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Perspective/Review

The biosynthesis and biological role of lipopolysaccharide O-antigens of pathogenic *Yersiniae*

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Abstract

Lipopolysaccharide (LPS) is the major component of the outer leaflet of the outer membrane of Gram-negative bacteria. The LPS molecule is composed of two biosynthetic entities: the lipid A—core and the O-polysaccharide (O-antigen). Most biological effects of LPS are due to the lipid A part, however, there is an increasing body of evidence indicating that O-antigen (O-ag) plays an important role in effective colonization of host tissues, resistance to complement-mediated killing and in the resistance to cationic antimicrobial peptides that are key elements of the innate immune system. In this review, we will discuss: (i) the work done on the genetics and biosynthesis of the O-ags in the genus *Yersinia*; (ii) the role of O-ag in virulence of these bacteria; (iii) the work done on regulation of the O-ag gene cluster expression and; (iv) the impact that the O-ag expression has on other bacterial surface and membrane components.

Keywords: Lipopolysaccharide; *Yersinia*; Virulence; Regulation; Biosynthesis; O-Antigen

Contents

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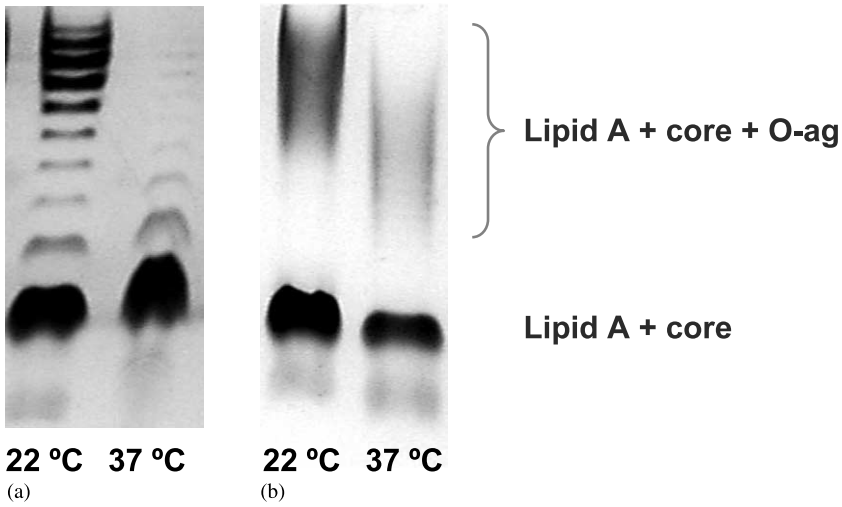


Fig. 1. Silver-stained LPS of *Ye* O:8 (A) and *Ye* O:3 (B) grown at 22 and 37 °C separated in deoxycholate-polyacrylamide-gel electrophoresis. The regions where different parts of LPS migrate are indicated at the right.

1. Introduction

1.1. Outline of the structure and biosynthesis of LPS

1.1.1. LPS structure. Bacteria carry on their outer membrane a heterogeneous population of LPS molecules. This is clearly seen when isolated LPS is analysed in polyacrylamide gel electrophoresis; a wide range of LPS molecules of different sizes are present (Fig. 1). The smallest LPS molecules are composed of lipid A and (inner) core moieties, the slightly larger molecules have lipid A and complete core structure and the largest contain lipid A, a complete core and a variable number of O-ag repeat units (O-unit). Therefore, these LPS populations appear as a ladder in the stained gel. If the repeating unit is a single sugar, the O-ag is called homopolymeric (Fig. 1B), if it is composed of 2 or more different sugar residues, the O-ag is called heteropolymeric (Fig. 1A).

1.1.2. LPS biosynthesis. The biosynthesis of O-ag requires numerous enzymatic activities that take care of the biosynthetic pathways in the cytoplasm leading to individual NDP-activated precursor sugars and transfer the sugar residues from the NDP-sugars in a linkage and sugar-specific manner to the growing oligosaccharide structure. In addition are required the O-unit flippase, O-ag polymerase and O-chain length determinant. Based on this enzymatic mode of biosynthesis, LPSs isolated from bacteria carry a heterogeneous population of O-ag molecules; some do not carry any O-ag while others have variation in the O-ag chain lengths (for more details see Whitfield and co-workers).[1]

The biosynthesis of LPS takes place as two biosynthetic entities:

i) lipid A is synthesized on the cytoplasmic leaflet of the inner membrane. The core sugar residues are sequentially transferred on it by specific glycosyltransferases, and the completed lipid A core is translocated to face the periplasmic side of the inner membrane, and;
ii) the heteropolymeric O-units are synthesized on a lipid carrier molecule, undecaprenyl phosphate (Und-P). A complete O-unit is flipped to the periplasmic face of the inner membrane where Wzy (the O-ag polymerase) polymerizes the O-units and Wzz (the O-ag chain length determinator) controls the length of the O-ag although in a yet not understood manner.[1–3] The homopolysaccharide O-chains synthesized onto Und-P are completed to their full lengths in the cytoplasm after which they are translocated to the periplasmic space by the Wzm and Wzt proteins that are components of an ATP binding cassette transporter system.

