Progress in Understanding the Assembly Process of Bacterial O-Antigen

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Abstract

The discovery that surfaces of the Gram-negative bacteria often carry unique polysaccharide signatures predates most seminal discoveries of molecular biology and biochemistry of the 20th century. The O-antigen component of the lipopolysaccharide has been one of the most intensely studied bacterial polysaccharide surface structures for over 80 years. Yet, many questions about the mechanism of biosynthesis of the O-antigen and its transport to the cell surface still remain unanswered. In this review we provide an overview of how the molecular basis of the O-antigen assembly and trafficking were unraveled in a historical context. We pay particular attention to the emergence of novel technological approaches and how they fueled the elucidation of the O-antigen maturation process. Moreover, we provide a brief perspective on the biosynthesis of enterobacterial common antigen (ECA) and underline the similarities and differences between the pathways used to assemble these two surface polysaccharides. Finally, we highlight key discoveries that led to the understanding of the mechanistic basis of bacteriophage-induced O-antigen modifications. We place special emphasis on the regulation of the length of O-antigen polymers and provide a detailed overview of the models explaining the O-antigen length determination. Finally, we underscore outstanding questions which need to be addressed both structurally and functionally to advance the understanding of the O-antigen assembly, trafficking and export within cellular and molecular contexts.

Introduction

Lipopolysaccharide (LPS) molecules form the outer leaflet of the outer-membrane of many Gram-negative bacteria and are essential components of the bacterial cell envelope. They are comprised of a lipid portion, an oligosaccharide core, and a highly variable polysaccharide known as the O-antigen. The latter is composed of repeating sequences of three to six sugar residues (known as an O-unit). These polymers are remarkably variable due to the chemical nature of the individual carbohydrate subunits as well as their chemical linkages. The LPS O-antigen is one of the essential components of the bacterial survival toolkit, employed in the hostile territories of mammalian tissues. A number of human obligate and opportunistic pathogens such as *Salmonella enterica*, *Francisella tularensis*, *Burkholderia cepacia* and others utilize the O-antigen to avoid phagocytosis and to resist the lytic action of the complement system (Murray, *et al.*, 2003, Murray, *et al.*, 2006, Duerr, *et al.*, 2009, Saldias, *et al.*, 2009). O-antigens have also become a basis for vaccine development against several human pathogens including *Shigella* species (Levine, *et al.*, 2007). Moreover, O-antigens are clinically and epidemiologically important for differentiating the various isolates (serotypes). Having a clear road map of molecular mechanisms employed by the protein machinery involved in O-antigen production is essential for the development of a next generation of therapeutic agents. This, however, requires a well-conserved target mechanism across various bacterial species; hence, understanding the precise molecular basis underlying O-antigen assembly and controlling its maturation in key human pathogens is of utmost biomedical importance.

Overview of O-antigen assembly processes

The building blocks of the O-antigen polymers are oligosaccharide subunits referred to as the O-units. Depending on the bacterial species, the O-unit is composed of three to six sugar residues, which may be arranged in an unbranched or branched fashion. An individual O-unit is assembled on a lipid carrier embedded in the inner membrane and the process takes place in the cytosol. The first sugar residue is added *via* the action of the polyisoprenyl-phosphate *N*-acetylaminosugar-1-phosphate transferases (PNPT) or polyisoprenyl-phosphate hexose-1-phosphate transferases (PHPT) protein family members and further extension occurs by the sequential action of specific glycosyl transferases (Samuel & Reeves, 2003). The polymerization of the O-units into an O-antigen mostly occurs by two distinct pathways: a Wzy polymerase-dependent, an ABC transporter-dependent mechanism (Valvano, 2003) (Figure 1A,B). The third pathway for the O-antigen biosynthesis is known as the synthase-dependent mechanism and so far is known to be present only in one Gram-negative species (*Salmonella enterica* serovar Borreze O:54) and little is presently know about its details (Keenleyside & Whitfield, 1996). It will be briefly presented further in the context of its discovery.

The Wzy-dependent pathway of the O-antigen assembly is named after an inner-membrane resident oligosaccharyltransferase encoded by a *wzy* gene. The individual repeat units bound to the lipid anchor are transported from the cytoplasmic side to the periplasmic side of the inner membrane by the Wzx flippase where they become joined by the Wzy glycosyl transferase/polymerase into a long polysaccharide chain (Valvano, 2003). The polysaccharide O-antigen is then transferred to the inner-core oligosaccharide of the lipid A by the WaaL ligase (Figure 1A). The length of the O-antigen molecules on the bacterial surface is not strictly uniform. When subjected to SDS-PAGE analysis, purified O-antigens exhibit ladder-like pattern

of electrophoretic mobilities with the different bands corresponding to the polymers of varying number of O-units (Jann et al, 1975). This property in the Wzy-dependent system is regulated by members of the polysaccharide co-polymerase (PCP) protein superfamily. Remarkably, Wzy-dependent assembly pathway of the O-antigen and other complex glycans operate in the periplasmic environment, which is devoid of the ATP energy source.

In the ABC-dependent O-antigen biosynthesis pathway the mode of polymerization is notably different. Rather than using Wzy polymerase, the entire polymer is being assembled in the cytosol by dedicated glycosyl transferases and is translocated across the inner membrane by a specialized ATP binding cassette (ABC) transporter (Willis & Whitfield, 2013). Unlike the Wzy-dependent pathway, the regulation of length of the O-antigen polymer in this pathway relies on a very different mechanism, which will be described below (Figure1 B).

Identification and chemical characterization of the carbohydrate component of the LPS

The discovery of the role that bacterial surface polysaccharides play in pathogenicity was realized at the onset of modern molecular microbiology and dates back to the studies done on the causative agent of bacterial pneumonia at the beginning of the 20th century. Seminal work carried out in Oswald Avery's laboratory led to the identification of the material now known to represent the capsule of the *Streptococcus pneumoniae* (Dochez & Avery, 1917). Avery pursued the chemical characterization of the capsule further and together with Heidelberger identified its carbohydrate nature (Heidelberger & Avery, 1923). Several years later Avery together with Dubos convincingly demonstrated the importance of the polysaccharide capsule for the virulence by *S. pneumoniae* (Avery & Dubos, 1930). The current understanding of the biogenesis of the capsular polysaccharides has been reviewed in recent years (Whitfield, 2006, Willis & Whitfield,

2013). Here, we provide an overview of the landmark discoveries underlying the biosynthesis of the O-specific polysaccharide (also referred to as the O-antigen) of Gram-negative bacteria.

The first steps to establish the chemical nature of the component of the Gram-negative bacteria, which are toxic to humans even in minute amounts dates back to the 1930s. The first breakthrough in studying this toxin was made by Boivin and Mesrobeanu, who were able to extract bacterial endotoxin from several bacterial species (Boivin & Mesrobeanu, 1933, Boivin & Mesrobeanu, 1934, Boivin & Mesrobeanu, 1934, Boivin, *et al.*, 1935, Boivin & Mesrobeanu, 1937). This allowed the chemical characterization of antigenic material and it was discovered that the endotoxin was largely made up of polysaccharides and did not contain any protein components (Mesrobeanu & Boivin, 1937). The non-proteinaceous nature of the bacterial antigen was also confirmed by Raistick and Topley (Raistrick & Topley, 1934, Topley, *et al.*, 1937) and by Morgan (Morgan, 1937), who employed a slightly different chemical approach of the endotoxin extraction. At about the same time Kauffmann recognized the remarkable diversity of bacterial surface complex carbohydrates when he realized that endotoxin molecules from various *Salmonella* strains contained unique polysaccharide signatures, now known as the O-antigens (Kauffmann, 1936). Kauffmann subsequently established that heat-inactivated bacteria could be used to immunize rabbits to raise the antibodies, which could then be used to recognize specific O-antigens of different bacterial species (Kauffmann, 1944, Kauffmann, 1949, Kauffmann, 1954). The antibodies raised by Kauffmann would lead to bacterial clumping or agglutination and this property gave rise to serological identification of bacteria, which still remains an important diagnostic tool in modern day clinical microbiology. A further advance was the realization by Morgan, while studying the extracted surface polysaccharides from *Shigella dysenteriae,* that the polysaccharide constitutes only about 50% of the pure bacterial

endotoxin (Morgan, 1937). The chemical nature of the remainder of the molecule emerged from the efforts of Luderitz and Westphal, who isolated the lipid portion of the endotoxin and coined the term lipid A (Westphal & Lüderitz, 1954), The complete chemical structure of lipid A was resolved by NMR almost 30 years later (Takayama, *et al.*, 1983). A detailed overview of the milestone discoveries fundamental to understanding the LPS biogenesis and transport has been recently provided by Ruiz and colleagues (Ruiz, *et al.*, 2009).

Kroger realized early on that a subset of *Salmonella* colonies exhibited a distinctly different morphology from the wild-type strains when grown on solid agar under laboratory conditions (Kroger, 1953). Owing to their unsmooth, flat and unconvex appearance, these colonies were termed 'rough'. Early work on rough mutants demonstrated that they were much more prone to phagocytosis as compared to their smooth counterparts (Skurski, *et al.*, 1959, Slopek, *et al.*, 1959) and that they exhibited a strikingly different sensitivity to certain bacteriophages (Fukasawa & Nikaido, 1960). In an effort to characterize surface polysaccharide signatures of rough mutants, Kauffmann isolated the endotoxins from 25 different rough *Salmonella* strains belonging to different serogroups and noticed that they were completely devoid of a surface serotype-specific O-antigen chains. He also discovered that a portion of the extracted LPS represented an oligosaccharide and that its chemical composition was identical among all of the strains analyzed. The oligosaccharide material was of an identical composition irrespective of the serogroup the rough mutant was derived from and was found to contain glucose, galactose, N-Acetylglucosamine (GlcNAc), and 3-Deoxy-D-manno-oct-2-ulosonic acid (KDO) (Kauffmann, *et al.*, 1961). These discoveries established that in addition to containing a highly diverse O-antigen polymer, the lipopolysaccharide also carries an oligosaccharide core, which is highly conserved in bacteria belonging to different serogroups (Mäkelä & Stocker,

1969). It is now recognized that core oligosaccharides exhibit certain heterogeneity in some bacterial species. At least five different core structures have been described for *E. coli* (Reviewed in (Raetz & Whitfield, 2002)).

