. thaliana, and has been reported to be effective against a broad spectrum of plant pathogens, including fungi, bacteria and viruses [53].

1.2.2. LPS and symbiosis

Increasing evidence suggests that there is a subtle difference between symbiosis, represented by the Rhizobium-legume interaction and pathogenicity, as successful microsymbionts must also either evade or neutralize the plant defense systems. Specific constituents of the rhizobial cell wall, including LPS, are required for an effective symbiosis. The correct LPS structure is essential for root hair infection, nodule invasion, for avoiding host defense responses (suppression of host defense responses during these infections) and for physiological adaptation to the endophytic microenvironment. The function of LPS also appears different for indeterminate and determinate nodules. Determinate development leads to nodules that cease development at maturity and maintain a relatively static spherical structure until senescence. Indeterminate nodules generate a meristem that allows growth and extension of defined developmental regions to proceed indefinitely, usually producing an elongated, cylindrical nodule. However, many other developmental and anatomical differences are associated with these modes of development [53,54].

In this section, the requirements for O-antigen in a successful plant-bacterium interaction will be illustrated at different stages of nodulation.

- Colonization (adhesion):

  In Rhizobium leguminosarum bv. viciae and trifolii, LPS was postulated to play a role in the very early recognition steps with their respective hosts, particularly in root adhesion [55,56]. Host-encoded lectins and their interaction with LPS may play an important role in the attachment of the symbiont to the host. The LPS of R. leguminosarum bv. trifolii were found to bind trifolitin A, the white clover lectin which mainly accumulates on the tips of root hairs. This specific interaction of the trifolitin A-binding glycoform of R. leguminosarum bv. trifolii LPS and white clover root hairs induced rapid changes in their cytoskeleton activity and the levels of several specific root hair proteins, designated ‘infectin’ [57]. The lectin-mediated binding is most likely a host-specific event because microscopy revealed that treatment of white clover roots with LPS from heterologous wild-type rhizobia (e.g. R. leguminosarum bv. viciae or Sinorhizobium meliloti) resulted in incompatible root hair responses [57]. In addition, specific LPS structures may be important in the early recognition steps. Recently, mutant CE166 of R. etli CE3 was reported to produce LPS structurally different from that of the wild-type by lacking quinovosamine (2-amino-2,6-dideoxyglucose) and with a decreased level of LPS I [58]. The decreased level of LPS I but not the absence of N-acetylquinovosamine (Qui-
Infection thread initiation and elongation:
As infection starts, the presence of O-antigen becomes critical, especially on determinate hosts, because LPS I appears to be required for formation of stable narrow infection threads. Infection of determinate nodulating plants with LPS mutants lacking the O-antigen (i.e. LPS II mutants) showed a premature abortion of infection threads. This may be related to the direct contact of the bacteria with the host plasma membrane in these narrow structures. The close proximity of the plant and mutant bacterial cell surfaces in the infection thread of determinate nodules may have a detrimental effect. Consistent with this suggestion, LPS I is not required for formation of broad infection threads present in indeterminate nodules. In these latter nodules, LPS mutants disturb nodulation at a later stage [53].

Infection threads are composed of plant material, although rhizobia may contribute to the matrix material found inside the threads [53]. Using monoclonal antibody MAC265 as a probe, a plant glycoprotein has been identified as a component of the luminal matrix of infection threads in pea nodules. This glycoprotein is secreted by the plant as an early response to *Rhizobium* infection. Because it attaches to the surface of bacteria through ionic binding and is itself susceptible to oxidative cross-linking, this glycoprotein may be involved in limiting the progress of microbial infections. It is suggested that this binding to the bacterial cell surface may be inhibited by LPS through masking the highly charged groups that lie closer to the surface of the bacterial outer membrane [61]. Moreover, Perotto et al. [62] observed a different amount of the MAC265-responding plant glycoprotein accumulating in nodule tissues in response to infection by wild-type or mutant rhizobia. Overproduction of this MAC265-reacting matrix glycoprotein was observed in pea nodules formed by LPS-defective strains [62]. Similarly, LPS may act as a fence or barrier to prevent toxic plant compounds, such as coumestrol, from penetrating to targets on the rhizobial cell [63]. It is possible that the volume of the remaining lumen within the narrow infection thread of determinate nodules is so small that relatively high concentrations of root-exuded antimicrobial compounds, such as phytoalexins, accumulate. Phenotypes of LPS mutant rhizobia in this type of nodules could then be explained by increased sensitivity towards toxic compounds in these mutants, resulting in the observed abortion of infection threads. Alternatively or in addition, it could be likely that the O-antigen covers compounds that induce plant defenses, such as the LPS core or outer membrane proteins. Several features of the aberrant nodule development caused by O-antigen mutants suggest that deleterious host defense responses occur throughout the invasion process and may be the principal cause of the symbiotic failure. Detailed cytological analysis in pea nodules induced by LPS mutants revealed that the walls of infection threads show secondary modifications and possibly callus deposition, accumulation of an intercellular matrix largely composed of the above mentioned MAC265-responding plant-derived glycoprotein, and sporadic cell death. These modifications have been interpreted as mild plant host defense reactions [62]. Another way of suppressing host defenses can be similar to the still unclarified manner that is proposed for the expolysaccharide (EPS) of *S. meliloti* [64]. For EPS, at least, this role may involve diffusion of defined EPS fragments to an unknown target [65]. However, it remains possible that LPS fractions might be cleaved off or become diverted from the normal pathway of synthesis for a similar purpose.

Bacteroid release and development:
In indeterminate nodules, LPS I appears to be essential at the stage of bacterial contact with the plasma membrane, i.e. during endocytotic uptake from the infection thread matrix (see Fig. 5). So far, the mechanisms of involvement of LPS are not clear. However, if uptake of rhizobia proceeds by a ‘membrane-zippering’ mechanism, as suggested for receptor-mediated phagocytosis by macrophages, the distribution of endocytotic factors on the rhizobial surface may determine whether a bacterium is completely endocytosed or not [53]. A model in which LPS play a role in attachment to the cytoplasmic membrane has some precedence in animal systems where the involvement of LPS in cell adhesion and invasion is under discussion [30].

Analysis of indeterminate nodules formed by *R. leguminosarum* LPS mutants on pea, vetch or clover has revealed that symbiosis is disrupted during and/or after release of the bacteria into the host cells. *R. leguminosarum* bv. *viciae* LPS mutants completely lacking the LPS O-chain develop nodules with abnormal infection threads and only sporadic release of bacteria into nodule cells [62,66,67]. The bacteroids senesce early and fix little or no nitrogen. In contrast, *S. meliloti* LPS mu-
tants form fully effective nitrogen-fixing nodules on *Medicago sativa* (alfalfa) [68]. However, a detailed analysis of a specific *S. meliloti* LPS mutant, designated Rm6963, showed that the alfalfa nodulation competence of this mutant is strongly reduced, compared with the *S. meliloti* wild-type strain [69]. The LPS of this mutant, although not fully characterized chemically, seem to contain a LPS O-chain, albeit a modified one [69]. Furthermore, a more recent study revealed that this mutant forms effective nodules with *M. sativa* but ineffective nodules with *Medicago truncatula* [70]. Analysis of the symbiotic defect indicated that the mutants are released from the infection thread but fail to multiply within the plant’s cytoplasm resulting in the formation of empty vesicles. In addition, infection threads were enlarged and encrusted with cell material. This was interpreted as the consequence of a plant defense reaction against the symbiont. The LPS mutant also induced another typical plant defense-associated reaction, namely increased auto-fluorescence of plant cells. The increased auto-fluorescence induced by Rm6963 was observed within the central tissue of the root nodule, in contrast to EPS-deficient *S. meliloti* mutants, in which the auto-fluorescence occurred in the cortical cell walls [71]. The authors propose that *S. meliloti* EPS act as a suppressor of the plant defense response during the infection thread initiation, while LPS take over this function after release of the bacteria from the infection thread. They also suggest a role for LPS during the infection thread initiation since nodulation is delayed when plants are inoculated with the LPS mutant. Rm6963 represents the first LPS mutant that alters the host range within one legume genus, meaning that there is a specific recognition of the LPS by the host plant, rather than a non-specific or structural function. This hypothesis is strengthened by the observation that a crude LPS fraction obtained from *S. meliloti* suppresses the elicitor-induced alkalization of alfalfa, but not of tobacco or tomato cell cultures [72]. Moreover, heterologous LPS from *R. leguminosarum*, *Salmonella* or *E. coli* showed no suppression of the elicitor-induced response in alfalfa cell cultures [73]. These data provide good evidence for a specific recognition of the *S. meliloti* LPS by the host plant which results in the suppression of the plant defense system, enabling the symbiont to establish an effective symbiosis.

In contrast, no LPS-impaired mutant rhizobia have been isolated that carry infection beyond the infection thread abortions observed in determinate nodules, making it difficult to assess LPS functions at later stages of cell invasion and development in this type of nodulation.