After synthesis, O-ag is transferred from the Und-P by WaaL (the O-ag ligase) to the preformed lipid A core molecule. The complete LPS molecule is then translocated onto the outer membrane by a still unknown mechanism.

1.2. The genus *Yersinia* and LPS

The genus *Yersinia* belongs to the family *Enterobacteriaceae* and includes 11 species, of which *Y. pestis*, *Y. pseudotuberculosis* and *Y. enterocolitica* are human pathogens. *Y. pestis* causes bubonic plague while *Y. pseudotuberculosis* and *Y. enterocolitica* cause yersiniosis that is usually a mild diarrheal disease. The genomic sequences of 2 *Y. pestis* strains, the *Y. pseudotuberculosis* serotype O:1b strain and the *Y. enterocolitica* serotype O:8 strain have been determined.

Research on the LPS O-ags of these organisms has involved several approaches. The earliest works used immune sera to type the isolated strains,[4–6] and since the O-ags are highly variable and strongly immunogenic, several serotypes were established for *Y. pseudotuberculosis*[7] and *Y. enterocolitica*[8] based on the variability. On the contrary, *Y. pestis* strains are serologically identical due to lack of O-ag and, as described later, this is explained by genetic studies.

Chemical approaches have elucidated the O-ag structures of a large number of different *Y. pseudotuberculosis* and *Y. enterocolitica* serotypes as well as those of the other *Yersinia* species.[9]

The genetic basis of the *Yersinia* O-ag variation has been studied only recently; the first nucleotide sequence was reported in 1993[10] and the first O-ag gene cluster cloning was done in 1991[11]. At present, a total of 17 O-ag gene cluster sequences have been determined of which 11 are from different *Y. pseudotuberculosis* serotypes[12], three from different *Y. pestis* strains[13–15] and three from different *Y. enterocolitica* serotypes.[10,16–18] Of these nine have been published.

Selected aspects of the biochemistry of the O-ag biosynthesis have been studied in a small number of studies,[19–21] as well as the role of the O-ag in virulence of the pathogenic *Yersinia* (see below). Finally, in some very recent studies the regulation of the O-ag expression has revealed intriguing new information (see below).

In the battle against infectious diseases, it is very important to know the molecular mechanisms by which bacteria cause the disease, and for the LPS work *Yersinia* serves as an exceptionally good study organism because there is a good animal model for the disease.

2. Overview of *Yersinia* LPS research

2.1. Earlier work on genus *Yersinia* LPS

In the genus *Yersinia* the genomic locus between the *hemH* and *gsk* genes is occupied by the genes required for the biosynthesis of the heteropolymeric O-ags.[22] In strains which express homopolymeric O-ags, such as *Y. enterocolitica* O:3 and O:9, the *hemH–gsk* locus is occupied by the outer core (OC) gene cluster.[23] In these strains, the O-ag gene cluster is located elsewhere in the genome.

Long range PCR using primers specific for the *hemH* (forward primer) and the *gsk* genes (reverse primer) produced approx 13 kb fragments from *Y. enterocolitica* serotype O:3, O:5,27, O:9 strains and strains of some other serotypes and approx 20 kb fragments from *Y. enterocolitica* serotype O:8 strain (Skurnik et al., unpublished results), from *Y. pseudotuberculosis* serotype O:1–O:15 strains and also from *Y. pestis*.[13,22] The latter result indicated that *Y. pestis* carries also the O-ag gene cluster even though it does not express any O-ag. We subsequently showed that the *Y. pestis* O-ag gene cluster is almost 100% identical to that of *Y. pseudotuberculosis* O:1b and that five of the 17 *Y. pestis* genes were inactivated (see below).