The chemistry and enzymology underlying the reactions controlling the O-unit assembly were carried out using radioactively labeled nucleotide sugar precursors and *Salmonella enterica* serovar Typhimurium (referred to as *S.* Typhimurium from here on in) mutants defective in the synthesis of such precursors (Nikaido, *et al.*, 1964, Zeleznick, *et al.*, 1965). These latter groups discovered that the synthesis of the O-antigen is critically dependent on the presence of the membrane fraction (Zeleznick, *et al.*, 1965). Further analysis of the O-unit synthesis by Weiner and colleagues led to the discovery that the intermediate products of the O-unit synthesis remain covalently bound to lipid (Weiner, *et al.*, 1965). Finally, the exact nature of this lipid was delineated by Wright and colleagues who established that the lipid carrier is a C55 polyisoprenoid compound containing a pyrophosphate (Wright, *et al.*, 1967). Further investigations of the O-antigen maturation mechanism had demonstrated that the polymerization occurs in such a way that the growing polysaccharide chain is being transferred from the lipid undecaprenyl-phosphate to the non-reducing end of a new O-unit (Bray & Robbins, 1967, Robbins, *et al.*, 1967).

Although it was clear that the O-antigen must be present on bacterial surface to be agglutinated by the antibodies, the very first unequivocal evidence for its precise cellular localization was obtained by Shands (Figure 3) (Shands, 1965). Shands had used the electron microscopy analysis of the negatively stained sections of both *E. coli* and *S.* Typhimurium, where the O-antigens were labeled by a ferritin-coupled O-antigen specific antibody (Shands, 1966). These studies revealed that some O-antigens extend as far as 150 nM away from the

bacterial surface. Interestingly, in addition to observing the O-antigens on the bacterial surface, Shands also detected the presence of O-antigens on the outer side of the bacterial inner membrane, within the periplasm. Mulford and Osborn later confirmed that this cellular compartment is the site of the O-antigen assembly by employing a combination of pulse-chase experiments with radioactively labeled galactose and visualization by transmission electron microscopy (Mulford & Osborn, 1983, McGrath & Osborn, 1991). Chemical analysis of the actual O-antigen building blocks has been greatly enhanced by the advent of the NMR spectroscopy throughout 1970s and particularly by its applications to the studies of the carbohydrate chemistry (reviewed in (Bubb, 2003)). This approach has truly transformed the ways of how the chemical structures of the O-unit oligosaccharides were being determined and, starting from early 80's, NMR analysis became an essential method of the carbohydrate "sequencing" toolbox (Lindberg, *et al.*, 1981, Kenne, *et al.*, 1983).

Development of the synthetic sugar substrate analogues in the late 80's constituted another major technological advance toward understanding the enzymatic basis of the synthesis of O-antigen building blocks, the O-units (Palcic, *et al.*, 1988). Defined synthetic sugar nucleotide acceptor substrates corresponding to various O-unit intermediates could be used in *in vitro* glycosyl transferase reactions containing activated nucleotide sugar donors and purified proteins. Synthetic sugar acceptors were conjugated to the hydrophobic moieties, which provided a convenient means of purifying the products of such reactions for subsequent analysis by NMR. This methodology has proven to be particularly useful in studies characterizing glycosyl transferases that operate on the lipid linked sugars such as undecaprenol-(GlcNAc-PP), where the intermediate acceptors are difficult to obtain from the natural source or to synthesize chemically (Montoya-Peleaz, *et al.*, 2005, Brockhausen, *et al.*, 2008, Greenfield, *et al.*, 2012).

The development of the synthetic approaches toward generating sufficient amounts of stable acceptor substrates opened up a way to probe glycosyl transferase specificities, greatly aiding the functional assignment of this class of enzymes in the O-antigen biosynthetic clusters across various species (Montoya-Peleaz, *et al.*, 2005, Brockhausen, *et al.*, 2008, Xu, *et al.*, 2011).

Identification of Wzy-dependent O-antigen biosynthesis gene cluster

The early period of discovery of genes involved in surface polysaccharide biosynthesis saw parallel efforts of multiple labs working on a variety of organisms that resulted in multiple names for homologous genes and made following the progress of the field rather difficult. A new, unified gene nomenclature was introduced in 1996 (Reeves, *et al.*, 1996) to systematize the field. In the following we adhere to this naming convention and, when appropriate, also provide the original names.

Genes encoding the biosynthetic machinery that is responsible for the production of the polysaccharide portion of the LPS were first characterized using genetic linkage analysis employing bacterial conjugation and phage transduction in *S.* Typhimurium species. Subbaiah and Stocker identified two distinct genetic loci referred to as *rfa* and *rfb*, where mutations at these loci gave rise to rough *S.* Typhimurium devoid of their surface O-antigens (Subbaiah & Stocker, 1964). Further investigations led to the discovery that *rfa* mutants are deficient in the synthesis of the LPS core oligosaccharide (Nikaido, *et al.*, 1964) whereas the mutants in the *rfb* locus were defective in the synthesis of the carbohydrate moieties making up the O-antigen polymer (Beckmann, *et al.*, 1964). Furthermore, Beckman discovered that strains containing mutations in the *rfa* locus were fully capable of the O-antigen synthesis, yet the polymers were of lower molecular weight than those normally covalently bonded to the rest of the LPS (Beckmann, *et al.*, 1964). These studies indicated that the synthesis of the two distinct

carbohydrate portions of the LPS molecule (the oligosaccharide core and the O-antigen, respectively) was controlled by distinct cellular mechanisms. It was also realized that the machinery responsible for the transfer of mature O-antigen to the outer core sugars of the LPS molecule requires a complete core oligosaccharide, which was compromised in the *rfa* mutants (Beckmann, *et al.*, 1964). However, one of the rough *Salmonella* mutants discovered by Subbaiah and Stocker contained a complete core region, yet the O-antigen polymer accumulated in the cell interior instead of populating the cell surface (Kent & Osborn, 1968, Kent & Osborn, 1968). The mutations responsible for this observation were mapped to a gene *waaL* (formerly *rfaL*) within the *rfa* locus and it was proposed that the product of this gene performs the actual transfer of the O-antigen from the lipid-precursor to the sugar residues making up the LPS oligosaccharide core (Kent & Osborn, 1968, MacLachlan, *et al.*, 1991). Subsequent studies using both *in vitro* reconstituted O-antigen ligation reaction as well as *in vivo* functional studies have confirmed that *waaL* is indeed the O-antigen ligase responsible for the ligation of the O-antigen to the LPS outer core sugars (Abeyrathne & Lam, 2007, Han, *et al.*, 2012, Ruan, *et al.*, 2012). E*v*en within the same bacterial species, *waaL* genes frequently exhibit low sequence conservation between strains but the fine points of the catalytic mechanism of the WaaL-mediated ligation are not currently known. O-antigen ligase was recently proposed to function as a metal-independent inverting oligosaccharyltransferase, which utilizes an arginine and a histidine residues located within its periplasm-exposed regions to catalyze the transfer of the undecaprenol-linked polysaccharide to the outer core of the LPS with formation of a β-anomeric linkage (Han, *et al.*, 2012, Ruan, *et al.*, 2012).

WaaL ligase exhibits relaxed substrate specificity with respect to the reducing-end sugar residues (GlcNAc *vs.* Gal) and under some circumstances was even shown to ligate the colanic

acid (containing glucose at its non-reducing end) to the outer LPS core (Meredith, *et al.*, 2007, Han, *et al.*, 2012, Ruan, *et al.*, 2012). It is therefore not surprising that the expression of entire gene clusters for the O-unit synthesis and polymerization in a heterologous host frequently leads to functional O-antigen production (Manning, *et al.*, 1986, Morona, *et al.*, 1991, Falt, *et al.*, 1993). The specificity of WaaL ligase appears to be determined by the nature of the acceptor sugar residues comprising the outer LPS core (Heinrichs, *et al.*, 1998, Abeyrathne, *et al.*, 2005, Schild, *et al.*, 2005).

Relying on bacterial conjugation and interrupted mating experiments, which was a widely-used tool for genetic mapping analysis at the time, Mäkelä established that the *rfb* locus contained all the genes necessary for biosynthesis of the individual O-antigen building blocks, (oligosaccharide O-units). She demonstrated that *rfb* locus is both necessary and sufficient to confer the chemical composition of the O-antigen of the host serotype to the *Salmonella* species of a different serotype (Mäkelä, 1965, Stocker, *et al.*, 1966). The sequence of the entire gene cluster was reported in 1991 by Reeves' group and was found to contain 16 genes encoding mainly enzymes necessary for the nucleotide sugar precursor biosynthesis and the corresponding glycosyl transferases (Jiang, *et al.*, 1991, Samuel & Reeves, 2003). Mäkelä and colleagues also recognized that some *S.* Typhimurium strains contained only a single O-unit attached to the core oligosaccharide, which they termed semi-rough strains (Naide, *et al.*, 1965). They reasoned that these mutants must be deficient in their ability to polymerize the corresponding O-units into mature polymer. Genetic analysis led to the successful identification of a gene found outside of the *rfb* locus and therefore termed *rfc* (now known as *wzy*), which she presumed to be responsible for the polymerization activity (Mäkelä, 1966). The unequivocal confirmation of *wzy* being a *bone fide* O-antigen polymerase was provided some 44 years later by P.G. Wang and co-

workers, who succeeded in reconstituting the O-antigen assembly pathway *in vitro* (Woodward, *et al.*, 2010). Subsequent sequencing of the *rfb* loci from other bacteria revealed that in most analyzed *E. coli* and *Salmonella* species the Wzy polymerase is predominantly found as part of the *rfb* locus, hence the *wzy* gene originally identified by Mäkelä is atypical (Stevenson, *et al.*, 1994, Lukomski, *et al.*, 1996, Fitzgerald, *et al.*, 2003, Fitzgerald, *et al.*, 2006).