Whether or not the lipid A portion of LPS also plays an important role in symbiosis is unknown. Que and co-workers, who recently re-evaluated the structure of lipid A in *R. etli* CE3 [2,3], favor the view that the unusual structure of lipid A in *R. etli* somehow facilitates symbiosis by masking the potential immunostimulatory ac-

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**Fig. 5. Membrane topography of the symbiosome.** The intimate contact of the *Rhizobium* outer membrane with the plant peribacteroid membrane could involve remodeling of specific membrane proteins and lipids. Diagram adapted from [213].
tivity that might be associated with more conventional (phosphate-containing) lipid A structures. Perhaps, the plant does not reject the \textit{R. etli} endosymbiont because \textit{R. etli} lipid A is not recognized by the innate-like immune system of the plant. In this scenario, the plant could still respond to infections by Gram-negative pathogens that possess a conventional phosphate-containing lipid A moiety, while retaining the nitrogen-fixing endosymbiont. However, the lethality and cytokine-inducing activity of LPS obtained from nodulating bacteria, \textit{R. leguminosarum} and \textit{Mesorhizobium loti}, were compared to those of \textit{S. typhimurium} LPS [74]. The activity of \textit{R. leguminosarum} LPS was almost comparable to \textit{Salmonella} endotoxin in terms of lethality and in vivo TNF-\alpha, IL-1\beta, IL-6, and interferon-\gamma induction capacity. In contrast to high lethal toxicity of \textit{Rhizobium} LPS, the lethality of LPS isolated from \textit{M. loti} was more than 10\(^3\)-fold lower. The examined overall chemical composition of LPS indicates a considerable distinction in their lipid A regions. Lipid A as obtained from \textit{R. leguminosarum} and \textit{M. loti} differed from their enterobacterial counterpart with respect to lipid A sugar backbone, its phosphate content as well as the type and distribution of hydrophobic acyl residues [74]. Interestingly, the LPS of \textit{Rhizobium} and \textit{Bradyrhizobium} carry long-chain (28-C) fatty acyl substituents [75], which probably provide extra stability to the bacterial outer membrane and might be considered an adaptation to the physiological stresses of the endophytic environment [76].

2. Inter- and intrastrain LPS heterogeneity

Most of the heterogeneity in LPS molecules is found in the O-antigen polysaccharide. The variation in O-antigen lies in the sugar composition and the linkages between monosaccharides and gives rise to an enormous structural diversity of O-antigens. The chemical composition and structure of the O-antigen is strain-specific, hence its antigenicity forms the basis of O-serotyping for species of Gram-negative bacteria. \textit{Salmonella} spp. produce more than 1000 chemically and structurally distinct variants, whereas 173 O-antigens have been reported for \textit{E. coli} [77]. Although the majority of O-antigens are heteropolymers, some were found to consist of a single monosaccharide.

Moreover, the LPS molecules extracted from a given bacterial culture are often heterogeneous in structure. This LPS heterogeneity within a strain gives rise to the existence of multiple glycoforms in one population, which makes the elucidation of the complex chemical structures of LPS an exciting research challenge. The recent re-evaluation of the lipid A structure in \textit{R. etli} CE3 confirms the difficulties which are encountered in determining the precise LPS structure [78]. The development of new chromatographic methods led to a more complete structural picture of \textit{R. etli} lipid A. Analysis of intact lipid A molecules isolated from whole cells by hydrolysis at pH 4.5 has revealed that lipid A of \textit{R. etli} consists of a mixture of at least six structurally related but distinct components [2,3].

The LPS heterogeneity within one bacterial strain can be achieved through variations of the O-antigen at different levels. First of all, O-polysaccharides can be modified non-stoichiometrically with sugar moieties, such as glucosyl and fucosyl residues. The addition of non-carbohydrate substituents, i.e. acetyl or methyl groups, to the O-antigen can also occur with regularity, but in most cases these modifications are again non-stoichiometric. In addition, a change, either positional or anomeric, in the linkage between sugars in the O-units can occur. Finally, the length of O-antigens (i.e. the number of repeat units) can vary within a strain. In the Enterobacteriaceae, which commonly produce smooth LPS, the LPS molecules are capped by O-antigens of varying chain lengths. The extent of this heterogeneity is readily appreciated in samples separated by SDS–PAGE. This procedure is especially useful for determining the number and distribution of O-antigen repeats, which are displayed as a ladder of bands after silver staining. Also in Rhizobiaceae the number of repeats in O-chain varies greatly for some strains, resulting in a ‘ladder’ pattern on SDS–PAGE [12]. For other strains the number of repeats is restricted to a narrow range and PAGE analysis results in only a few major bands [79]. In \textit{Pseudomonas aeruginosa} LPS are also a heterogeneous mixture of molecules of different polysaccharide lengths [80] and with variable levels of substitutions at specific sites [6]. In addition, LPS isolates from many serotypes of \textit{P. aeruginosa} contain two chemically and antigenically distinct forms of O-antigen, known as a common A band (homopolymer) and a serotype-specific B band (heteropolymer) [81].

Alterations in LPS structures often occur in response to different host environments and stages in the pathogenesis or symbiosis. These modifications occur at the level of the lipid A portion as well as the O-antigen portion. Therefore, structural analysis based on LPS isolated from cells after repeated passage of in vitro growth may not be a reliable measure for the situation in vivo.

3. O-antigen structural variations in pathogens

Understanding LPS structural variation is important because several studies have indicated that the composition or size of the O-antigen might be a reliable indicator of virulence potential and that these important features often differ within the same bacterial strain. For instance in \textit{Salmonella enteritidis}, it was shown that virulent isolates obtained from chick spleens produce LPS with larger O-chain:core ratios, indicating a higher number of O-chain repeats in the LPS molecules, as compared to avirulent...
isolation [82]. It is believed that it is possible to monitor emerging virulence of serovar enteritidis, and possibly other Salmonella serovars, by analyzing LPS structural heterogeneity during epidemiological investigations.

Contrary to the LPS studies in bacteria–animal models, investigations on LPS of plant pathogens are largely in the descriptive stage. It is not known whether LPS are structurally modified in response to environmental stresses experienced by bacteria within plant habitats during pathogenic interactions and therefore plant pathogenic bacteria will not be discussed in this section.

Possible genetic mechanisms for O-antigen variation in animal/human pathogens and the possible role of these variations will be discussed.

3.1. Genetic mechanisms involved in O-antigen variation

There are many reports of modifications of O-antigen, resulting either from altered gene expression, from lysogenic conversion or from lateral gene transfer followed by recombination. In most cases, the mechanisms underlying these changes have not been resolved. However, in recent studies some progress in understanding has been made.

3.1.1. Lateral gene transfer followed by recombination

Extensive studies have been undertaken to reveal the genetic basis of O-antigen variation. These studies are particularly based on analysis and comparison of O-antigen controlling gene sequences, mostly in Salmonella enterica and E. coli [83]. Genes involved in the biosynthesis of O-antigen are generally arranged in clusters. Depending on the complexity of the saccharide, the number of genes in the clusters varies from approximately six to nine in any cluster. It has been found that in general, O-antigen genes are of low G+C content (usually less than 40%), compared to the other genes on the chromosome, indicating that the O-antigen DNA originated in species with low G+C content and was captured in other species by lateral gene transfer. Although it is obvious that O-antigen gene clusters of S. enterica have undergone lateral gene transfer, the mechanism for such transfer is not clear. The finding of plasmid-borne O-antigens in S. enterica O54 [84] and E. coli Sonnei [85] suggests that plasmids could be the vehicle. Shepherd and coworkers [86] also found evidence for an interspecies transfer of an entire O-antigen gene cluster. Moreover, the G+C content within the O-antigen clusters greatly differs from gene to gene, indicating that the gene clusters were assembled from several sources over a much longer time.

By sequencing and comparison of O-antigen gene clusters, it was proven that DNA recombination events between O-antigen gene clusters within S. enterica and between E. coli and Klebsiella played a role in the formation of new O-antigen forms. In cases when comparable O-antigen gene clusters have been sequenced from different species or serovars, the gene products toward the center of the cluster are less strongly conserved whereas the gene products at the ends are more strongly conserved. If each cluster is considered to be made up of a series of interchangeable modules, this arrangement of conserved regions flanking a non-conserved region may facilitate the exchange of modules within the clusters as a means of generating diversity while preserving basic biosynthetic functions.

The O-antigens of S. enterica and E. coli are extremely diverse, with 54 and 190 known forms, respectively, recognized in their typing schemes. The low proportion of identical forms present in both species suggests that there has been extensive turnover of O-antigens, presumably by lateral gene transfer since divergence of the two species, because the majority of O-antigens, found in only one of the species, must have been gained or lost by one of them since divergence. Simple interstrain gene transfer and recombination could then replace one set of O-antigen biosynthetic genes with another, generating derivative strains with new serotypes [87]. The relatively higher level of transfer of O-antigen gene clusters within or between species is believed to reflect the advantage from time to time of an alternative O-antigen for bacterial adaptation to new niches, followed by natural selection for recombinants [88].