2.2. Genetic setup of O-ag gene clusters of *Y. pseudotuberculosis* and *Y. pestis*

The genetic setup of the O-ag gene clusters of different *Y. pseudotuberculosis* serotypes has been recently elucidated in the laboratories of Peter Reeves and Mikael Skurnik both by gene-specific PCRs and by sequencing.[12,13,24–26] Details of the gene clusters will be available in the Bacterial Polysaccharide Gene Database (http://www.mmb.usyd.edu.au/BPGD/default.htm) and the genetic organization of a representative set along with known O-antigen structures is presented in Fig. 2A and B. The gene clusters carry biosynthetic gene modules and the O-ag variability is based on the module make up. The known structures of the *Y. pseudotuberculosis* O-ags carry 4 or 5 sugar residues (Fig. 2), however, some of the sugars (such as glucose, galactose and *N*-acetylglucosamine) are also present in other oligo- or polysaccharides, thus their biosynthetic genes may not be within the O-antigen gene clusters. Characteristic biosynthetic gene modules are those carrying genes involved in the biosynthesis of CDP-dideoxyhexoses (the *ddhABCD* and *prt*, *tyv*, *ascEF* or *abe* genes), GDP-mannose (the *manBC* genes) and GDP-fucose (the *gmd* and *fcl* genes). In Fig. 2A are collected gene clusters of serotypes that contain mannose and fucose (except O:4a that does not have fucose) in their O-antigens. Thus, all of them have the GDP-mannose and GDP-fucose modules (except O:4a that is missing the GDP-fucose module). Those serotypes that have *N*-acetylgalactosamine in their structures (O:2b, O:3, O:5a, O:5b and O:4a) carry the *gne* gene encoding for UDP-*N*-acetylglucosamine-4-epimerase that will provide the bacteria with UDP-*N*-acetylgalactosamine. Serotype O:5a and O:5b gene clusters are identical except that the *ddhBC-ascEF* module present in O:5a (responsible for the biosynthesis of CDP-ascarylose) is replaced by the *wby?-wbyR-wbyH* module (responsible for the biosynthesis of CDP-6-deoxyaltrofuranose). This latter module is also present in the serotype O:14 gene cluster and, as the O-antigen structure of O:14 is not known, we can speculate that it most likely contains 6-deoxyaltrofuranose. Furthermore, as the O:14 gene cluster is otherwise almost identical to that of serotype O:1b we can quite safely suggest that the backbone of the O:14 structure is identical to that of O:1b, only the paratose residue of O:1b is replaced by the 6-deoxyaltrofuranose residue. The modules may also include genes for the specific glycosyltransferases (such as *wbyI*, *wbyK* or *wbyL* in the serotype O:1b and O:14