In the *E. coli* species, genetic analysis of O-antigen biosynthesis was carried out by the pioneering work of Ørskovs in the early1960s (Ørskov & Ørskov, 1962, Ørskov, *et al.*, 1977). They discovered loci similar to the *Salmonella rfb*. However, Ørskov could not offer an explanation for the incompetency of K-12 strain to make the O-antigen (Ørskov & Ørskov, 1962). The genetic basis for this observation was not uncovered until some ~30 years later when the *rfb* locus of *E. coli* was sequenced and it was established that the open reading frame encoding a rhamnosyl transferase gene contains an insertional element (Liu & Reeves, 1994, Stevenson, *et al.*, 1994). The lack of a functional rhamnosyl transferase gene voids the second step of the O16 O-unit assembly in K-12 strains such as W3110 and MG1655. Another independent mutation was also discovered to occur within the *rfb* locus in the less frequently used WG1 lineage of *E. coli* K-12. It was mapped to the *rmlC* gene involved in the synthesis of the activated nucleotide derivative dTDP-L-rhamnose (Stevenson, *et al.*, 1994).Thus by the late sixties, many of the genes involved in Wzy-dependent O-antigen biosynthesis had been identified and the inner membrane and the periplasm emerged as principal cellular sites of the O-antigen assembly.

Discovery of the O-antigen biosynthesis initiation enzymes

While studying phage-sensitivity of *S. enterica* serovars Minnesota and Montevideo, Mäkelä also reported the identification of yet another O-antigen mutant. While synthesis of the

oligosaccharide core of the LPS was not affected in these mutants, the O-antigen polysaccharides were lacking altogether. (Mäkelä, *et al.*, 1970). The mutation was mapped to the locus distinct from any of the ones previously characterized, which was designated as *rfe*. Mäkelä reasoned that the product of this gene somehow primed the lipid carrier for the O-unit assembly but did not provide any evidence for this claim (Mäkelä, *et al.*, 1970). The product of this particular gene was later found to encode an enzyme capable of transferring an N-acetyl-D-glucosamine-1 phosphate moiety (GlcNAc-P) to the undecaprenyl-phosphate carrier and is currently known as WecA (Figure 2A) (Meier-Dieter, *et al.*, 1992). The same enzyme catalyzes the first step of biosynthesis of another cell-surface polysaccharide present in enteric bacteria, the enterobacterial common antigen (ECA), which will be discussed in more detail below. The O-antigen of *S.* Typhimurium LT2 does not contain GlcNAc sugar but instead the O-unit assembly is primed by transfer of galactose-1-phosphate to the undecaprenyl phosphate. The gene associated with the initiation reaction in this organism was determined by Reeves' group (Wang and Reeves, 1994). They identified a gene within the *rfb* locus, referred to as *wbaP* (formerly *rfbP*), encoding an enzyme, capable of catalyzing a transfer of a galactose-1-phosphate moiety to the undecaprenyl phosphate (Figure 2B) (Wang & Reeves, 1994). WecA and WbaP belong to the PNPTs (polyisoprenyl-phosphate *N*-acetylaminosugar-1-phosphate transferases) and PHTPs (polyisoprenyl-phosphate hexose-1-phosphate transferases) families, respectively, and are presently the only known enzymes responsible for the initiation of complex surface polysaccharide biosynthesis on a lipid anchor in all enterobacteria.

Discovery of the O-antigen flippase

The O-antigen flippase gene, critical to the assembly of O-antigen polymers in Wzy-dependent systems, escaped detection by genetic mapping analysis of the early years.

Sequencing the complete *rfb* loci from several different organisms in early 1990-ties opened effective ways of probing the function of the individual genes using reverse genetics approaches (Liu, *et al.*, 1996). Comparison of the *rfb* gene clusters from different organisms identified a gene named *wzx* (formerly *rfbX*) that encoded a putative inner membrane protein with at least 12 transmembrane domains, but with highly divergent sequences across the species (Liu, *et al.*, 1996). Deletion of this gene led to the accumulation of undecaprenyl-diphosphate linked O-units in the cytosol. This strongly implied that the *wzx* gene encoded a transporter responsible for shuttling O-units across the inner membrane (Liu, *et al.*, 1996). The mechanistic details of how Wzx accomplishes translocation of the lipid-linked O-units to the periplasm have remained obscure. A more detailed understanding started to emerge only recently from a series of ingenious experimental approaches undertaken in J.S. Lam's group on *P. aeruginosa* (Islam, *et al.*, 2010, Islam, *et al.*, 2012). Its Wzx contains 12 transmembrane helices (Islam, *et al.*, 2010) and was proposed to translocate the undecaprenyl-linked O-units *via* an internal cavity rich in positively charged residues (Islam, *et al.*, 2012) by a proton-dependent antiporter-like mechanism (Islam, *et al.*, 2013). Wzx proteins were assumed to exhibit relaxed substrate specificity with respect to the chemical structure of the lipid-linked O-units that they transport (Alaimo, *et al.*, 2006, Marolda, *et al.*, 2006). However, a recent report from Reeves' group indicates that the chemical structure of the repeat O-unit cargo is being recognized by the flippase as part of the translocation mechanism (Hong, *et al.*, 2012). The reader is referred to a recent review by Islam and Lam to learn more about the Wzx flippases (Islam & Lam, 2013).

Discovery of the O-antigen chain length regulation.

The fact that the O-antigens differ not only by their chemical composition but also by the number of repeat units incorporated in the polysaccharide chain was realized once the LPS

molecules were subjected to SDS-PAGE analysis (Jann, *et al.*, 1975). This analysis demonstrated that O-antigen polymers extracted from the same species contained neither the same number of repeat units nor a random distribution of lengths but instead consisted of a population of molecules of similar but not identical lengths (Jann et al, 1975). The molecular basis for such a modal length distribution was attributed to the presence of a specific gene identified by Batchelor and colleagues in 1991. This gene was named *rol*, for Regulator of O-antigen Length (Batchelor, *et al.*, 1991), or *cld*, for Chain Length Determinant (Bastin, *et al.*, 1993), and is now referred to as *wzzB*. The gene encodes a 36 kDa protein containing two transmembrane helices flanking a domain exposed to the periplasmic space (Batchelor, *et al.*, 1991). Shortly thereafter, Reeves' group reported identification of the gene encoding the O-antigen chain length regulator responsible for the very long O-antigen assembly in the *Shigella flexneri* species (Stevenson, *et al.*, 1995). This gene was found within a stably maintained plasmid and exhibited a high sequence identity to the *fepE* open reading frame found in the operon encoding the iron uptake system in the *E. coli* K-12 strain (Stevenson, *et al.*, 1995). Several years later, Morona and colleagues demonstrated that Wzz^{FepE} indeed regulates the assembly of very long O-antigens in *S.* Typhimurium (Murray, *et al.*, 2003).

The question of how Wzz proteins perform the task of controlling the length of the O-antigen arose soon after their discovery. What made this question even more intriguing was the observation that closely related chain length regulators can be expressed in related bacteria and impose the O-antigen length distribution characteristic of their original hosts (Batchelor, *et al.*, 1992, Morona, *et al.*, 1995). Thus, it was clear that the regulation of the O-antigen length was being intrinsically defined by the chain-length regulator protein, in spite of the differences in the chemical structures of the corresponding carbohydrate constituents and despite low sequence

conservation among the other components of the polysaccharide trafficking machinery (flippase, ligase and polymerase).

In 2008, the first crystal structures of several O-antigen chain length regulators were reported by Cygler and colleagues who solved the structures of the periplasmic domains of both WzzB and Wzz^{FepE} as well as the PCP family member involved in the ECA (enterobacteria common antigen) synthesis (Wzz^{ECA}) (Tocilj, *et al.*, 2008). These crystal structures demonstrated that chain length regulators assemble into the bell-shaped oligomers of varying composition, ranging from pentamers (for WzzB) to octamers (for WzzE) to nonamers (for Wzz^{FepE}). The structural analysis also revealed that all these chain length regulators adopt a similar three-dimensional fold in spite of sharing very little sequence identity (Tocilj, *et al.*, 2008). This finding strongly suggested a common molecular mechanism utilized by these molecules for glycan chain length control.

Proposed models of the O-antigen chain-length regulation in the Wzy-dependent assembly

Several models were put forth to explain how chain length regulation may take place. Prior to the discovery of the chain-length regulator Goldman and Hunt proposed, based on a mathematical modeling approach, that the polymerase and the ligase exhibit certain specificity toward the O-antigens of certain lengths (Goldman & Hunt, 1990). However, work by McGrath and Osborn demonstrated that O-antigens achieve their proper lengths even if they cannot be ligated to the core oligosaccharide; this excluded the notion of the ligase influence on chain length determination (McGrath & Osborn, 1991). Similarly, Daniels and Lam studying *P. aeruginosa* came to the conclusion that polymerization of modal chain length-distributed O-antigen occurred before ligation to the lipid A core (Daniels, *et al.*, 2002). Furthermore,

identification of the gene encoding the O-antigen chain regulator protein indicated that the activity of the polymerase is likely to be influenced by a new factor not accounted for in Goldman and Hunt's model. Reeves and colleagues proposed another model whereby Wzz proteins were proposed to control the activity of the *wzy* polymerase by switching between the extension (polymerization) and transfer modes. They speculated that Wzz molecules from different species are capable of interacting with different O-antigen polymerases, however, they provided no experimental evidence to back this claim up. Another model was proposed by Morona and colleagues who suggested that the length of the O-antigen chain is being determined by virtue of Wzz acting as a chaperone capable of organizing Wzy (the polymerase) and WaaL (the ligase) in a certain fashion (Morona, *et al.*, 1995). Morona reasoned that different Wzz molecules could influence the kinetics of O-antigen polymerization *via* their ability to differentially organize multiprotein complexes resulting in different stoichiometric ratios of the O-antigen ligase and the O-antigen polymerase (Morona, *et al.*, 1995).