However, genes for O-antigen biosynthesis in H. pylori are not clustered in one locus as is the case in other bacteria, but are distributed throughout the genome. Only rfbM, rfbD and wbcJ, the genes involved in the synthesis of the GDP-fucose, are clustered [89]. Berg et al. [90] suggest that special features of the H. pylori–host interaction may result in relatively little selection for the ability to exchange sets of LPS biosynthetic genes en bloc.

3.1.2. Phage-induced conversion

Another way pathogens may alter their O-antigen structure is by acquisition of new genetic material from phages via transduction. Lysogeny may generally have a role in bacterial survival in animal hosts, and perhaps in patho-
genesis. On the one hand, some genes from bacteriophage lambda, which are expressed during lysogeny, encode entirely new host cell envelope proteins which may confer virulence to the host cell [91]. One of these is lom. Its gene product is found in the bacterial outer membrane, and is homologous to virulence proteins of two other enterobacterial genera. This lom gene is also involved in adhesion of E. coli K12 lysogens to human buccal epithelial cells [92]. Another gene is designated bor. Expression of bor significantly increases the survival of the E. coli host cell in animal serum. This property is also a well-known bacterial virulence determinant.

On the other hand, the integration of bacteriophages into the host chromosome can also lead to serotype conversion or modifications of the basic O-antigen structure of the parent strain. These variations are due to either glucosyl or O-acetyl residues being added at specific positions on the repeat unit through enzymes expressed by the prophage. The modification itself is probably a protection mechanism for the prophages to exclude other homologous phages from entering lysogenized cells by altering the adsorption sites. These serotype-converting genes from bacteriophages may also play an important role in virulence of the host cell.

In this section, three different strategies for O-antigen modification conferred by bacteriophages will be discussed. Each case will be illustrated with one example of bacteriophage-host interaction.


In S. flexneri, the basic O-antigen (serotype Y) consists of repeating units of the N-acetylmuramic acid–rhamnose–rhamnose–rhamnose tetrasaccharide. Bacteriophage-mediated glucosylation was found on all three rhamnose residues and also on the N-acetylmuramic acid (GlcNAc) residue, depending on the bacteriophage (for review see [93]). For instance, S. flexneri bacteriophage X (SfX) is responsible for converting serotype Y to serotype X through its serotype X-specific glucosyltransferase (GtrX, formerly Gtr) by the addition of one glucosyl group to the first rhamnose residue of the tetrasaccharide repeating unit (Fig. 6).

Besides the gtrX gene, two other genes in the gtr locus of SfX are also involved in this O-antigen modification process. While GtrX alone can only mediate a partial conversion, gtrX together with the two other genes are responsible for the full O-antigen conversion. The first gene in the cluster (gtrA) encodes a small highly hydrophobic protein, which might play a role in the translocation of lipid-linked glucose across the cytoplasmic membrane. The second gene in the cluster (gtrB) encodes an enzyme catalyzing the transfer of the glucose residue from UDP-glucose to a lipid carrier. The third gene (gtrX) in the O-antigen modification operon encodes the bacteriophage-specific glucosyltransferase (Gtr) which is largely responsible for the final step, i.e. attaching the glucosyl molecules onto the correct sugar residue of the O-antigen repeating unit. When the amino acid sequences of the Gtr proteins from different bacteriophages are compared, there is little similarity. This diversity of the serotype-specific Gtr proteins is in sharp contrast to the high degree of conservation of GtrA and GtrB between the bacteriophages. This could result from the fact that each Gtr protein has evolved to recognize a different target, to which it attaches a glucosyl group via a specific linkage [93].

Based on the identification of the three-gene cluster, a three-step model for the glucosylation of bacterial O-antigen mediated by S. flexneri bacteriophage SfX has been proposed by Guan et al. [94] (see Fig. 7).

In the first step, GtrB catalyzes the formation of the UndP-Glc intermediate from UDP-Glc and UndP (undecaprenyl phosphate). Like the formation of O-antigen repeat units, this step takes place at the cytoplasmic side of the cytoplasmic membrane. In the second step, UndP-Glc is translocated to the periplasmic face either by GtrA alone or by GtrA associated with GtrX. Finally, the glucosyl group is positioned on the correct sugar residue in the growing polysaccharide side chain by GtrX while this chain is still linked to the antigen carrier lipid (ACL). Since the glucosylation appears to happen only from the second repeat unit (or subsequent repeat units) from the growing end [95,96], the proximal repeat unit from the ACL is not altered by this modification. The mode of biosynthesis [97] of the O-antigen by adding new single repeating units to the proximal end (later to be transferred to the LPS core) of the growing chain would indeed predict that at least two and perhaps several of the most proximal units would be non-glcosylated [98]. Therefore, the same O-antigen polymerase called Wzy is still able to
catalyze O-antigen polymerization and subsequently the same ligase called WaaL can still transfer the polysaccharides to the core oligosaccharide.

When only gtrX is introduced into the genome of S. flexneri SFL124, both serotype Y- and serotype X-specific monoclonal antibodies can bind upon agglutination testing, indicating that GtrX can mediate a partial conversion when incorporated into the chromosome. Therefore, it is postulated that there must be other host-encoded glucose transportation pathways in this strain. This pathway can ineffectively complement the defect of GtrA and GtrB, therefore resulting in partial O-antigen conversion [94].

Some naturally occurring serotypes contain multiple glucosyl modifications at different positions within the tetrasaccharide repeating unit. So far, little is known on how multiple glucosyl transfers interact within a single host. The addition of a glucosyl group to the tetrasaccharide repeat unit is likely to change its conformation and, consequently, the conformation of the target for a Gtr, suggesting that the individual Gtrs can tolerate a degree of flexibility in target formation [93].

Sequence analysis of phage P22, which upon infection also glucosylates the Salmonella spp. O-antigen, revealed the presence of GtrA and GtrB homologs [99,100]. These data suggest that the mechanism and factors involved in the first step of O-antigen modification are conserved between Salmonella spp. and S. flexneri [93]. The Salmonella O-antigen is normally glucosylated at the galactose residue in an α-1,4 linkage. After P22 enters the cell and establishes lysogeny, the prophage specifies glucosylation in α-1,6 linkage resulting in the appearance of O-antigen 1. Mutants defective for O-antigen conversion were mapped to the con region of the P22 genome. Franko [101] sequenced this region and found three closely linked, open reading frames (ORFs) which she called conABC, most likely corresponding to the gtrA, gtrB and gtrC genes described in the recent paper of Vander Byl and Kropinski [100]. Mutants in either conB or conC failed to convert the O-antigen.

3.1.2.2. O-acetylation of O-antigen: S. flexneri bacteriophage Sf6. Bacteriophage Sf6 antigenically converts S. flexneri serotype Y strains to type 3b strains by means of a phage-directed O-acetylation of the third rhamnose residue within the tetrasaccharide repeating unit (Fig. 8) [102,103].

The O-acetyltransferase protein (Oac) is 333 amino acids long and is relatively hydrophobic, with nine putative transmembrane regions predicted [103]. Comparison of Oac with other proteins in the protein databank revealed similarity to a variety of proteins involved in the O-acetylation of O-antigen, LPS, EPS and macrolide antibiotics [93]. It has been proposed that these proteins define a family of inner membrane trans-acylases [104].

It is unclear where acetylation of the O-antigen takes place. There are several examples where an acetyl residue is part of the basic O-antigen repeat unit, and acetylation is thus likely to take place in the cytoplasm. Comparison of the S. flexneri serotype Y and serotype 3b O-antigen by SDS-PAGE, however, reveals that shifts in mobility are not obvious for O-antigen chains less than approximately six units in size, suggesting that only the larger O-antigen chains are acetylated [102]. Western immunoblots of glucosylated LPS also suggest that only larger O-antigen chains are modified [105,106]. O-acetylation and glucosylation of only larger O-antigen chains would, however, contradict the results obtained from early chemical analysis, which indicated that the ratio of the sugars in the tetrasaccharide unit to the added O-acetyl or glucosyl residue was 1:1 [93,107]. Further analysis of the mechanism and extent to which the S. flexneri O-antigen is modified is required to resolve these discrepancies [93].

3.1.2.3. Change in the bonding between the O-antigen repeats: P. aeruginosa bacteriophage D3. It has been reported that the O-antigen-specific D3 bacteriophage causes lysogenic conversion in P. aeruginosa PA01 from serotype O5 to an O-acetylated form of serotype O16 (Fig. 9). The corresponding lysogen loses the ability to adsorb this phage, owing to a modification of the side chains of LPS consisting in the acetylation of position 4 of fucosamines, and the change in the glycosidic linkage of the FucNac residue from α to β [108]. Newton et al. [109] identified three ORFs encoding a β-polymerase (wzyp), an inhibitor of α-polymerase (iap) and an O-acetylase (oac) [108–110]. The α-polymerase inhibitor (Iap) is capable of inhibiting the assembly of the serotype-specific O5 B-band LPS and allows the phage-encoded β-polymerase (Wzyp) to form new β-linked B-band LPS. The paper of Newton et al. [109] is the first report illustrating a three-component system by which a bacteriophage alters the LPS structure of a bacterium.