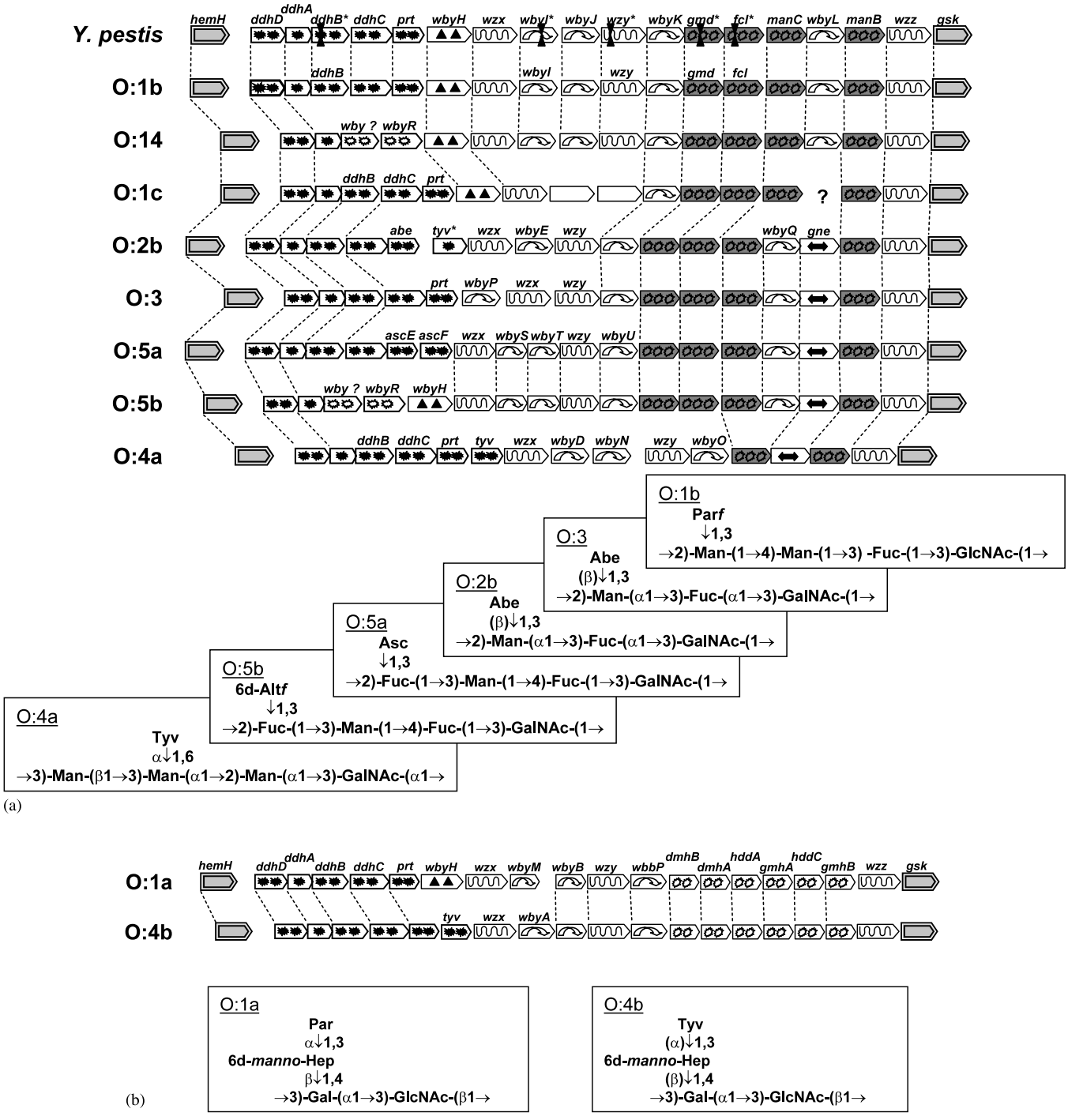
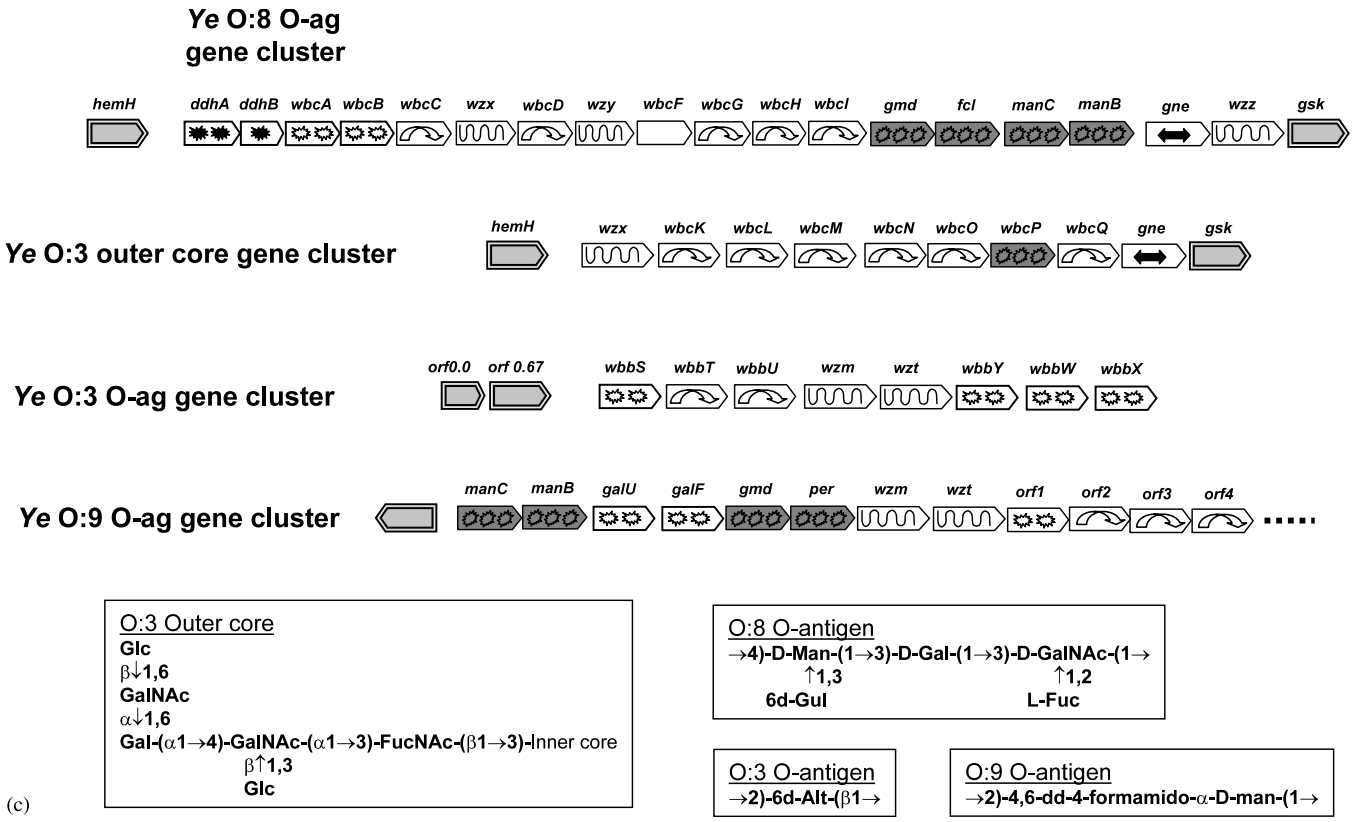