Despite the availability of the Wzz structures, insight into the mechanism of chain length regulation was limited. The observation that the chain length regulators assembled into oligomers of varying composition in crystals provided further support for the Morona model. It was suggested that the differences in the O-antigen chain length distribution were related to the oligomeric composition of the specific Wzz protein, with Wzz molecules forming larger oligomers, thus being capable of recruiting greater numbers of Wzy polymerases for continuous polymerization of the O-antigen (Morona, *et al.*, 2009). Nevertheless, the stoichiometric cellular distribution of Wzz with respect to Wzy-polymerases does not align well with the proposed mechanism since it is well established that Wzz proteins are a lot more abundant than the Wzy-polymerase (Wong, *et al.*, 1999, Carter, *et al.*, 2009).

The proposed models explaining how the length of the O-antigen may be established center around Wzz and Wzy interplay. Reconstitution of the complete O-antigen biosynthesis pathway by Wang and colleagues *in vitro* using purified components provided further experimental evidence to support this notion (Woodward, *et al.*, 2010). This study demonstrated that the length of the mature O-antigen depends solely on the presence of the Wzy polymerase and Wzz chain length regulator and is independent of any other protein components of the O-antigen biosynthetic machinery.

Moreover, increasingly more clues obtained in several recent *in vivo* studies point to the existence of the Wzz-Wzy protein complex. Recent work on the D3 bacteriophage of *P. aeruginosa* revealed that the serotype switching mechanism is critically dependent on a small hydrophobic 3kDa protein (Iap), which mimics the N-terminal transmembrane domain of the Wzz proteins and uses it to compete with chain-length regulators for binding to the endogenous Wzy-polymerase (Taylor, *et al.*, 2013). In addition, an extensive mutational analysis of Wzy-polymerase from *P. aeruginosa* by Islam and Lam identified several mutations within the C-terminal cytosolic loops of Wzy polymerase, which influenced the proper length specification of O-antigen polymers (Islam & Lam, 2013). These discoveries imply that the N-terminal transmembrane domain of the chain-length regulator proteins and the C-terminal regions of Wzy may form a critical interface of the Wzy-Wzz protein complex. However, in spite of an accumulating body of evidence for the existence of such complexes, no physical associations between the polymerase and the chain length regulator have been reported to date (Carter, *et al.*, 2009) (Valvano personal communication, Kalynych and Cygler unpublished data; Morona unpublished data).

Discovery of the ABC-dependent pathway for O-antigen biosynthesis

In 1975 Kopmann and Jann reported that not all enteric bacteria assembled their O-antigens in the same fashion. The *E. coli* serotypes O9 and O8 were found to use a distinctly different mechanism from the Wzy-dependent pathway known at the time (Kopmann & Jann, 1975, Flemming & Jann, 1978). The O-units in these strains were found to be entirely composed of mannose residues (Kopmann & Jann, 1975, Prehm, *et al.*, 1976). The O-antigen assembly did not require a polymerase protein (Wzy) and occurred on the lipid acceptor, which was identified to also be undecaprenol (Weisgerber & Jann, 1982). Synthesis of the polymer was found to be critically dependent on the WecA protein, which was initially proposed to catalyze the formation of a gluco-lipid intermediate (α-glucosyl di-phospho-undecaprenol), on which the rest of the mannose homo-polymer is assembled (Weisgerber, *et al.*, 1984). The initiation reaction was later revisited by Rick and coworkers who demonstrated that the acceptor of mannose resides for the *E. coli* O8 O-antigen is actually the GlcNAc pyrophosphoryl-undecaprenol (Rick, *et al.*, 1994). This was more consistent with the function of the WecA as a GlcNAc-1-phosphate transferase established a few years before (Meier-Dieter *et al*, 1992). The *rfb* locus of the *E. coli* O9 strain was reported in 1995 and it was realized that this gene cluster contained the genes encoding the ATP-dependent transporter of the ABC-2 superfamily (Kido, *et al.*, 1995). Other groups made similar observations. Zhang and colleagues sequenced the O-antigen biosynthesis cluster from *Yersinia enterocolitica* O:3 a few years earlier and identified two genes essential for the export of the O-antigen (Zhang, *et al.*, 1993). The sequence of both proteins was strikingly similar to those involved in the ABC-dependent export of the capsular polysaccharide in *E. coli* and it was concluded that the export of the O-antigen proceeds in an

ATP-dependent fashion (Zhang, *et al.*, 1993). The same observation was also made by Whitfield and coworkers, who discovered a consensus ATP-binding domain in one of the genes of the *rfb* locus in *Klebsiella pneumoniae* O1 species (Bronner, *et al.*, 1994). The ABC transporters mediating export of the O-antigen polysaccharide belong to the family of two-component transporters where the nucleotide binding domain (the ATP hydrolyzing engine) and the actual transporter (the substrate channel) are encoded by two separate genes exemplified by *wzt* and *wzm* of *E. coli* O8, O9, and O9A, and O52 (reviewed in (Greenfield & Whitfield, 2012)). The same configuration is found in the transporters mediating the export of class II capsular polysaccharides in other bacterial species such *Haemophilus influenzae*, *E. coli*, *Neisseria meningitidis*, and others (Whitfield, 2006, Cuthbertson, *et al.*, 2010, Willis & Whitfield, 2013).

The strongest evidence to date proving further support of this type of O-antigen export was shown by the Whitfield group. They used electron microscopy to demonstrate accumulation of O-linked products in the cytosol of the *Klebsiella pneumoniae* O1 cells devoid of genes encoding the transporters (Bronner, *et al.*, 1994). For a long time, the ABC-dependent transport was considered to be limited to the export of O-antigens comprised of homo-oligosaccharides only. However, it was later discovered that a number of species rely on this pathway for the export of hetero-oligosaccharide O-antigens as well (Bronner, *et al.*, 1994, Saigi, *et al.*, 1999, Izquierdo, *et al.*, 2003, Feng, *et al.*, 2004).

It appears that the ABC-dependent transport of O-antigen polysaccharides is much less prevalent than the Wzy-dependent mechanism. Interestingly, in *P. aeruginosa*, both pathways of O-antigen biogenesis operate in parallel (Kintz & Goldberg, 2008, Lam, *et al.*, 2011). The Wzy-dependent pathway gives rise to the serotype-specific O-antigen known as the B-band while the ABC-transporter mediating the assembly of the common antigen referred to as the A-band,

giving rise to two populations of LPS molecules, which co-exist in the same cell (Lam, *et al.*, 2011). Recently, sequencing of the *Vibrio de* O31 genome also revealed the presence of both gene clusters in this microorganism (Aydanian, *et al.*, 2011).

ABC-dependent O-antigen assembly machinery does not employ Wzz proteins but utilizes a completely different mode of chain length regulation. The length of the O-antigen in this system is regulated either by a covalent modification of the terminal carbohydrate residues, as exemplified by methylation in *E. coli* O9a (Clarke, *et al.*, 2004), or appears to depend on stoichiometry of the ABC-transporter relative to other components of the O-antigen biosynthetic machinery, as was shown to be the case in *Klebsiella pneumoniae* O2 (Kos, *et al.*, 2009).

Discovery of the Synthase-dependent O-antigen assembly mechanism

In addition to the two pathways utilized for O-antigen export and assembly described above, Whitfield and Keenleyside identified yet another O-antigen assembly pathway operational in the *Salmonella enterica* O54 serotype (Keenleyside & Whitfield, 1996). In this organism, the O-antigen appears to be polymerized by a protein from a processive glycosyltransferase family known as the synthase, which also includes proteins involved in the synthesis of various bacterial polysaccharides, such as hyaluronan and chondroitin, as well as bacterial cellulose (Romling, 2002, Weigel & DeAngelis, 2007). Molecular details of this mode of O-antigen assembly still remain elusive. Synthase-dependent O-antigen polymerization in *Salmonella enterica* O54 is WecA dependent and proceeds on undecaprenyl-pyrophosphate (Keenleyside, *et al.*, 1994). The details of the chain-length regulation of the synthase-made O-antigens are not currently established but in Gram-positive organisms the length of the polymer appears to be controlled by the cytosolic levels of specific nucleotide-sugar precursors (Forsee, *et*

al., 2009). The synthase is assumed to be involved in the simultaneous export and polymerization of the carbohydrate polymer operating on a range of lipid acceptors. Moreover, different synthases appear to extend the polymer chain at either the reducing (Tlapak-Simmons, *et al.*, 2005) or non-reducing end (Forsee, *et al.*, 2009). It remains to be seen which molecular mechanisms underlie the function of the synthase involved in the O-antigen assembly in Gram-negative enteric bacteria.

Overview of the ECA biosynthesis

Yet another highly abundant polysaccharide decorating bacterial surfaces was discovered in the 1960s. Unlike the O-antigen, this polysaccharide was found to be remarkably conserved across all Enterobacteria (Kunin, *et al.*, 1962, Kunin, 1963) and was thus named the Enterobacterial Common Antigen or ECA (Mäkelä & Mayer, 1976). Its chemical structure was fully elucidated in 1983 when it was determined that it consists of a trisaccharide repeat unit composed GlcNAc, N-acetyl-D-mannosaminuronic acid (ManNAcA), and 4-acetamido-4,6-dideoxy-D-galactose (Fuc4NAc) (Mänel & Mayer, 1978, Lugowski, *et al.*, 1983). The pathway for the ECA polysaccharide assembly was found to share many similarities with the O-antigen biosynthesis. Individual repeat units of both polymers are assembled on the same C55 isoprenoid lipid carrier, undecaprenol diphosphate (Rick, *et al.*, 1998). Both pathways utilize the same initiating enzyme (WecA in *E. coli*) for the formation of the undecaprenyl-diphosphate linked sugar intermediate, which serves as a primer for the subsequent repeat unit assembly (Barr & Rick, 1987). Furthermore, it is widely accepted that the ECA biosynthesis pathway utilizes the same general mechanism of chain elongation mediated by the WzyE polymerase (Barr, *et al.*, 1999, Kajimura, *et al.*, 2005). Moreover, much like the O-antigen, the length of the ECA chains

exhibit a modal length distribution which is being regulated by a dedicated ECA chain-length regulator protein encoded by a *wzzE* gene (Barr, *et al.*, 1999).