Another example is the Salmonella group E phage ε15 which was among the first O-antigen-converting phages to be described. In uninfected Salmonella, the Gal-Man linkage occurs in the α configuration while after infection by
Mechanistically, this variable gene expression, leading to changes in the amount and composition of bacterial surface components in general, is controlled at the DNA level by cis- or trans-acting regulatory elements. cis-Acting control mechanisms include DNA rearrangement and slipped-strand DNA synthesis mechanisms leading to phase or antigenic variation. trans-Acting control mechanisms require the intervention of positive and negative transcriptional regulatory proteins which convert the environmental signals into altered gene expression. This phenomenon is also termed ‘phenotypic modulation’.

3.1.3.1. Phase variation or on-off switching of genes leading to O-antigen modifications. A mechanism used by several pathogens to increase heterogeneity is phase variation. Phase variation (also called antigenic variation) is the occurrence of spontaneous, high frequency (up to 0.5%), reversible on-and-off switching of surface epitopes, e.g. those present on adhesins or LPS. Genetically, this is paralleled by on-and-off switching of specific genes encoding these surface structures. The expression or non-expression of particular genes (phase variation) or the alteration of microbial structures, especially surface structures such as pili or outer membrane proteins (antigenic variation), in vivo represent paradigms of microevolution or short-term alterations [114]. Typically, bacterial infections occur within a matter of hours in which bacteria in vivo encounter host landmarks of such diversity that prescriptive strategies, such as two-component sensory transducer systems or classical gene regulation, may be inadequate to encompass the plethora of potential variables [115].

Recently, non-embryonic bacterial surface structures also have been shown to be phase variable, including H. influenzae LOS [125−127], N. meningitidis LOS, H. pylori LPS, Coxiella burnetii LPS [128], Neisseria gonorrhoeae Opa proteins [129−131], and E. coli Ag43 [132]. Many of these corresponding genes have evolved sequences containing runs of repetitive DNA [131,133]. Nucleotide repeats (microsatellites) are highly mutable, because of their propensity to undergo slippage [134,135]. The resulting mutations, involving spontaneous loss or gain of nucleotides, result in reversible, high frequency on-off switching of genes through altered transcription, altered binding of RNA polymerase to promoters, or translation frameshifts [136]. Given that there are several such genetic loci in a single pathogen genome and that the mutations occur at random and independently of one another, the combinatorial effect on the phenotypic diversity of the pathogen population can be substantial. These genes have been called contingency loci, to emphasize their potential to enable at least a few bacteria in a given population to adapt to unpredictable and precipitous contingencies within, and in transmission between, different host environments [115,137].

Four phenotypic changes in O-antigen structure mediated by phase variation, including fucosylation, glucosylation, acetylation and changes in O-antigen size, are the focus of the following section and are illustrated in each case for one bacterium.

3.1.3.1.1. Fucosylation: H. pylori. Recently, there has been significant progress in understanding the genetic determinants and mechanisms involved in phase variation of the O-antigens in H. pylori. The LPS of most H. pylori strains contain fucosylated oligosaccharides that mimic Lewis antigens present in humans and the expression of these antigens can be phase-variable in the population of a single strain. As in mammalian cells, the final steps of
Lewis antigen synthesis require fucosyltransferases (FucTs) to add fucose to the precursor molecules, as shown in Fig. 10.

Expression of alternative Lewis antigens in a given strain is apparently due to the particular activities of the respective FucTs. It was demonstrated that the synthesis of Le\(^a\) in *H. pylori* occurs in the same way as observed in mammalian cells, namely, addition of fucose (in the form of GDP-fucose) onto the type 2 precursor molecule LacNAc by α\(_{1,3}\) FucT [138]. Furthermore, α\(_{1,2}\) FucT was shown to be the enzyme that can synthesize the difucosylated Le\(^\alpha\) antigen from the monofucosylated Le\(^\alpha\) antigen. The presence of some cis-elements in these FucT-encoding genes could allow *H. pylori* to produce complicated and interchangeable LPS antigens. These cis-elements can explain why, from a single strain, several variants (serotypes) can be isolated, all expressing different LPS structures and having different FucT levels.

- **Molecular genetic basis for the variable expression of Le\(^\alpha\) antigen in *H. pylori* strain NCTC 11637: analysis of cis-elements in the α\(_{1,3}\) fucT gene:**

Type strain *H. pylori* NCTC 11637 possesses two α\(_{1,3}\) FucT genes, *futA* and *futB*, encoding two FucTs with different specificities. The role of the *futA* and *futB* gene products was confirmed through the structural-chemical and serological analysis of mutant strains in which one or both α\(_{1,3}\) FucT genes were inactivated [139]. The data of Appelmelk et al. [139] suggested that in *H. pylori* strain NCTC 11637, the *futB* gene product efficiently fucosylates LacNAc at the terminus of the O-antigen chain, while the *futA* gene product preferentially fucosylates internal LacNAc. Thus, only strains with an intact *futB* reading frame contain terminal mono- and oligomeric Le\(^\alpha\). The presence of terminal Le\(^\alpha\) is required for α\(_{1,2}\) fucosylation in *H. pylori*, and hence strains that have an active *futB* gene also strongly express Le\(^\alpha\).

Both α\(_{1,3}\) FucT genes, *futA* and *futB* (and also the α\(_{1,3}\) FucT genes in other *H. pylori* strains), contain two common sequence features: a polyA-polyC tract in the 5′ end and a 21-mer repeat region in the 3′ end. As a result of the different length of the polyA-polyC tract, the gene can be in the ‘off’ status (encoding a truncated FucT) or in the ‘on’ status (encoding an intact FucT). The length of the polyA-polyC tract can vary between the two homologous genes (*futA* and *futB*) within a single strain as well as between different isolates. The addition or deletion of one or more Cs during DNA replication causes the difference in length of the polyC tract as the result of DNA polymerase slippage. This frameshift mutation may provide a mechanism for switching between the on and off status of the gene at a frequency of < 1%.

Recently, Appelmelk et al. [139] detailed how the alteration of the polyC length in the *futA*futB (see Fig. 11) led to the phase variation of Lewis antigen expression in *H. pylori* strain NCTC 11637 (phenotype: Le\(^{\alpha+}\)). A variant of this strain (K4.1) lacking expression of Le\(^\alpha\) contained the parental *futB* (*C*\(9^{\text{off}}\)) and a mutated *futA* (from the parental C10\(\text{on}\) to the C11\(\text{off}\)). Subsequently, another variant, K5.1, was derived from K4.1 that had switched back to the parental *futAfutB* genotype and Le\(^{\alpha+}\) phenotype. Finally, a variant 1c was isolated with Le\(^{\alpha+}\) and Le\(^{\alpha+}\) phenotype. Sequence analysis revealed that both α\(_{1,3}\) FucT genes were intact (on) in this variant, whereas in NCTC 11637, only *futA* was functional, of which the gene product preferentially fucosylates internal LacNac. Changes in the α\(_{1,3}\) FucT gene status appear to influence the expression of an α\(_{1,2}\) fucosylated epitope. It may be that having both genes switched on in variant 1c increases the availability of terminal Le\(^\alpha\), a precursor of Le\(^\beta\).

- **Molecular genetic basis for the variable expression of Le\(^\beta\) antigen in *H. pylori* strain 26695: analysis of cis-elements in the α\(_{1,2}\) fucT gene:**

While α\(_{1,3}\) FucT is necessary for Le\(^\alpha\) synthesis, both α\(_{1,2}\) and α\(_{1,3}\) FucTs are involved in Le\(^\beta\) synthesis. The publication of the whole genome sequence of *H. pylori* 26695 in 1997 [140] also revealed the existence of two copies of the α\(_{1,3}\) *fucT* gene, whereas no putative α\(_{1,2}\) *fucT* gene had been annotated. In the subsequent commentary by Berg et al. [90], the two ORFs, HP0093 and HP0094, were predicted as the putative α\(_{1,2}\) *fucT* gene (also called *fucC*) in *H. pylori* strain 26695 (Fig. 12). Wang and coworkers [141] reported a detailed analysis of the α\(_{1,2}\) *fucT* gene from *H. pylori* strains 26695 and UA802, which led to the identification of possible mechanisms regulating the phase-variable expression of this gene. Contrary to strain 26695, *H. pylori* strain UA802 contains a single ORF of 300 amino acids with 95% nucleotide sequence similarity to the region