Fig. 2. The genetic organization of the O-ag gene clusters of genus *Yersinia.* Panels A and B. O-ag gene clusters of *Y. pestis* and several *Y. pseudotuberculosis* serotypes[12,13,24–26] and the corresponding chemical structures (structures are not known for serotypes O:1c and O:14). Individual genes are drawn as arrows but not to scale. The *hemH* and *gsk* genes are flanking the O-antigen gene clusters. Homologous genes in the gene clusters are joined by vertical dashed lines. The names of the genes are given above the joined genes. Filling of the gene arrows indicates functions, for example, the CDP-dideoxyhexose pathway genes contain black explosions while the GDP-mannose and GDP-fucose pathway genes are indicated by shading and triple explosions. In the O:1c cluster the open arrows indicate that two unknown genes are present there; the question mark indicates that PCR with *manC* and *manB* specific primers gave no product indicating the presence of a large insertion or severe rearrangement. Panel C. The O-ag gene clusters of *Y. enterocolitica* serotypes O:3, O:8 and O:9 (incomplete in the downstream region) and the OC gene cluster of serotype O:3 and the corresponding chemical structures. The putative functions of the deduced polypeptides of the O:9 open reading frame are: Orf1, perosamine synthesis; Orf2, mannosyltransferase-like; Orf3, mannose formyl transferase-like; Orf4, mannosyltransferase-like.



(c)

Fig. 2. (*Continued*)

clusters) and for the O-unit structure-specific proteins Wzx and Wzy. The serotype O:1a and O:4b gene clusters represent serotypes that contain 6-deoxy-*manno*-heptose in their O-antigens, and the gene clusters have the *wbyB–gmhB* region dedicated for the biosynthesis of GDP-6-deoxy-*manno*-heptose and the necessary glycosyltransferases. These serotypes differ from each other by their dideoxyhexoses, paratose in O:1a and tyvelose in O:4b, and this is reflected by the gene set-up upstream of the *wbyB* gene (Fig. 2B).

In the *Y. pestis* O-ag gene cluster that is >95% identical to that of *Y. pseudotuberculosis* serotype O:1b, five genes are inactivated either by frame shift mutations (four genes) or a small deletion of 62 bp (one gene)[13] (Fig. 2A). The mutations prevent the biosynthesis of GDP-fucose and CDP-paratose, the GDP-mannose pathway is however, intact. The biological significance of these mutations is not clear at present.

Nucleotide sequence analyses of the *Y. pseudotuberculosis* O-ag gene clusters revealed signs of very active horizontal gene transfer of the biosynthetic modules that most likely has involved homologous recombination events and gene transfer by bacteriophages and/or conjugative plasmids carrying IS-elements. For a more detailed treatment of this subject, see Refs. [12, 24, 25].

2.3. Genetics and biosynthesis of *Y. enterocolitica* O-antigens and the OC hexasaccharide of *Y. enterocolitica* serotype O:3

2.3.1. O-Antigen of serotype O:8. The O-ag polysaccharide of *Y. enterocolitica* serotype O:8 (hereafter *Ye* O:8) contains branched pentasaccharide O-units of *N*-acetyl-galactosamine (GalNAc), L-fucose (Fuc), D-galactose (Gal), D-mannose (Man) and 6-deoxy-D-gulose (6d-Gul). The repeat units are linked together by a (1→4) glycosidic bond between GalNAc and Man residues (Fig. 2C).[27] The O-ag gene cluster contains 18 genes which are sufficient to express the O-ag in a heterologous host like *Escherichia coli*. The NDP-activated sugar precursors needed for the *Ye* O:8 O-unit biosynthesis are: (i) CDP-6-deoxygulose, synthesized enzymatically from glucose-1-phosphate by the action of DdhA, DdhB, WbcA and WbcB; (ii) GDP-mannose, synthetized from fructose-1-phosphate by ManA, ManB and ManC; (iii) **GDP-fucose**, synthetized from GDP-mannose by Gmd and Fcl; (iv) UDP-*N*-acetylgalactosamine, synthetized from UDP-*N*-acetylglucosamine by Gne;[21] and (v) UDP-galactose, synthetized from UDP-glucose by GalE. The glycosyltransferases needed to assemble the O-unit on Und-P are WecA (transfers the *N*-acetylgalactosamine residue to Und-P), WbcC, WbcG,