Unlike the O-antigen, however, ECA molecules bound to the lipid A can only be observed in the bacterial strains incapable of producing the O-antigen (Mäkelä & Mayer, 1976). In a vast majority of bacterial species, the ECA present in the outer membrane is found covalently bound to the phosphoglyceride *via* a phosphodiester linkage (ECA_{PG}) (Kuhn, *et al.*, 1983). Another subpopulation of ECA molecules is found in the periplasm in a water-soluble lipid-free form and is referred to as ECA_{cyc} owing to its cyclic nature (Dell, *et al.*, 1984, Kajimura, *et al.*, 2005). Both forms of ECA are assembled by the same enzymatic machinery, consisting of dedicated flippase and polymerase proteins (encoded by *wzxE* and *wzyE* genes respectively) (Rick, *et al.*, 2003, Kajimura, *et al.*, 2005).

Bacteriophage-induced O-unit modifications

Even before the biochemical basis of the O-antigen biosynthesis was uncovered, it was realized that the chemical structure of the monosaccharides making up the O-antigen repeat unit can be modified as a result of the prophage integration in several *Salmonella* species (Iseki & Sakai, 1953). This phage-induced conversion of the O-antigen structure was thought to confer the resistance to other homologous phages, which may use the O-antigen as a recognition receptor for cellular entry (Lerouge & Vanderleyden, 2002). O-antigen modifications range from the addition of the O-acetyl and glycosyl groups to selected sugar residues, to the alternation of the chemical linkage between the consecutive repeat unit oligosaccharides (reviewed in (Lerouge & Vanderleyden, 2002)).

Early studies of phage-mediated serotype conversion revealed that phages rely on their own enzymatic machinery to accomplish modification of the O-units. Robbins and colleagues

had shown that the lack of the O-unit acetylation in *Salmonella anatum* upon the infection with the lysogenic phage ε15, was due to repression of the endogenous trans-acetylase protein (Robbins, *et al.*, 1965). The same ε15 phage was also known to change the nature of the glycosidic linkage between the consecutive repeat O-units from α-galactosyl to β-galactosyl type. Losick demonstrated that the phage utilizes an inhibitor of the native bacterial polymerase and employs its own polymerase to alter the type of the anomeric linkage (Losick, 1969). A strikingly similar type of the O-unit modification mechanism was found deployed by the lysogenic phage D3 involved in the *P. aeruginosa* serotype conversion some thirty years later (Newton, *et al.*, 2001). The D3 bacteriophage-encoded short hydrophobic peptide known as Iap was found to suppress the long O-antigen production by associating with the endogenous Wzy polymerase through the transmembrane helix-mediated interactions (Taylor, *et al.*, 2013).

Further understanding of the molecular mechanisms underlying phage-mediated rewiring of the O-antigen biosynthetic pathways has been greatly advanced as a result of the work carried out on *Shigella flexneri* throughout the 1990s and 2000s. In 1991 Verma had identified a phage protein responsible for the O-acetylation of the third rhamnos**e** residue within the tetrasaccharide repeat unit. This protein was an inner membrane O-acetyl transferase (Verma, *et al.*, 1991).

Glucosylation was recognized to be yet another common phage-induced modification of the *Shigella flexneri* O-antigen repeat unit and, depending on the bacteriophage, can occur on any one of the four monosaccharides making up the O-unit. The prophage proteins responsible for this type of modification were identified in the late 1990s. Morona and colleagues (Mavris, *et al.*, 1997) and Huan and colleagues (Huan, *et al.*, 1997) demonstrated that glucosylation is not catalyzed by a single gene product but is rather dependent on three integral membrane proteins known as the GtrA, GtrB, and GtrX. The first two genes are fairly well conserved and are

exchangeable among the different serotypes but the latter is very divergent and encodes a serotype specific glucosyl transferase (Guan, *et al.*, 1999). Guan and colleagues proposed a model based on the experimental evidence, which states that the glucose residue is transferred to the growing O-antigen in the periplasmic space from the undecaprenyl-phosphate-glucose intermediate via the action of GtrX. GtrB was demonstrated to catalyze the transfer of glucose-phosphate to the undecaprenyl lipid carrier on the cytosolic face of the inner membrane, while GtrA was shown to mediate the transfer of the undecaprenyl-phosphate-glucose conjugate to the periplasm.

Interestingly, bacteriophage induced O-antigen glucosylation has been directly linked to the invasiveness of *Shigella flexneri* in the rabbit model of shigellosis (West, *et al.*, 2005). West and colleagues established that the Gtr protein mediated glucosylation of the O-antigen building blocks results in more compact and overall shorter O-antigen chains, which greatly enhances the function of the type III secretion system used to deliver effector virulence factors into the host cells (West, *et al.*, 2005). This finding further underscores the importance of an in-depth understanding of the molecular basis for the O-antigen modifications by bacteriophages as it may have far reaching therapeutic implications.

Future Directions

Despite a significant progress over the past 60 years in understanding the genetic, biochemical and structural basis for O-antigen biosynthesis (Figure 4), a number of critically important questions remain to be answered.

The precise mechanism governing the O-antigen chain length control still remains elusive. Interaction of the chain-length regulator proteins with the growing O-antigen chain

needs to be rigorously investigated to gain some insights into how these proteins regulate the length of the growing chain with little dependence on the chemical structure of O-units. The existence of the Wzy-Wzz protein complex has been a long-lasting uncertainty and to date their physical association in the inner membrane has not been resolved. Are these the only interactions among the components of O-antigen synthesizing machinery? Based on genetic interactions, the Wzx flippase has also been proposed to be part of the O-antigen biosynthesis multiprotein assembly (Marolda, *et al.*, 2006). This has to be investigated further. Biochemical characterization of these protein-protein interactions will be essential to gaining insights into the organization of the cellular O-antigen biosynthesis apparatus. A major high throughput proteomic effort is currently underway, aimed at identification of the membrane-bound protein complexes in *E. coli* K-12 by TAP-tagging and mass spectrometry (A. Emili and M. Babu, personal communication). This approach holds great potential for confirming or refuting the presence of physical associations among the aforementioned components of the O-antigen processing machinery.

Structural analysis of other proteins involved in the assembly of the O-antigen, aside from the polysaccharide co-polymerases, so far has been limited either to the topology mapping or to the modeling approaches (Islam, *et al.*, 2010, Islam, *et al.*, 2012). These difficulties have been mostly attributed to the inner membrane-residence of many of the key players involved. Yet the precise understanding of the O-antigen maturation will require establishing atomic-level snapshots of the O-antigen assembly process including reaction intermediates. High resolution atomic models of the Wzy-polymerase, Wzx flippase and WaaL ligase along with their substrates would constitute a major breakthrough toward understanding the catalytic mechanisms of these oligosaccharyltransferases. Recent advances in membrane protein crystallography

including more effective expression and purification methods (Wagner, *et al.*, 2006), novel crystallization approaches (Cherezov, 2011, Ujwal & Bowie, 2011), along with the rapid development of micro-focus synchrotron beamlines allowing data collection from smaller crystals, make structural studies of these proteins achievable.

Another unanswered question has to do with the molecular mechanism of O-antigen transport to the bacterial surface. The components responsible for the LPS transport have been identified in *E. coli* K-12, which lacks O-antigen polymers (Wu, *et al.*, 2006, Sperandeo, *et al.*, 2007, Sperandeo, *et al.*, 2008). They were proposed to shuttle LPS to the surface using the energy of the cytosolic ATP hydrolysis through a trans-envelope spanning translocation channel (Okuda, *et al.*, 2012). It appears, however, that they recognize the lipid A portion only and are 'ignorant' as to the presence of O-antigen polymers decorating their cargo (Sperandeo, *et al.*, 2009). It is not clear as to how the transport system can accommodate such a high molecular weight modification; this will require establishing a better understanding of the intracellular architecture of the LPS transport machinery at the atomic level. Work by the Kahne group utilizing incorporation of the unnatural amino acid and UV cross linking demonstrates that the LPS molecules are shielded from the hydrophilic environment of the periplasm by binding within the β-jellyroll fold of LptA and LptC proteins with core oligosaccharides presumably facing the solvent (Okuda, *et al.*, 2012). A structural snapshot of the LptA/C-LPS complex would clarify how the LPS transport machinery may accommodate O-antigen polysaccharides. The same question applies to the outer-membrane LPS exit tunnel formed by the plugged β-barrel complex consisting of the LptD (β-barrel) and LptE (the plug) proteins (Freinkman, *et al.*, 2011). LPS molecules traverse the outer membrane through the LptD-LptE channel before being incorporated into the outer leaflet of the outer membrane and, indeed, the purified LptE protein

was shown to bind LPS (Chng, *et al.*, 2010). Teasing out the structural details of LptE-LPS interaction would be necessary to rationalize how O-antigen bearing LPS molecules may be exported to the cell surface.

Precise spatial organization of the O-antigen assembly factories is not currently well understood. Early work by Muhlardt and colleagues utilizing whole-cell electron microscopy-based analysis of *S.* Typhimurium indicated that the newly made LPS molecules emerge at specific sites found on bacterial surface before being evenly distributed throughout the outer membrane. Interestingly, the authors noticed a co-localization of the newly synthesized LPS molecules with adhesion sites connecting the outer and inner membrane (Muhlradt, *et al.*, 1973), frequently referred to as Bayer junctions (Bayer, 1979, Bayer, *et al.*, 1987, Bayer, 1991). The trans envelope spanning passage formed by the LPS transporting Lpt proteins may in fact represent adhesion sites between an outer and an inner membrane observed by Muhlradt and Bayer (Bayer, 1979, Bayer, *et al.*, 1987, Bayer, 1991). However, the very existence of Bayer junctions has been controversial and could not be unequivocally confirmed by other electron microscopy studies (Matias, *et al.*, 2003). Recent technological advances in cryo-electron microscopy and cryo-electron tomography (Rigort, *et al.*, 2012, Grigorieff, 2013, Lucic, *et al.*, 2013) should allow the resolution of these intriguing outstanding questions. By obtaining three dimensional views of various bacterial sub-compartments and docking high resolution atomic structures of individual components into the tomography maps, understanding of the spatial organization of the O-antigen assembly and export machinery in the enteric bacteria will be greatly advanced.