![Fig. 10. Synthesis of Lewis antigens in *H. pylori* UA802. Abbreviations: LacNAc, N-acetyllactosamine; Gal, galactose; Fuc, fucose [89].](image-url)
containing both HP0094 and HP0093 in strain 26695. Like futA/B genes, the H. pylori futC contains a polyC tract as well as imperfect TAA repeats (or so-called A-rich sequence) at the mid-region of the gene that mediate slipped-strand mispairing. The futC genes from various isolates exhibited highly divergent sequences (addition/deletion of the repeat units) within this region (Fig. 12) [141]. In strain 26695, the addition of two C residues in the polyC tract lead to the ‘off’ status of the futC gene because this frameshift leads to the occurrence of a TGA stop codon shortly after the polyC region. However, strain 26695 with the hypothetical ‘off’ status of the futC gene has the Le^- phenotype and is able to produce the full-length FucT protein in vitro, as can do strain UA802. A second element within the futC gene of H. pylori 26695 seems to be responsible for the fact that the translation could be shifted back to the prototype reading frame of UA802 at a high frequency, producing functional proteins. Wang et al. [141] identified within the futC gene of H. pylori 26695 a slippery sequence for ribosomal translation, which is located immediately behind the hypermutable polyC region. This slippery sequence contributing to the –1 ribosomal frameshift in the translation was first identified within E. coli dnaX, the gene for the τ subunit of DNA polymerase III. It includes an internal Shine–Dalgarno-like context and a heptamer (AAAAAAG) followed by a potential stem-loop structure (see Fig. 12). During translation, when the ribosome encounters the A AAA AAG sequence of the mRNA (in the indicated reading frame, but not in the other two reading frames), it can slip to the –1 reading frame (AAA AAA G) at a frequency of approximately 50%. The presence of the Shine–Dalgarno-like sequence and the stem-loop structure enhances this process of frameshifting by interacting with the components of the ribosome. The occurrence of frameshifting also allows the strain 26695 to produce functional Fut proteins, indicating that this mechanism may be a common feature in H. pylori strains.
rance of this mechanism of translational frameshifting in certain *H. pylori* strains, such as 26695 and UA1182, is supported by the ability of the *H. pylori* 26695 furC with the predicted truncated ORFs (HP0094-0093) to express the intact α1,2 FucT protein in vitro, accounting for its Leα phenotype [142]. Interestingly, owing to different repeat numbers of polyC residues, the slippery sequence in strain UA802 is not in frame (AA AAA AG), thus it is not functional. Therefore, this strain is characterized by the prototype reading frame, producing functional proteins.

Other glycosyltransferases in *H. pylori* which have been shown to be involved in generating phase variants are β3-galactosyltransferase and β3-N-acetyl-D-glucosaminyltransferase [20].

Based on these data, phase variation clearly contributes to the heterogeneity of *H. pylori* and may explain the finding that from one patient, several highly related yet different isolates may be obtained. Further studies are required to assess the biological relevance of this phase variation in *H. pylori*.

3.1.3.1.2. O-acetylation: *H. pylori*. In the previous section, it was shown that some DNA repeats, such as the polyC tract in *H. pylori*, play an important role in phase variation. These and other types of repetitive elements in other bacteria have been used as markers for searching gene libraries using Southern hybridization in an attempt to identify novel genes involved in phase variation [143]. However, the availability of whole-genome sequence data now allows this type of searching to be conducted in silico. Saunders and coworkers [144] described an integrated system for the analysis of DNA sequence motifs within complete bacterial genome sequences. With their integrated software system for prokaryotic genome analysis, they now allows this type of searching to be conducted in silico.

The possibility of two different LPS within one bacterial culture has been first reported by Helander et al. [95] for *S. typhimurium*. When applying a phenol/chloroform/petroleum ether method for extraction of LPS from smooth-type *S. typhimurium*, two LPS fractions were isolated with a different extent of glucosylation of the repeating O-antigen units. The only differentiating factor between these fractions seems to be the presence or absence of O-antigen factor 12:2, represented by α-glucose linked to position C4 of the α-galactose of the O-antigen saccharide backbone and recognized by specific antiserum (Fig. 13). If the chromosomal gene controlling the expression of 12:2 is ‘on’, the specific glucosyltransferase is produced and the LPS of the cell is glucosylated [149]. In most *Salmonella* strains the 12:2-positive form is rare [150], but variants have been described in which the regulatory gene is permanently ‘on’ and all cells are 12:2-positive [151].

The molecular basis of this form variation has so far not been identified. However, a gene oafR [154], close to purE at min 19 of the *Salmonella* chromosome [150], was found responsible for the 12:2 fixation in a strain of *S. enteridis*. This locus was also shown to determine whether a variable strain, with oafR+, of *S. abony* or *S. typhimurium* LT2 was in the 12:2-positive or -negative state [150]. Interestingly, this oafR locus is located outside the LPS biosynthetic (waa and wba) operons [152].

Significantly, quantitative sugar analysis of the LPS fraction containing the highly glucosylated O-antigen revealed that this O-antigen was not totally glucosylated. The results of an SDS–PAGE rather favor the possibility that glucosylation appears at the seventh repeat, suggesting that only the larger O-antigen chains are glucosylated [95]. The regulation of this glucosylation and the point at which it occurs in LPS biosynthesis are not known; however, it has been reported that glucosylation takes place after the repeating units have been synthesized and part-
ally polymerized [149]. So far, it is difficult to understand how modification of only the larger O-antigen chain might be implemented. It is tempting to speculate that interaction with Rol, the factor involved in regulating O-antigen chain length, is involved [93]. There is also some indication that temperature and other environmental conditions alter the preference of the Osfr enzyme for O-chain, as growth at ambient temperatures enhances glucosylation of low-molecular-mass LPS compared to that at 37°C [152].

Based on a study revealing differences in O-antigen structure of a virulent and an avirulent isolate of *S. enteridis*, it appears that virulence in *S. enteridis* may be associated with a large increase in the production of a glucosylated O-antigen repeating unit resulting in LPS with the structure identical to that for the highly pathogenic *S. typhi* [82]. High-molecular-mass LPS from both virulent and avirulent isolates were compared and revealed that the ratio of the non-glucosylated O-antigen repeating unit to the glucosylated repeating unit is 7:1 in the avirulent isolate, while for the virulent isolate, this ratio is 1:1. So it is believed that it will be possible to monitor emerging virulence of serovar *enteridis*, and possibly other *Salmonella* serovars, by analyzing LPS structural heterogeneity during epidemiological investigations [152].

Occasionally, O-antigen conversion and phase variation can occur simultaneously. The above-mentioned phase P22 specific O-antigen conversion in *S. typhimurium* by the con region involves O-antigen modification by glucosylation of the Gal residues in an α1-6 linkage as opposed to the *Salmonella* factor 12 system which is synthesized in the α1-4 linkage [151]. Another interesting aspect about this O-antigen conversion is that it is subject to phase variation [101]. Differential methylation is involved in regulation of this phase variation. Examination of the DNA upstream of *conA* revealed four closely spaced GATC sites available for DNA adenine methylase (Dam) methylation. Differential Dam methylation is known to be involved in the phase-variable regulation of the *E. coli* pap operon [153]. Also phase variation of fimbriae in *E. coli* [154,155] and *S. typhimurium* [156] has been shown to involve variable DNA methylation through the action of the Dam and the leucine-responsive regulatory protein (Lrp) [118,157]. Franko [101] explored the possibility that Dam methylation plays a role in phase variation by placing the con region in the background of a dam host. It was found that phase variation did not occur in this genetic background. Additionally, Franko [101] mutated the four upstream GATC sites, independent of one another, and showed that phase variation was altered. Finally, Tillney [111] has shown that the GATC methylation pattern is different for cultures in the on and off phases.

### 3.1.3.1.4. Size of O-antigen: *S. typhimurium* 798

*S. typhimurium* 798 is known to phase vary from a non-adhesive to an adhesive phenotype. A clinical isolate of *S. typhimurium* 798 obtained from a pig with diarrhea could be grown in two phases with markedly different phenotypes. The two phenotypes initially were identified based on adhesiveness to porcine enterocytes and, thus, were termed adhesive and non-adhesive. The effect of phenotypic switching can also be visualized by changes in colony morphologies and the presence of 10–15 proteins in the envelopes of cells in the adhesive phenotype. Mutants previously constructed with cells in the adhesive phenotype and the transposon *Tn*phaA were screened to identify mutants lacking one or more of the unique proteins. One mutation was cloned and sequenced, and the mutation was shown to be in *rfaL*, the gene that encodes O-antigen ligase. O-antigen ligase is an enzyme required for the ligation of O-antigen polymer to the lipid A–core moiety [158,159]. To demonstrate that *rfaL* expression was controlled by phase variation, phase variants of the *rfaL::Tn*phaA mutant were sought. The phase variants were identified by the characteristic differences in colony morphologies. Cells from each phase subsequently were plated on medium containing 5-bromo-4-chloro-3-indolyl phosphate. Colonies containing cells in the adhesive phase were blue, indicating the production of PhoA, and thus RfAL, while colonies containing cells in the non-adhesive phase were white. The rate of shift from the adhesive to the non-adhesive phenotype was approximately 10⁻³, which is characteristic of this strain. Whether the phase-variable production of O-antigen is related to variable expression of *rfaL* or some other gene in the *rfa* operon still needs to be determined [160]. The adhesive strain expressed an O-antigen that was at least eightfold longer (up to 18 subunits) than that for the non-adhesive strain (only one or two subunits).