WbcH and WbcI. The cluster also contains the genes encoding for Wzx and Wzy (previously known as WbcE[21]) both required to build up the heteropolymeric O-antigen. The Wzz protein, also present in the cluster, is required to control the O-ag length which, in *Ye* O:8, is of 7–10 repeats.[16,17,21] At present, we do not have a function for the *wbcF* gene product. Thus, all the genes needed are located in the O-ag gene cluster except *manA*, *galE* and *wecA*. The enzymatic activities of the three latter gene products are used in other cellular processes in addition to the O-ag biosynthesis.

Promoter cloning experiments revealed that the O-ag gene cluster contains two promoters, one promoter upstream of *ddhA*, the first gene in the O-ag cluster, and another one between the *manB* and *gne* genes (designated P_{wb1} and P_{wb2}, respectively).[28] These promoters are expressed under a complex regulatory circuit (see below).

2.3.2. O-Antigen and OC serotypes O:3 and O:9. The structures of the LPSs of *Ye* O:3 and O:9 have some peculiarities rarely seen in other enterobacteria. Fig. 2C shows the genetic organization of the O-ag and OC gene clusters of serotype O:3 and that of the O-ag gene cluster of O:9. Based on PCR-analysis, the O:9 OC gene cluster is very closely related to that of O:3. In both serotypes, the OC gene clusters are located between the *hemH* and *gsk* genes while the genomic location of the O-ag clusters are presently unknown. The serotype O:3 O-ag is a homopolymer of (1→2)-linked 6-deoxy-L-altrose[29] and that of O:9, a homopolymer of *N*-formylperosamine,[30] that are attached to the inner core region of the LPS. In addition to the O-ag of these serotypes also the OC hexasaccharide is attached to the inner core thus forming a short branch in the LPS molecule. This peculiar structure has made it possible to construct mutants that are missing either the O-antigen, the OC or both, and that has been extensively studied with serotype O:3.[23,31,32]

2.4. *Yersinia* O-antigen and virulence

Our first efforts were devoted to characterize the role of *Y. enterocolitica* O-ag in virulence. To this end, two rough mutants were isolated from *Ye* O:8 and *Ye* O:3 by resistance to lysis by bacteriophages and they were used to infect mice orogastrically.[17,33] Significantly, both mutants were attenuated with LD_{50} values 50–100 times higher than the wild type (wt) strains. To pinpoint the phase of infection in which the mutants manifest the attenuation, mice were co-infected with the wt strains and each of the rough strains. Neither of the mutants colonized the Peyer's patches as efficiently as the wt strains and they did not multiply in spleen, liver and mesenteric lymph nodes. The importance of *Ye* O:8 O-ag as virulence factor has been further corroborated by signature-tagged transposon mutagenesis (STM) experiment as 23% of the attenuated mutants had insertions inactivating genes in the O-antigen gene cluster.[34]

We have also studied the relative contribution of OC to *Ye* O:3 virulence. The LD_{50} of the OC mutant was approx 1000 times higher than the wt in orogastrically infected mice.[32] However, in contrast to the rough mutant, co-infection experiments revealed that the OC mutant did colonize the Peyer's patches as efficiently as the wt but it was much less efficient in colonizing deeper organs and at 5 days postinfection it was completely eliminated from Peyer's patches. It is then clear that *Ye* O:3 OC and O-ag play different roles during infection. It seems that O-ag is needed during the first hours of infection whereas the OC is required for prolonged survival of the bacteria in Peyer's patches and for invasion of deeper tissues like liver and spleen.

Interestingly, O-ag also plays a role in virulence of *Y. pseudotuberculosis*. Using signature-tagged transposon mutagenesis (STM), Mecsas and co-workers found that 15% of the isolated mutants do not express O-ag.[35] Similar to *Y. enterocolitica* rough strains, these *Y. pseudotuberculosis* mutants were unable to reach the Peyer's patches and, furthermore, they did not invade epithelial cells as efficiently as the wild type.

On the contrary, O-ag does not play any role in *Y. pestis* virulence since this species does not express O-ag.[13,36–39] Interestingly, expression of a heterologous O-ag in *Y. pestis* did not affect its virulence.[40] However, we cannot at present rule out that expression of *Y. pestis* homologous O-ag may affect the virulence of this species.