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References

Abeyrathne PD & Lam JS (2007) WaaL of *Pseudomonas aeruginosa* utilizes ATP in *in vitro* ligation of O antigen onto lipid A-core. *Mol Microbiol* **65**: 1345-1359.

Abeyrathne PD, Daniels C, Poon KK, Matewish MJ & Lam JS (2005) Functional characterization of WaaL, a ligase associated with linking O-antigen polysaccharide to the core of *Pseudomonas aeruginosa* lipopolysaccharide. *J Bacteriol* **187**: 3002-3012.

Alaimo C, Catrein I, Morf L, *et al.* (2006) Two distinct but interchangeable mechanisms for flipping of lipid-linked oligosaccharides. *EMBO J* **25**: 967-976.

Avery OT & Dubos R (1930) The specific action of a bacterial enzyme on *Pneumococci* of type Iii. *Science* **72**: 151-152.

Aydanian A, Tang L, Morris JG, Johnson JA & Stine OC (2011) Genetic diversity of O-antigen biosynthesis regions in *Vibrio cholerae*. *Appl Environ Microbiol* **77**: 2247-2253.

Barr K & Rick PD (1987) Biosynthesis of enterobacterial common antigen in *Escherichia coli*. *In vitro* synthesis of lipid-linked intermediates. *J Biol Chem* **262**: 7142-7150.

Barr K, Klena J & Rick PD (1999) The modality of enterobacterial common antigen polysaccharide chain lengths Is regulated by *o349* of the *wec* gene cluster of *Escherichia coli* K-12. *J Bacteriol* **181**: 6564-6568.

Bastin DA, Stevenson G, Brown PK, Haase A & Reeves PR (1993) Repeat unit polysaccharides of bacteria: a model for polymerization resembling that of ribosomes and fatty acid

synthetase, with a novel mechanism for determining chain length. *MolMicrobiol* **7**: 725-734.

Batchelor RA, Haraguchi GE, Hull RA & Hull SI (1991) Regulation by a novel protein of the bimodal distribution of lipopolysaccharide in the outer membrane of *Escherichia coli*. *J Bacteriol* **173**: 5699-5704.

Batchelor RA, Alifano P, Biffali E, Hull SI & Hull RA (1992) Nucleotide sequences of the genes regulating O-polysaccharide antigen chain length (rol) from *Escherichia coli* and *Salmonella* Typhimurium: protein homology and functional complementation. *J Bacteriol* **174**: 5228-5236.

Bayer ME (1979) The fusion sites between outer membrane and cytoplasmic membrane of bacteria: their role in membrane assembly and virus infection. *Bacterial outer membranes, biosynthesis*,(Inouye M, ed.^eds.), p.^pp. 167-202. John Wiley, New York.

Bayer ME (1991) Zones of membrane adhesion in the cryofixed envelope of *Escherichia coli*. *J Struct Biol* **107**: 268-280.

Bayer ME, Bayer MH, Lunn CA & Pigiet V (1987) Association of thioredoxin with the inner membrane and adhesion sites in *Escherichia coli*. *J Bacteriol* **169**: 2659-2666.

Beckmann I, Subbaiah TV & Stocker BA (1964) Rough mutants of *Salmonella* Typhimurium. Ii. Serological and chemical investigations. *Nature* **201**: 1299-1301.

Boivin A & Mesrobeanu L (1933) *C r Soc BioI Paris* **112**: 76.

Boivin A & Mesrobeanu L (1934) *C r Soc BioI Paris* **115**: 304.

Boivin A & Mesrobeanu L (1934) *C r Soc BioI Paris* **115**: 309.

Boivin A & Mesrobeanu L (1937) *C r Soc BioI Paris* **124**: 442.

Boivin A, Mesrobeanu L & Mesrobeanu I (1935) *Arch roum Path exp Microbiol* **8**: 45.

Bray D & Robbins PW (1967) The direction of chain growth in *Salmonella anatum* O-antigen biosynthesis. *Biochem Biophys Res Commun* **28**: 334-339.

Brockhausen I, Larsson EA & Hindsgaul O (2008) A very simple synthesis of GlcNAc-alpha-pyrophosphoryl-decanol: a substrate for the assay of a bacterial galactosyltransferase. *Bioorg Med Chem Lett* **18**: 804-807.

Bronner D, Clarke BR & Whitfield C (1994) Identification of an ATP-binding cassette transport system required for translocation of lipopolysaccharide O-antigen side-chains across the cytoplasmic membrane of *Klebsiella pneumoniae* serotype O1. *Mol Microbiol* **14**: 505-519.

Bubb WA (2003) NMR spectroscopy in the study of carbohydrates: Characterizing the structural complexity. *Concepts in Magnetic Resonance Part A* **19A**: 1-19.

Carter JA, Jimenez JC, Zaldivar M, Alvarez SA, Marolda CL, Valvano MA & Contreras I (2009) The cellular level of O-antigen polymerase Wzy determines chain length regulation by WzzB and WzzpHS-2 in *Shigella flexneri* 2a. *Microbiology* **155**: 3260-3269.

Cherezov V (2011) Lipidic cubic phase technologies for membrane protein structural studies. *Curr Opin Struct Biol* **21**: 559-566.

Chng SS, Ruiz N, Chimalakonda G, Silhavy TJ & Kahne D (2010) Characterization of the two-protein complex in *Escherichia coli* responsible for lipopolysaccharide assembly at the outer membrane. *PNAS*.

Clarke BR, Cuthbertson L & Whitfield C (2004) Nonreducing terminal modifications determine the chain length of polymannose O antigens of *Escherichia coli* and couple chain termination to polymer export via an ATP-binding cassette transporter. *J Biol Chem* **279**: 35709-35718.

Cuthbertson L, Kos V & Whitfield C (2010) ABC transporters involved in export of cell surface glycoconjugates. *Microbiol Mol Biol Rev* **74**: 341-362.

Daniels C, Griffiths C, Cowles B & Lam JS (2002) *Pseudomonas aeruginosa* O-antigen chain length is determined before ligation to lipid A core. *Environ Microbiol* **4**: 883-897.

Dell A, Oates J, Lugowski C, Romanowska E, Kenne L & Lindberg B (1984) The enterobacterial common-antigen, a cyclic polysaccharide. *Carbohydr Res* **133**: 95-104.

Dochez AR & Avery OT (1917) The elaboration of specific soluble substance by *Pneumococcus* during growth. *J Exp Med* **26**: 477-493.

Duerr CU, Zenk SF, Chassin C, Pott J, Gutle D, Hensel M & Hornef MW (2009) O-antigen delays lipopolysaccharide recognition and impairs antibacterial host defense in murine intestinal epithelial cells. *PLoS Pathog* **5**: e1000567.

Falt IC, Schweda EK, Weintraub A, Sturm S, Timmis KN & Lindberg AA (1993) Expression of the *Shigella dysenteriae* type-1 lipopolysaccharide repeating unit in *Escherichia coli* K12/*Shigella dysenteriae* type-1 hybrids. *Eur J Biochem* **213**: 573-581.

Feng L, Senchenkova SN, Yang J, *et al.* (2004) Synthesis of the heteropolysaccharide O antigen of *Escherichia coli* O52 requires an ABC transporter: structural and genetic evidence. *J Bacteriol* **186**: 4510-4519.

Fitzgerald C, Gheesling L, Collins M & Fields PI (2006) Sequence analysis of the rfb loci, encoding proteins involved in the biosynthesis of the *Salmonella enterica* O17 and O18 antigens: serogroup-specific identification by PCR. *Appl Environ Microbiol* **72**: 7949-7953.

Fitzgerald C, Sherwood R, Gheesling LL, Brenner FW & Fields PI (2003) Molecular analysis of the rfb O antigen gene cluster of *Salmonella enterica* serogroup O:6,14 and development of a serogroup-specific PCR assay. *Appl Environ Microbiol* **69**: 6099-6105.

Flemming HC & Jann K (1978) Biosynthesis of the O9 antigen of *Escherichia coli*. Growth of the polysaccharide chain. *Eur J Biochem* **83**: 47-52.

Forsee WT, Cartee RT & Yother J (2009) A kinetic model for chain length modulation of *Streptococcus pneumoniae* cellubiuronan capsular polysaccharide by nucleotide sugar donor concentrations. *J Biol Chem* **284**: 11836-11844.

Freinkman E, Chng SS & Kahne D (2011) The complex that inserts lipopolysaccharide into the bacterial outer membrane forms a two-protein plug-and-barrel. *Proc Natl Acad Sci U S A* **108**: 2486-2491.

Fukasawa T & Nikaido H (1960) Formation of phage receptors induced by galactose in a galactose-sensitive mutant of *Salmonella*. *Virology* **11**: 508-510.

Goldman RC & Hunt F (1990) Mechanism of O-antigen distribution in lipopolysaccharide. *J Bacteriol* **172**: 5352-5359.

Greenfield LK & Whitfield C (2012) Synthesis of lipopolysaccharide O-antigens by ABC transporter-dependent pathways. *Carbohydr Res* **356**: 12-24.

Greenfield LK, Richards MR, Li J, Wakarchuk WW, Lowary TL & Whitfield C (2012) Biosynthesis of the polymannose lipopolysaccharide O-antigens from *Escherichia coli* serotypes O8 and O9a requires a unique combination of single- and multiple-active site mannosyltransferases. *J Biol Chem* **287**: 35078-35091.

Grigorieff N (2013) Direct detection pays off for electron cryo-microscopy. *eLife* **2**: e00573.

Guan S, Bastin DA & Verma NK (1999) Functional analysis of the O antigen glucosylation gene cluster of *Shigella flexneri* bacteriophage SfX. *Microbiology* **145**: 1263-1273.