It is hypothesized that the adhesive phenotype is the in vivo, virulent form, while the non-adhesive phenotype is the environmental, avirulent form. By modulating the fraction of cells in each phase, long-term colonization of pig intestines by *S. typhimurium* resulting in persistent asymptomatic infections can be promoted.

Interestingly, smooth to rough phase variations were also observed in *Brucella* [161,162]. However, the molecular mechanism for the phase variation in *Brucella* has not been defined.

#### 3.1.3.2. Transcriptional regulation by environmental factors

Several studies have been performed to examine the influence of environmental parameters on LPS synthesis. Analysis of LPS isolates from cells grown under different conditions showed that high, near-growth-limiting salt concentrations, high, near-growth-limiting temperatures, and low, near-growth-limiting pH induce dramatic decreases in the length of the O-specific LPS in several Gram-negative species. Several reports indicate that the LPS structure is not static but rather modifiable in response to environmental parameters.

The first report of a specific O-antigen biosynthesis gene induced at the transcription level by exposure to acid came from McGowan and coworkers who used a subtractive
RNA hybridization approach to identify genes in *H. pylori* of which expression is induced at low pH [163]. This led to the isolation of the *wbcJ* gene whose product is similar to enzymes involved in the conversion of GDP-d-mannose to GDP-L-fucose used in LPS O-antigen biosynthesis in other enteric bacteria [164]. An isogenic *wbcJ* null mutant strain failed to express O-antigen (and expression of Lewis determinants) and was more sensitive to acid stress than was the wild-type strain [163]. The basis of this acid-sensitive phenotype conferred by the *wbcJ* mutation is not known, but in some way O-antigen expression seems to contribute to *H. pylori* acid survival, maybe by providing a barrier, reducing proton influx and thereby helping to maintain periplasmic and intracellular pH within a non-lethal range. In any case, acid resistance must play an important role in the pathogenesis of *H. pylori* because the human stomach and duodenum are the usual sites of colonization. Likewise, the acid-induced upregulation of *wbcJ* expression in *H. pylori* suggested that acid stimulates LPS biosynthesis in this organism. Qualitative differences in LPS profiles were observed in *H. pylori* cells grown at pH 5 compared with pH 7, which suggests that *H. pylori* may alter its LPS structure in response to acidic pH in vivo.

Although phenotypic O-antigen modifications in response to environmental stresses have been reported, the regulatory mechanisms governing these structural changes have often not been determined. For a better understanding of these regulatory networks, further work in this area should be directed at determining how these environmental conditions influence LPS synthesis at the molecular level. Some reports indicate that known positive and negative transcriptional regulatory proteins implicated in the environmental control of virulence in diverse bacterial species also seem to influence LPS expression.

In the case of salmonellae, many virulence factors are regulated via PhoP/PhoQ, a two-component signal transduction system. PhoP/PhoQ-activated genes promote intracellular survival within macrophages and resistance to cationic antimicrobial peptides, whereas PhoP-repressed genes promote entrance into epithelial cells and macrophages by macroinocytosis and stimulate epithelial cell cytokine production. PhoP-activated genes include those that alter the cell envelope through structural alterations of LPS. Both chemical analysis and SDS–PAGE analysis showed a remarkable difference between the LPS of *S. typhimurium* C5 wild-type and its *phoP* (PhoP-constitutive phenotype) derivative. *PhoP* strains appeared to have much shorter O-antigen chains than the wild-type strain [165,166]. More investigations were done on lipid A modifications regulated by the *phoP-phoQ* genes [165]. *S. typhimurium* lipid A is also a dynamic structure that is modified in different environments. Analysis by mass spectrometry revealed that *S. typhimurium* PhoP-PhoQ regulon altered structural modifications of lipid A by the addition of aminoarabinose and 2-hydroxy-myristate under low Mg²⁺-conditions, a condition that activates PhoP-PhoQ.

In several *Bordetella bronchiseptica* strains, LPS production is affected by growth temperature and in some of these cases the global regulator of virulence factors, BvgAS, was involved in this regulation of LPS biosynthesis [167].

### 3.2. Possible roles of O-antigen variations in pathogenesis

The fact that *H. pylori* can give rise to a diversity of diseases, such as gastritis, gastric glandular atrophy, duodenal and gastric ulcer, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma, is remarkable. The phenomenon of phase variation described for this bacterium certainly contributes to the adaptation capacity of this bacterium. This ability to rapidly produce or modify specific structures on the cell surface may be crucial in the establishment of infection, in particular with respect to the variety of biological niches within the host in which some organisms are found. The result of phase variation is a more versatile, more heterogeneous microorganism that can cope better with a variety of different environments: with one set of genes switched on, the microorganism may be able to adhere to mucosal cells of the nasopharynx but not to survive complement attack in serum; with that particular set switched off, the reverse may apply. For example, the presence of a capsule renders *N. meningitidis* resistant to host defense mechanisms, but it hinders invasion into epithelial cells. Similarly, adhesins are necessary for tissue colonization, but they delay the crossing of the bacteria through mucus and can also mediate binding to phagocytic cells that destroy the bacteria [114]. However, the role of antigenic phase variation in *H. pylori* is unclear at this point as it has not been definitively demonstrated that any Lewis antigen is dominant in any disease state, adhesive mechanism or gastric location [89].

In general, O-antigen modifications seem to play an important role at several (at least two) stages of the infection process, including the colonization (adherence) step and the ability to bypass or overcome host defense mechanisms.

#### 3.2.1. Better colonization

The LPS on Gram-negative bacteria with the potential structural diversity to mediate specific adherence probably function as an adhesin. Several alterations of the O-antigen may enhance the binding capacity of this ‘adhesin’.

- **O-antigen modifications mediated by prophage D3 in Pseudomonas:**
  Adhesion of *P. aeruginosa* to eukaryotic cells appears to be a complex phenomenon, since several bacterial components have been implicated experimentally in the adherence of this bacterium to distinct target cells. Recently, it was shown that bacteriophage D3 can increase adhesion of *P. aeruginosa* to human buccal epithelial cells after integration in the host chromosome...
3.2.2. Evasion of host defenses

3.2.2.1. Antigenic variation. The O-polysaccharide is antigenic, and the usual basis for antigenic variation in Gram-negative bacteria rests in differences in their O-polysaccharides. One way bacteria can avoid forces of the host immune response is to periodically change antigens, i.e., to undergo antigenic variation. Antigenic variation is an important mechanism used by pathogenic microorganisms for escaping the neutralizing activities of antibodies and thus evading pre-existing host immunity. Antigens may vary or change within the host during the course of an infection, or alternatively antigens may vary among multiple strains (antigenic types) of a parasite in the population mediated by one of the mechanisms described above.

3.2.2.2. Molecular mimicry. Depending on the Lewis phenotype of the host, the infecting H. pylori strain expressed mainly Le\(^a\) or mainly Le\(^b\). These data suggest that H. pylori LPS Lewis antigen expression may change, depending on the host. This phenotypic change might mediate evasion by the microorganism of host immune attack and allow colonization to persist. Data from experimental infection studies of monkeys clearly suggest an adaptive role [169]. Four monkeys were colonized with the same strain. Bacteria of that strain isolated from animals that expressed Le\(^b\) in their gastric mucosa also strongly expressed Le\(^b\) in their LPS; bacteria isolated from monkeys that expressed Le\(^a\) also strongly expressed Le\(^a\). These data suggest that the Lewis phenotype of the pathogen can vary and can adapt to that of the host. Whether this is also the case in humans is controversial: one study reported a relationship between the Lewis phenotype of the host and that of the colonizing strain of H. pylori [171]. However, this was not confirmed in another study [172].

3.2.2.3. Higher resistance to antibacterial activities of human complement (and antibody). As described earlier, O-antigens are known to confer resistance to serum complement by inhibiting the activation of complement or sterical hindrance of the formation of an effective MAC [173]. Long O-side chains preferentially bind C3 and lead to the formation of weakly bound C5b-9 complexes, which are sterically hindered from integrating into and disrupting the hydrophobic outer membrane [174-176]. This occurs by activating complement at a distance from the bacterial membrane and MAC is then shed from the bacterial cell without effect. Subsequently, phase-variable expression of O-antigen production in S. typhimurium 798 leading to longer O-antigen chains for the adhesive strain will enhance resistance to serum which was confirmed by Kwan and Isaacson [160]. Furthermore, serum resistance is also influenced by O-antigen structure and the amount of O-antigen substituted on core-lipid A [177]. Interestingly, the phase-variable expression of a putative galactosyltransferase that adds a terminal galactose on Galα(1-4)Gal of the core of H. influenzae LOS also contributes to killing resistance by naturally acquired antibody and complement present in human serum [178]. As Galα(1-4)Gal is also found on human glycolipids, it appears that decoration of the cell surface with this host-like antigen blocks antibody-mediated serum bactericidal activity (molecular mimicry). Genetic analysis of the H. influenzae strain within the human respiratory tract demonstrated that Galα(1-4)Gal may not be expressed during carriage but may be advantageous for the organism in inflammatory states such as pneumonia. The phase variable expression of this LOS epitope in H. influenzae is in part directly dependent upon the number of copies of S′-CAAT-3′ within the biosynthesis gene lic2A which encodes the putative galactosyltransferase. Also the lic1A gene contains multiple copies of the tetranucleotide, CAAT, in the S′ portion of the gene. The lic1 locus which comprises four genes licA, B, C and D) is required for the synthesis of choline phosphate (ChoP), a component of H. influenzae LOS [179]. Through slippage, lic1A switches on and off at high frequency which results in loss or gain of ChoP\(^+\) and ChoP\(^-\) LOS glycoforms. In an infant rat model, ChoP\(^+\) variants colonize the nasopharynx more efficiently than ChoP\(^-\) variants. However, ChoP\(^+\) variants are more susceptible to C-reactive protein-dependent, complement-mediated killing and variants found in blood
and cerebrospinal fluid are almost exclusively ChoP−. Thus, the switching of this contingency gene confers an adaptive strategy that is selectively advantageous to the survival of H. influenzae at different stages in its pathogenesis [178].