We can now only speculate on the specific role of O-ag in virulence. It could be that O-ag prevents the access of harmful molecules into the outer membrane as has been demonstrated for many pathogens. For example, it is generally accepted that O-ag is involved in the resistance to complement-mediated killing and to antimicrobial peptides. However, our initial results indicated that this is not the case in *Y. enterocolitica*.[32] It is also conceivable that some virulence factors located in the outer membrane require the presence of the O-ag for its proper expression or functionality and hence the O-ag role in virulence would be indirect (see below).

2.5. Regulation of O-antigen expression in *Yersinia*

An old dogma with *Y. enterocolitica* is that it expresses less O-ag when grown at 37 °C. This has been demonstrated by bacteriophage susceptibility tests,[41] exposure of antigenic determinants to antibodies,[42] and by chemical analysis of isolated LPS,[43] also clearly shown in the DOC-PAGE analysis in Fig. 1. By Northern blot analysis, we could reveal that O-ag gene cluster transcription was down-regulated at 37 °C.[28,44] We have

constructed reporter strains in which *lucFF* was introduced downstream of each of the promoters identified in the O-ag gene clusters to study more closely the regulation of O-ag expression.[28,45] These in vitro studies have further confirmed that O-ag expression is down-regulated at 37 °C and that there are signals in addition to temperature that modulate O-ag expression. So far, these signals are the bacterial growth phase, low pH, iron concentration, oxygen tension and ionic strength[45] (Bengoechea et al. unpublished results). It is worth noting that these signals are likely to be found in vivo which led us to speculate whether or not *Y. enterocolitica* expresses O-ag in vivo. Recent experiments have demonstrated that *Ye* O:8 O-ag expression is higher in vivo than in vitro at 37 °C. It is also noteworthy that expression is tissue specific being maximal in Peyer's patches and almost absent in spleen and liver (Bengoechea et al. unpublished results). This finding might indicate that O-ag is indeed expressed during infection at least during colonization of the gut.

2.5.1. Identification of loci involved in temperature regulation of O-antigen expression. The data summarized above clearly indicate that O-ag expression is highly regulated although the molecular basis is largely unknown. Therefore, we have recently started the identification of loci that could be involved in the *Ye* O:8 O-ag regulation. Rather unexpectedly, we found out that the cosmid clones carrying the *Ye* O:8 O-ag gene cluster also expressed the O-ag in a temperature-dependent manner in *E. coli*.[28] Thus, we reasoned that these cosmids might contain loci involved in the regulation of the O-ag expression. Using a genetic approach, construction of cosmid deletions followed by *trans*-complementation experiments, we mapped a region downstream of the O-ag gene cluster containing an operon formed by the *rosA* and *rosB* genes (for regulation of O-ag synthesis) that conferred O-ag temperature dependent expression. These studies also revealed that the RosAB system required the expression of *Ye* O:8 Wzz, the O-ag chain length determinant, to exert its regulatory effect. Expression of RosAB without Wzz did not down-regulate O-ag expression at 37 °C.[26] RosA is a proton motive force-driven efflux pump involved in resistance to amphipathic compound and RosB is a potassium antiporter that together with RosA are also involved in the resistance to antimicrobial peptides.[46] However, all the previous findings were obtained in an *E. coli* background and it was possible that similar results would not be obtained in the *Yersinia* background. To address the role of RosAB and Wzz in O-ag regulation in *Yersinia*, we constructed a *rosA–rosB* double mutant (YeO8-RosA$^-$B$^-$) and a *wzz* mutant (YeO8-ΔWzzGB) and analysed O-ag expression.[28] The *rosAB* double mutant but also *rosA* or *rosB* non-polar mutants expressed significantly more O-ag than *Ye* O:8 when grown at 37 °C. Analysis of O-ag expression by the *Ye* O:8 *wzz* mutant demonstrated that, in addition to displaying a random distribution of O-ag chain length, this mutant also expressed more O-antigen than when the wt was grown at 37 °C.[26]

2.5.2. RosAB and Wzz modulate the O-ag transcription. Taking into consideration that O-ag expression is transcriptionally regulated; it was quite feasible to postulate that RosAB affects the transcriptional regulation of the O-ag gene cluster. Indeed, transcriptional analysis using promoter fusions to of each of the two promoters identified in the O-ag gene cluster, P_{wb1} and P_{wb2}, demonstrated that only the activity of the second promoter was higher in the *rosAB* background than in the wt background.[28] Thus, in the *rosAB* strain at 37 °C the P_{wb2} activity is derepressed leading to over-expression of both Gne and Wzz which is linked to an increase in the O-ag expression. In good agreement, over-expression of *gne* or *wzz* in the wt strain caused an increase in O-ag expression at 37 °C.[26]