Han W, Wu B, Li L, *et al.* (2012) Defining function of lipopolysaccharide O-antigen ligase WaaL using chemoenzymatically synthesized substrates. *J Biol Chem* **287**: 5357-5365.

Heidelberger M & Avery OT (1923) The soluble specific substance of *Pneumococcus*. *J Exp Med* **38**: 73-79.

Heinrichs DE, Yethon JA & Whitfield C (1998) Molecular basis for structural diversity in the core regions of the lipopolysaccharides of *Escherichia coli* and *Salmonella enterica*. *MolMicrobiol* **30**: 221-232.

Hong Y, Cunneen MM & Reeves PR (2012) The Wzx translocases for Salmonella enterica O-antigen processing have unexpected serotype specificity. *Mol Microbiol* **84**: 620-630.

Huan PT, Bastin DA, Whittle BL, Lindberg AA & Verma NK (1997) Molecular characterization of the genes involved in O-antigen modification, attachment, integration and excision in Shigella flexneri bacteriophage SfV. *Gene* **195**: 217-227.

Iseki S & Sakai T (1953) Artificial transformation of 0 antigens in *Salmonella* E group. II. Antigen-transforming factor in bacilli of subgroup Es. PI-oc. *Proc Japan Acad* **29**: 127-131.

Islam ST & Lam JS (2013) Wzx flippase-mediated membrane translocation of sugar polymer precursors in bacteria. *Environ Microbiol* **15**: 1001-1015.

Islam ST, Taylor VL, Qi M & Lam JS (2010) Membrane topology mapping of the O-antigen flippase (Wzx), polymerase (Wzy), and ligase (WaaL) from *Pseudomonas aeruginosa* PAO1 reveals novel domain architectures. *mBio* **1**.

Islam ST, Fieldhouse RJ, Anderson EM, Taylor VL, Keates RA, Ford RC & Lam JS (2012) A cationic lumen in the Wzx flippase mediates anionic O-antigen subunit translocation in *Pseudomonas aeruginosa* PAO1. *Mol Microbiol* **84**: 1165-1176.

Islam ST, Eckford PD, Jones ML, Nugent T, Bear CE, Vogel C & Lam JS (2013) Proton-dependent gating and proton uptake by Wzx support O-antigen-subunit antiport across the bacterial inner membrane. *mBio* **4**: e00678-00613.

Izquierdo L, Merino S, Regué M, Rodriguez F & Tomás JM (2003) Synthesis of a *Klebsiella pneumoniae* O-antigen heteropolysaccharide (O12) requires an ABC 2 transporter. *J Bacteriol* **185**: 1634-1641.

Jann B, Reske K & Jann K (1975) Heterogeneity of lipopolysaccharides. Analysis of polysaccharide chain lengths by sodium dodecylsulfate-polyacrylamide gel electrophoresis. *Eur J Biochem* **60**: 239-246.

Jiang XM, Neal B, Santiago F, Lee SJ, Romana LK & Reeves PR (1991) Structure and sequence of the rfb (O antigen) gene cluster of *Salmonella* serovar Typhimurium (strain LT2). *Mol Microbiol* **5**: 695-713.

Kajimura J, Rahman A & Rick PD (2005) Assembly of cyclic enterobacterial common antigen in *Escherichia coli* K-12. *J Bacteriol* **187**: 6917-6927.

Kauffmann F (1936) Untersuchungen uber die Korperantigene in der *Salmonella*-Gruppe. *Z Hyg Infektionskr* **117**: 778-791.

Kauffmann F (1944) Zur Serologie der *Coli*-Gruppe. *Acta Pathologica Microbiologica Scandinavica* **21**: 20-45.

Kauffmann F (1949) On the Serology of the *Klebsiella* group. *Acta Pathologica Microbiologica Scandinavica* **26**: 381-406.

Kauffmann F (1954) *Enterobacteriaceae*. E. Munksgaard.

Kauffmann F, Krueger L, Lüderitz O & Westphal O (1961) [On the immunochemistry of the O-antigen of Enterobacteriaceae. VI. Comparison of the sugar components of

polysaccharides from S and R forms of Salmonella] (in German). *Zentralbl Bakteriol* **182**: 57-66.

Keenleyside WJ & Whitfield C (1996) A novel pathway for O-polysaccharide biosynthesis in *Salmonella enterica* serovar Borreze. *J Biol Chem* **271**: 28581-28592.

Keenleyside WJ, Perry M, Maclean L, Poppe C & Whitfield C (1994) A plasmid-encoded rfbO:54 gene cluster is required for biosynthesis of the O:54 antigen in Salmonella enterica serovar Borreze. *Mol Microbiol* **11**: 437-448.

Kenne L, Lindberg B, Soderholm E, Bundle DR & Griffith DW (1983) Structural studies of the O-antigens from *Salmonella greenside* and *Salmonella adelaide*. *Carbohydr Res* **111**: 289-296.

Kent JL & Osborn MJ (1968) Properties of the O-specific hapten formed *in vivo* by mutant strains of *Salmonella* Typhimurium. *Biochemistry* **7**: 4396-4408.

Kent JL & Osborn MJ (1968) Haptenic O-antigen as a polymeric intermediate of *in vivo* synthesis of lipopolysaccharide by *Salmonella* Typhimurium. *Biochemistry* **7**: 4419-4422.

Kido N, Torgov VI, Sugiyama T, *et al.* (1995) Expression of the O9 polysaccharide of *Escherichia coli*: sequencing of the *E. col*i O9 rfb gene cluster, characterization of mannosyl transferases, and evidence for an ATP-binding cassette transport system. *J Bacteriol* **177**: 2178-2187.

Kintz E & Goldberg JB (2008) Regulation of lipopolysaccharide O antigen expression in *Pseudomonas aeruginosa*. *Future microbiology* **3**: 191-203.

Kopmann HJ & Jann K (1975) Biosynthesis of the 09 Antigen of *Escherichia coli*. *Eur J Biochem* **60**: 587-601.

Kos V, Cuthbertson L & Whitfield C (2009) The *Klebsiella pneumoniae* O2a antigen defines a second mechanism for O antigen ATP-binding cassette transporters. *J Biol Chem* **284**: 2947-2956.

Kroger E (1953) Experimentelle Untersuchungen zum morphologischen S/R- und antigenen O/o-Formenwechsel gramnegativer Darmbakterien. *Zeitschrift für Naturforschung* **8b**: 133-141.

Kuhn HM, Neter E & Mayer H (1983) Modification of the lipid moiety of the enterobacterial common antigen by the "Pseudomonas factor". *Infect Immun* **40**: 696-700.

Kunin CM (1963) Separation, characterization and biological significance of a common antigen in *Enterobacteriaceae*. *The Journal of Experimental Medicine* **118**: 565-586.

Kunin CM, Beard MV & Halmagyi NE (1962) Evidence for a common hapten associated with endotoxin fractions of *E. coli* and other *enterobacteriaceae*. *Proc Soc Exp Biol Med* **111**: 160-166.

Lam JS, Taylor VL, Islam ST, Hao Y & Kocincova D (2011) Genetic and functional diversity of *Pseudomonas aeruginosa* lipopolysaccharide. *Frontiers in microbiology* **2**: 118.

Lerouge I & Vanderleyden J (2002) O-antigen structural variation: mechanisms and possible roles in animal/plant-microbe interactions. *FEMS Microbiol Rev* **26**: 17-47.

Levine MM, Kotloff KL, Barry EM, Pasetti MF & Sztein MB (2007) Clinical trials of *Shigella* vaccines: two steps forward and one step back on a long, hard road. *Nat Rev Microbiol* **5**: 540-553.

Lindberg B, Lindh F & Lonngren J (1981) Structural studies of the O-specific side-chain of the lipopolysaccharide from *Escherichia coli* O55. *Carbohydr Res* **97**: 105-112.

Liu D & Reeves PR (1994) *Escherichia coli* K12 regains its O antigen. *Microbiology* **140**: 49-57.

Liu D, Cole RA & Reeves PR (1996) An O-antigen processing function for Wzx (RfbX): a promising candidate for O-unit flippase. *J Bacteriol* **178**: 2102-2107.

Losick R (1969) Isolation of a trypsin-sensitive inhibitor of O-antigen synthesis involved in lysogenic conversion by bacteriophage epsilon-15. *J Mol Biol* **42**: 237-246.

Lucic V, Rigort A & Baumeister W (2013) Cryo-electron tomography: the challenge of doing structural biology in situ. *J Cell Biol* **202**: 407-419.

Lugowski C, Romanowska E, Kenne L & Lindberg B (1983) Identification of a trisaccharide repeating-unit in the enterobacterial common-antigen. *Carbohydr Res* **118**: 173-181.

Lukomski S, Hull RA & Hull SI (1996) Identification of the O antigen polymerase (rfc) gene in *Escherichia coli* O4 by insertional mutagenesis using a nonpolar chloramphenicol resistance cassette. *J Bacteriol* **178**: 240-247.

MacLachlan PR, Kadam SK & Sanderson KE (1991) Cloning, characterization, and DNA sequence of the rfaLK region for lipopolysaccharide synthesis in *Salmonella* Typhimurium LT2. *J Bacteriol* **173**: 7151-7163.

Mäkelä PH (1965) Inheritance of the O antigens of *Salmonella* groups B and D. *J Gen Microbiol* **41**: 57-66.

Mäkelä PH (1966) Genetic determination of the O antigens of *Salmonella* groups B (4,5,12) and C1 (6,7). *J Bacteriol* **91**: 1115-1125.

Mäkelä PH & Stocker BAD (1969) Genetics of polysaccharide biosynthesis. *Annu Rev Genet* **3**: 291-322.

Mäkelä PH & Mayer H (1976) Enterobacterial common antigen. *Bacteriol Rev* **40**: 591-632.

Mäkelä PH, Jahkola M & Lüderitz O (1970) A new gene cluster *rfe* concerned with the biosynthesis of *Salmonella* lipopolysaccharide. *J Gen Microbiol* **60**: 91-106.

Mänel D & Mayer H (1978) Isolation and chemical characterization of the enterobacterial common antigen. *Eur J Biochem* **86**: 361-370.