3.2.3. Enhanced survival under environmental stress conditions

Analysis of LPS isolates from cells grown under different conditions showed that stress conditions (high temperature, various osmotic conditions) often induce dramatic changes in the length of the O-specific LPS which may lead to a higher resistance to these stress conditions. The induced expression of wbcL at pH 5 or less in H. pylori resulting in stimulation of LPS biosynthesis may contribute to acid survival. H. pylori, just like other mucosal pathogens which normally enter the body through the alimentary tract, must contend with the harsh acid conditions (pH less than 2) of the stomach during digestion. In that context, LPS may provide a barrier, reducing proton influx and thereby helping to maintain periplasmic and intracellular pH within a non-lethal range. The composition of the O-antigens also may affect the bacterial surface charge, which could alter membrane permeability under changing pH conditions. Markedly, loss of O-antigen expression is likely to affect the conformation and function of various surface proteins. For example, a complete LPS molecule is necessary for the proper processing and insertion of PhoE [180,181] and OmpF [182] outer membrane proteins in E. coli. S. flexneri mutants lacking O-antigen exhibit aberrant surface localization and decreased secretion of IcsA (see also Section 1.2.1) [38,183].

In addition, further definition of the mechanism of lipid A modifications will help to clarify their role in bacterial pathogenesis. The above-described lipid A modifications mediated by PhoP-PhoQ may also take place within host cells because phagosomes harboring Salmonella are known to be low in Mg2+. A higher substitution of the ester-linked acidic phosphate group in the lipid A portion of the LPS by amino-deoxy-arabinose results in a lower surface negative charge of the lipid A of the LPS, and therefore gives a greater resistance to cationic peptides [184]. This can explain why S. typhimurium is resistant to polymyxin B during growth in Mg2+-limited environments due to the lower overall negative charge of the modified LPS. Secondly, these lipid A modifications can also alter the LPS-mediated host response by lowering cytokine and chemokine production [165] and thus may promote bacterial survival. These observations demonstrate that subtle changes in the structure of lipid A may have profound effects on pathogenesis.

4. O-antigen structural variations in plant symbionts

The recent elucidation of the complete O-antigen structure of R. etli CE3 grown under laboratory conditions revealed that the structural heterogeneity in the O-chain arises mainly from the O-acetyl and O-methyl substitutions [185]. Moreover, another recent report about the structure of lipid A of R. etli CE3 proclaimed the presence of a mixture of six different lipid A structures in this strain [2,3].

In addition to this observed LPS heterogeneity from cultured cells, the structure of LPS is also altered in response to signals from the plant during symbiosis. It appears to be part of a molecular communication between bacterium and host plant. Experiments ex planta suggest that the bacterium in the rhizosphere is preparing its LPS for symbiosis. The LPS structure changes in response to seed and root compounds and the lower pH at the root surface. Moreover, modifications in LPS induced by conditions associated with infection are another indication that specific structures are important. The O-antigen part clearly is important in this infection step. During the differentiation of the bacterium to a bacteroid, Rhizobium LPS undergoes changes in the composition of the O-antigen, presumably in response to the change of environment. Therefore, new efforts devoted to determining the basal and adaptive structures of this portion of the LPS should greatly help to understand the role of LPS in symbiosis.

4.1. Identification of O-antigen changes during symbiosis

Because a potential host plant challenges the bacteria with a series of different microenvironments, surface adaptations are to be expected. Therefore, LPS structural modifications and adaptations in rhizobia currently are investigated in detail.

4.1.1. Subtle changes in LPS antigenicity and size distribution

4.1.1.1. Changes of O-antigen ex planta. R. etli CE3 has been found to alter its LPS antigenically during growth ex planta in exudates of host seeds and roots [186]. These changes were observed using monoclonal antibodies against the O-antigenic part of the LPS. The inducers from the seeds are anthocyanins, compounds that also induce the nod genes of R. etli [187]. In addition, Reuhs et al. [188] have shown that Sinorhizobium fredii LPS also change in response to the presence of host root extract in the growth medium. Thus, although there is a structural conservation of LPS in the form found in cultured cells throughout the genus Sinorhizobium, this finding suggests that the cells may express different forms of LPS in the soil, for instance at the point of infection, which may have an impact on host range, including strain-cultivar specificity [189].

Other workers examining LPS modifications have investigated the effect of different soil environments. A halotolerant strain of S. meliloti was isolated from nodules of a
Melilotus plant grown in a salt marsh in Spain [190]. This strain showed alterations in LPS molecular size and antigenicity that could be related to ionic stress and osmotic pressure. In a similar fashion, Reuhs and coworkers observed that many changes in the expression of Sinorhizobium LPS are associated with growth under varied abiotic conditions, such as pH or temperature [189].

4.1.1.2. Changes of O-antigen in planta. Other immunological studies revealed that certain LPS epitopes appear and that others disappear during bacteroid differentiation. The LPS of pea bacteroids of R. leguminosarum 3841 exhibit a unique antibody epitope and an altered PAGE banding pattern compared to the LPS of this strain grown under typical laboratory conditions. These differences can be mimicked in planta through variation in growth conditions such as growth at acidic pH (pH 4.8) or under low oxygen concentration [191]. These and other conditions, such as high temperature or phosphate starvation of the cells, that may represent stresses to the bacteria, also induce changes in R. etli CE3 LPS that are detected as the loss of reactivity with certain monoclonal antibodies [192]. Bacteroids of this strain also react weakly to these monoclonal antibodies, but the PAGE banding pattern is not appreciably altered.

The monoclonal antibodies were also used as immunocytochemical probes for monitoring in planta the variations in LPS structure that occur during the development and functioning of bacteroids. These studies discovered the expression of some LPS epitopes at defined developmental stages. For example, in pea nodules the LPS epitope recognized by the monoclonal antibody MAC301 and the nitrogenase enzyme almost express simultaneously in time. However, this developmental stage-specific LPS epitope expression is apparently independent of nitrogenase expression since the MAC301 epitope is also expressed in nodules induced by rhizobial nifH mutants unable to fix nitrogen [193]. Additionally, this developmental stage-specific expression pattern can neither easily be explained as being a function of the nodule oxygen, nor could it be significantly mimicked under laboratory culture conditions using defined medium [193]. These findings indicate that LPS expression and molecular adaptation may be generated through mechanisms employing both physiological conditions and plant-specific molecular signals.

The adjustment of the LPS in symbiosis also depends on the type of nodule that a plant generates. Changes that occur in an indeterminate type of nodule can be different from those in a determinate one [194]. Comparison of LPS from bacteroids of pea (indeterminate) and bean nodules (determinate) showed that the LPS isolated from bacteria within pea or bean nodules differ in molecular size and in LPS I antibody reactivity from each other and from LPS of free-living bacteria [194].

Finally, immunoblot analysis of LPS extracts from alfalfa bacteroids of two strains of S. meliloti showed that smooth LPS production was modified in the bacteroids. S. meliloti NRG185 bacteroids were shown to produce distinct forms of smooth LPS (S-LPS) which includes the O-antigen. Interestingly, no S-LPS was detected in the polysaccharide preparation from the S. meliloti NRG247 bacteroids, showing that specific bacteroid structures may vary significantly from strain to strain in the same host plant [7].

4.1.2. Chemical basis of LPS changes
The structural basis for LPS adaptation in response to physiological factors and during symbiosis is not yet adequately characterized although several attempts have been made as described here.