To study the role of Wzz in the transcriptional regulation of the O-ag gene cluster, we followed a similar approach as before. Results showed that both promoter fusions were up-regulated in the *wzz* mutant background.[26] Since Wzz is over-expressed in the *rosAB* mutants, we studied the promoter activities in wt over-expressing Wzz. Somewhat unexpectedly, both promoter fusions were down-regulated the effect being more pronounced for the P_{wb2} than for the P_{wb1} promoter and more evident in bacteria grown at 37 °C than in bacteria grown at RT.[26] This finding was in sharp contrast to the LPS phenotype of *Ye* O:8 over-expressing Wzz that produced more O-antigen when grown at 37 °C.[28] Since more O-antigen is produced from a decreased number of transcripts, the efficiency of the post-transcriptional events in the O-antigen biosynthesis must be better. At present, we find two non-exclusive explanations for this: (i) since Wzz, Wzy, Wzx and perhaps also WaaL may form a complex involved in the O-antigen assembly/translocation, it is possible that overexpression of Wzz could facilitate the Wzy polymerase function. Thus, changes in the stoichiometry of the complex could modulate the efficiency of the O-antigen polymerization and translocation resulting in increased expression of O-antigen. (ii) Overexpression of Wzz could also affect the expression or function of systems that are involved in downregulation of the O-antigen expression at the post-transcriptional level. Supporting this explanation, the expression of *rosAB* was affected by Wzz.[28] Thus, it is tempting to speculate that, in addition to repression of the P_{wb2} promoter activity, the presence of the RosAB system compromises the efficiency of the O-ag biosynthetic machinery directly. In any case, at present we cannot rule out the possibility that other systems are affected as well.

2.6. Coordinate regulation of O-antigen and other *Yersinia* virulence factors

One hallmark of pathogenic bacteria is their ability to coordinate the expression of different virulence factors. Moreover, the synthesis and assembly of bacterial structures like the flagellum is energy demanding and consequently they have become subjects of stringent control. On the other hand, the outer membrane, and particularly the O-ag, plays a critical role in the bacterial interaction with the environment and hence it is likely that changes in the O-ag affect bacterial virulence as discussed in the preceding sections. In this context, we have put forward the hypothesis that the expression of other virulence factors could be coordinated with the O-ag regulation. Initially, we focused our attention in those factors located in the outer membrane and analyzed whether their expression is affected in *Ye* O:8 rough strain. Results have shown that *inv* expression was one-twelfth in the mutant as compared to that in the wt (Bengoechea et al., to be published). Of note, Inv is the main factor for penetration of the intestinal epithelium by *Y. enterocolitica*.[47,48] Significantly, this finding may account for the low colonization of Peyer's patches by the rough mutant. Indeed, invasion of epithelial culture cells by the rough mutant was severely impaired (Bengoechea et al. submitted). However, it is worth noting that the LD_{50} of an *inv* mutant is the same as that of the wt[47,48] suggesting that deficient inv expression cannot solely explain the attenuation of the rough strain. Our studies have also shown that the expression of the flagellar master regulatory operon, *flhDC*, was 60-fold higher in the rough mutant than in the wt (Bengoechea et al. to be published). Furthermore, the rough strain was hypermotile and expressed higher amounts of flagellins than the wt. Noteworthy, in *Yersinia* these two phenotypes have been previously linked to *flhDC* over-expression.[49] Interestingly, a recent report demonstrated that the *Yersinia* flagellum basal body functions as a type III secretion system and secretes proteins designated as Fops (flagellar outer proteins).[50] Moreover, the amount of secreted proteins correlates with *flhDC* expression. Thus, the increase in the secretion of flagellins by the rough mutant and the *flhDC* up-regulation led us to explore the possibility that secretion of Fops could be also higher in the rough mutant. One of these proteins is YlpA, a virulence-associated phospholipase A,[51] which, as we expected, was secreted in higher quantities by the rough strain than the wt (Bengoechea et al. to be published). It is worth noting that increased expression and secretion of YlpA, and most probably also of other Fops, did not enhance the rough strain virulence.

Altogether these findings give experimental support to our hypothesis and serve as evidence that the expression of *Ye* O:8 membrane components might be coordinated by a regulatory network. A tantalizing speculation could be that *Y. enterocolitica* somehow senses the O-ag status and this acts as a regulatory signal that, so far, affects the expression of outer membrane components. We are aiming to elucidate the molecular basis behind these regulatory connections.

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