- **R. etli CE3**: Bhat and Carlson [195] have shown that the O-chain of the LPS from R. etli CE3 grown at pH 4.8 has an altered methylation pattern. These changes correlate with the weak binding of two monoclonal antibodies to the LPS from bacteria cultured at low pH (4.8) compared with the strong binding when bacteria are cultured at neutral pH. This change in methylation pattern of LPS was also observed in the LPS from nodule bacteria [195]. Moreover, R. leguminosarum bv. viciae and trifolii transconjugants, producing LPS with the R. etli O-antigen sugars, but lacking the tri-O-methyl-fucose, do not bind to ‘anti-R. etli’ antibodies [196]. In addition to the low pH [195], also anthocyanins appear to be involved in triggering the methylation of particular O-antigen sugar residues in R. etli CE3 [197]. The structural change of LPS after induction of the R. etli CE3 with anthocyanins was first detected as the lack of binding to the LPS by monoclonal antibody JIM28. More recently, the sugar compositions of LPS purified after growth with and without anthocyanins have been determined. Growth in anthocyanins leads to approximately twice as much of a 2-O-methylated-6-deoxyhexose, which is believed to be 2-O-methylfucose (Fig. 14). Recently, a mutant strain CE395 that completely lacks the 2-O-methylated 6-deoxyhexose and binds antibody JIM28 much more strongly than the LPS of the wild-type grown without anthocyanins was identified. Therefore, increasing 2-O-methylation of this LPS sugar is correlated with increasingly suppressed JIM28 antibody binding.

- **R. leguminosarum 3841**: Kannenberg and Carlson [198,199] observed that during indeterminate nodule formation the LPS from R. leguminosarum 3841 are subject to a change in certain O-chain structural features as well as an increase in hydrophobicity. They discovered that the LPS from nodule R. leguminosarum bacteria, or from bacteria grown at low pH or low O2, are extracted into the phenol layer during hot phenol/water extraction rather than
largely into the aqueous layer as is found for the LPS from a laboratory culture. The chemical analysis of the hydrophobic LPS form revealed that it contains (i) higher proportions of long-chain fatty acids attached to their lipid A moieties, (ii) larger amounts of neutral sugars and reduced amounts of charged sugars in their O-chain moieties, as well as (iii) higher degrees of acetylation or methylation. Overall, LPS from low-oxygen-cultured rhizobia resembled those of nodule bacteria indicating that, during bacteroid development, a switch takes place from more hydrophilic to more hydrophobic LPS [200].

Interestingly, in addition to this increase in LPS hydrophobicity, Kannenberg and Carlson [199] found that the hydrophobicity of the entire bacterium increases during symbiosis. Thus, the relatively highly acidic nature of the surface of cultured bacteria due to acidic EPS, capsular polysaccharide CPS and the LPS core region, must be dramatically altered during symbiotic infection [199].

4.2. Genetic mechanisms involved in O-antigen changes

The abrupt transition in LPS epitope expression [193,201,202] casts some doubt on whether the observed modifications are manufactured by de novo biosynthesis of LPS molecules. Goosen-de Roo et al. [201], who investigated LPS I epitope expression of *R. leguminosarum* in *Vicia sativa* nodules, observed that epitopes present on rhizobia disappear very abruptly shortly before or during cell internalization. The authors suspected that an active degradation or modification of LPS I epitopes takes place rather than repression of their synthesis.

So far, the mechanisms by which the LPS epitope changes are regulated remain unclear. Neither *nodD* or any other gene on the pSym (*nod-nif* plasmid) is required for the LPS alterations induced by the plant compounds or other environmental parameters [186,192].

4.3. Possible roles of O-antigen variations in symbiosis

4.3.1. Adaptational role ex planta or in planta

The finding that rhizobia exhibit considerable heterogeneity not only in different plant microhabitats, but also in different soil environments, may indicate that the LPS heterogeneity ex planta can also serve the adaptational needs of the local microenvironments in the soil.

So far, whether the O-antigen changes in planta facilitate one or more stages of infection, or which role(s) these LPS epitope changes have in the functioning of the bacteroid, is not known. However, it is not surprising that the development of bacteroids is associated with many changes in cell wall composition compared with free-living rhizobia because of the very different physiological environments experienced by nodule bacteria and free-living forms. Moreover, these changes are not only restricted to changes in cell wall composition. Other physiological and biochemical changes associated with the differentiation of bacteroids include the induction of nitrogenase and hydrogenase, of nitrate reductase and changes in cytochrome levels, and of enzymes for heme biosynthesis [203].

4.3.2. Functional role

The observed differences in LPS of bacteroids versus free-living bacteria have been suggested to play a role in adapting to the conditions inside the symbiosome. Additionally, the differences observed between the LPS I of bacteroids from different legume hosts may reflect the fact that in pea nodules bacteroids are enclosed individually by a peribacteroid membrane, whereas in *Phaseolus* nodules the bacteroids are usually clustered in groups of about 10. Thus, in *Phaseolus* nodules, much less of the surface of bacteroids is in intimate contact with peribacteroid membrane and more of the surface is continuously bathed by peribacteroid fluid. Although the exact nature of the material in the peribacteroid space is not known, it
mediated by surface carbohydrates (Le x) and selectins, systems [204]. For example, primary adhesion of lympho- 
mediated adhesion is well documented in di¡erent animal 
cess is still unresolved, although this surface carbohydrate-
are involved directly or indirectly in such an adhesion pro-
structures [76]. However, the question of whether LPS 
roids derived from mutant rhizobia with modi¢ed LPS 
matter and the peribacteroid membrane is in-
membrane. It is possible that adhesion between the bacte-
membrane and the peribacteroid membrane is in-
volved in maintaining the synchronous division between 
the multiplying rhizobia and the peribacteroid membrane 
during the stage of intracellular multiplication in indeter-
minate nodules and that this process is disrupted in bacte-
roids derived from mutant rhizobia with modi¢ed LPS structures [76]. However, the question of whether LPS 
are involved directly or indirectly in such an adhesion pro-
cess is still unresolved, although this surface carbohydrate-
mediated adhesion is well documented in di¡erent animal systems [204]. For example, primary adhesion of lymphocytes to activated platelets or the endothelium of venules is mediated by surface carbohydrates (Le�) and selectins, specific lectins (see also Section 1.2.1)[204]. The release of bacteria from the infection thread and their enclosure by the peribacteroid membrane could involve similar mechanisms [64]. The function of the increase in LPS hy-
drophobicity may also be necessary for the invasion of 
host cells which occurs by an endocytotic process. Subtle structural changes may also be required for the exchange of metabolites between the bacterial and host symbionts. It is also possible that these structural changes are impor-
tant in providing increased resistance of the bacteroid to a plant defense molecule(s). The intensity of the host defense response is directly correlated with the degree of structural modification of LPS, shown by various mutants [62]. Addi-
tionally, it has been shown that growing \textit{Bradyrhizobium japonicum} under conditions which induce \textit{nod} genes con-
fers increased resistance to the soybean phytoalexin, gly-
ceollin [205].

5. Conclusions

In the interactions between animals/humans and bacte-
rial pathogens, LPS seem to have an important role for infection of and survival in the host and are therefore an important virulence factor. The virulence of LPS of many pathogens resides in the endotoxic activities of the lipid A component of the molecule and the ability of the polysac-
charide chain, i.e. the core and especially the O-antigenic polysaccharide, to provide the bacterium with resistance to host defense mechanisms such as opsonization and phago-
cytosis. Moreover, variations in the structural features of the O-antigen are often coupled with altered virulence. It is suggested that modifications of LPS in different environ-
ments of the host result in synthesis of new LPS structures 
that probably benefit the survival of the pathogen. How-
ever, the exact role of LPS and its modifications in animal pathogenicity is still a controversial matter. For instance, several studies initially supported the concept that \textit{H. pylori} is capable of molecular mimicry of its host through the (phase-variable) expression of specific O-antigen structures resembling the Lewis blood group antigens. However, Appelmelk et al. [20] recently stated that host evasion, although intuitively attractive, is still weak. Interestingly, several recent studies are trying to unravel the genetic mechanisms involved in the O-antigen variations associ-
ated with animal/human pathogens.

The effects of LPS on plant cells have been less well studied. In analogy with animal pathogenesis, LPS are thought to contribute to the restrictive membrane perme-
ability properties of the outer membrane, allowing bacterial growth and survival in harsh environments in planta. In the context of plant pathogenesis, this may involve the exclusion of preformed or induced antimicro-
bial substances of plant origin. In addition, LPS from several plant pathogens have been shown to activate a number of defense-related responses in plants. Furth-
more, whether LPS are structurally modified in response to environmental stresses in planta experienced by bac-
teria during pathogenic interactions remains to be deter-
dined.

The role of LPS and the occurring modifications in symbiosis are also subject to intensive research. On one hand, it appears that the O-antigen is required for success-
ful symbioses in many rhizobia, but variation in O-antigen 
is tolerated. Strains with the same host range often show a considerable variability in O-antigen compositions. On the other hand, other recent findings point to the possibility 
that, within a given host–bacterium interaction, specific structural features are important for infection and/or nodule 
development. As symbiotic infection goes along with structural changes in the LPS O-antigen, there is a strong indication that specific structures are important. Seed and root exudates have been shown to induce O-antigen mod-
ifications ex planta, but LPS have also the potential to be 
modified in planta. In addition to the physiological param-
eters associated within the nodule, unidentified host sub-
stances may be involved in these in planta modifications. In addition, their functions with regard to symbiotic de-
velopment are unknown. Currently, also no information is 
available on the molecular mechanisms in the symbiont 
triggering these alterations.

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