Manning PA, Heuzenroeder MW, Yeadon J, Leavesley DI, Reeves PR & Rowley D (1986) Molecular cloning and expression in *Escherichia coli* K-12 of the O antigens of the Inaba and Ogawa serotypes of the *Vibrio cholerae* O1 lipopolysaccharides and their potential for vaccine development. *Infect Immun* **53**: 272-277.

Marolda CL, Tatar LD, Alaimo C, Aebi M & Valvano MA (2006) Interplay of the Wzx translocase and the corresponding polymerase and chain length regulator proteins in the translocation and periplasmic assembly of lipopolysaccharide O antigen. *J Bacteriol* **188**: 5124-5135.

Matias VR, Al-Amoudi A, Dubochet J & Beveridge TJ (2003) Cryo-transmission electron microscopy of frozen-hydrated sections of *Escherichia coli* and Pseudomonas aeruginosa. *J Bacteriol* **185**: 6112-6118.

Mavris M, Manning PA & Morona R (1997) Mechanism of bacteriophage SfII-mediated serotype conversion in Shigella flexneri. *Mol Microbiol* **26**: 939-950.

McGrath BC & Osborn MJ (1991) Localization of the terminal steps of O-antigen synthesis in *Salmonella* Typhimurium. *J Bacteriol* **173**: 649-654.

Meier-Dieter U, Barr K, Starman R, Hatch L & Rick PD (1992) Nucleotide sequence of the Escherichia coli rfe gene involved in the synthesis of enterobacterial common antigen. Molecular cloning of the rfe-rff gene cluster. *J Biol Chem* **267**: 746-753.

Meredith TC, Mamat U, Kaczynski Z, Lindner B, Holst O & Woodard RW (2007) Modification of lipopolysaccharide with colanic acid (M-antigen) repeats in Escherichia coli. *J Biol Chem* **282**: 7790-7798.

Mesrobeanu L & Boivin A (1937) *C r Soc BioI Paris* **124**: 439.

Montoya-Peleaz PJ, Riley JG, Szarek WA, Valvano MA, Schutzbach JS & Brockhausen I (2005) Identification of a UDP-Gal: GlcNAc-R galactosyltransferase activity in *Escherichia coli* VW187. *Bioorg Med Chem Lett* **15**: 1205-1211.

Morgan WT (1937) Studies in immuno-chemistry: The isolation and properties of a specific antigenic substance from *B. dysenteriae* (Shiga). *Biochem J* **31**: 2003-2021.

Morona R, Van Den Bosch L & Manning PA (1995) Molecular, genetic, and topological characterization of O-antigen chain length regulation in *Shigella flexneri*. *J Bacteriol* **177**: 1059-1068.

Morona R, Brown MH, Yeadon J, Heuzenroeder MW & Manning PA (1991) Effect of lipopolysaccharide core synthesis mutations on the production of *Vibrio cholerae* O-antigen in *Escherichia coli* K-12. *FEMS Microbiol Lett* **66**: 279-285.

Morona R, Purins L, Tocilj A, Matte A & Cygler M (2009) Sequence-structure relationships in polysaccharide co-polymerase (PCP) proteins. *Trends BiochemSci* **34**: 78-84.

Muhlradt PF, Menzel J, Golecki JR & Speth V (1973) Outer membrane of salmonella. Sites of export of newly synthesised lipopolysaccharide on the bacterial surface. *Eur J Biochem* **35**: 471-481.

Mulford CA & Osborn MJ (1983) An intermediate step in translocation of lipopolysaccharide to the outer membrane of *Salmonella* Typhimurium. *Proc Natl Acad Sci U S A* **80**: 1159-1163.

Murray GL, Attridge SR & Morona R (2003) Regulation of *Salmonella* Typhimurium lipopolysaccharide O antigen chain length is required for virulence; identification of FepE as a second Wzz. *MolMicrobiol* **47**: 1395-1406.

Murray GL, Attridge SR & Morona R (2006) Altering the length of the lipopolysaccharide O antigen has an impact on the interaction of *Salmonella enterica* serovar Typhimurium with macrophages and complement. *J Bacteriol* **188**: 2735-2739.

Naide Y, Nikaido H, Mäkelä PH, Wilkinson RG & Stocker BAD (1965) Semirough strains of *Salmonella*. *Proceedings of the National Academy of Sciences* **53**: 147-153.

Newton GJ, Daniels C, Burrows LL, Kropinski AM, Clarke AJ & Lam JS (2001) Three-component-mediated serotype conversion in *Pseudomonas aeruginosa* by bacteriophage D3. *Mol Microbiol* **39**: 1237-1247.

Nikaido H, Mikaido K, Subbaiah TV & Stocker BA (1964) Rough mutants of *Salmonella* Typhimurium. III. Enzymatic synthesis of nucleotide-sugar compounds. *Nature* **201**: 1301-1302.

Okuda S, Freinkman E & Kahne D (2012) Cytoplasmic ATP hydrolysis powers transport of lipopolysaccharide across the periplasm in *E. coli*. *Science* **338**: 1214-1217.

Ørskov F & Ørskov I (1962) Behaviour of *E. coli* antigens in sexual recombination. *Acta Pathologica Microbiologica Scandinavica* **55**: 99-109.

Ørskov I, Ørskov F, Jann B & Jann K (1977) Serology, chemistry, and genetics of O and K antigens of *Escherichia coli*. *Bacteriol Rev* **41**: 667-710.

Palcic M, Heerze L, Pierce M & Hindsgaul O (1988) The use of hydrophobic synthetic glycosides as acceptors in glycosyltransferase assays. *Glycoconj J* **5**: 49-63.

Prehm P, Jann B & Jann K (1976) The O9 antigen of *Escherichia coli*. Structure of the polysaccharide chain. *Eur J Biochem* **67**: 53-56.

Raetz CR & Whitfield C (2002) Lipopolysaccharide endotoxins. *Annu Rev Biochem* **71**: 635-700.

Raistrick H & Topley WWC (1934) Immunizing fractions isolated from *Bact. aertrycke*. *Br J Exp Pathol* **15**: 113-130.

Reeves PR, Hobbs M, Valvano MA, *et al.* (1996) Bacterial polysaccharide synthesis and gene nomenclature. *Trends Microbiol* **4**: 495-503.

Rick PD, Hubbard GL & Barr K (1994) Role of the rfe gene in the synthesis of the O8 antigen in *Escherichia coli* K-12. *J Bacteriol* **176**: 2877-2884.

Rick PD, Barr K, Sankaran K, Kajimura J, Rush JS & Waechter CJ (2003) Evidence that the wzxE gene of *Escherichia coli* K-12 encodes a protein involved in the transbilayer movement of a trisaccharide-lipid intermediate in the assembly of enterobacterial common antigen. *J Biol Chem* **278**: 16534-16542.

Rick PD, Hubbard GL, Kitaoka M, *et al.* (1998) Characterization of the lipid-carrier involved in the synthesis of enterobacterial common antigen (ECA) and identification of a novel phosphoglyceride in a mutant of *Salmonella* Typhimurium defective in ECA synthesis. *Glycobiology* **8**: 557-567.

Rigort A, Bauerlein FJ, Villa E, Eibauer M, Laugks T, Baumeister W & Plitzko JM (2012) Focused ion beam micromachining of eukaryotic cells for cryoelectron tomography. *Proc Natl Acad Sci U S A* **109**: 4449-4454.

Robbins PW, Keller JM, Wright A & Bernstein RL (1965) Enzymatic and kinetic studies on the mechanism of O-antigen conversion by bacteriophage epsilon-15. *J Biol Chem* **240**: 384-390.

Robbins PW, Bray D, Dankert BM & Wright A (1967) Direction of chain growth in polysaccharide synthesis. *Science* **158**: 1536-1542.

Romling U (2002) Molecular biology of cellulose production in bacteria. *Res Microbiol* **153**: 205-212.

Ruan X, Loyola DE, Marolda CL, Perez-Donoso JM & Valvano MA (2012) The WaaL O-antigen lipopolysaccharide ligase has features in common with metal ion-independent inverting glycosyltransferases. *Glycobiology* **22**: 288-299.

Ruiz N, Kahne D & Silhavy TJ (2009) Transport of lipopolysaccharide across the cell envelope: the long road of discovery. *NatRevMicrobiol* **7**: 677-683.

Saigi F, Climent N, Pique N, *et al.* (1999) Genetic analysis of the Serratia marcescens N28b O4 antigen gene cluster. *J Bacteriol* **181**: 1883-1891.

Saldias MS, Ortega X & Valvano MA (2009) *Burkholderia cenocepacia* O antigen lipopolysaccharide prevents phagocytosis by macrophages and adhesion to epithelial cells. *J Med Microbiol* **58**: 1542-1548.

Samuel G & Reeves P (2003) Biosynthesis of O-antigens: genes and pathways involved in nucleotide sugar precursor synthesis and O-antigen assembly. *Carbohydr Res* **338**: 2503-2519.

Schild S, Lamprecht AK & Reidl J (2005) Molecular and functional characterization of O antigen transfer in *Vibrio cholerae*. *J Biol Chem* **280**: 25936-25947.

Shands JW (1965) Localization of somatic antigen on Gram-negative bacteria by electron microscopy. *J Bacteriol* **90**: 266-270.

Shands JW (1966) Localization of somatic antigen on Gram-negative bacteria using ferritin antibody conjugates. *Ann N Y Acad Sci* **133**: 292-298.

Skurski A, Slopek S, Michalska E & Obst B (1959) II. Phagocytosis and S-R dissociation of gram negative bacilli. *J Hyg Epidemiol Microbiol Immunol* **3**: 389-392.

Slopek S, Skurski A, Michalska E & Dabrowski L (1959) I. Phagocytosis and the antigenic structure of gram negative bacill. *J Hyg Epidemiol Microbiol Immunol* **3**: 382-388.

Sperandeo P, Dehï G & Polissi A (2009) The lipopolysaccharide transport system of Gram-negative bacteria. *BiochimBiophysActa* **1791**: 594-602.

Sperandeo P, Cescutti R, Villa R, Di Benedetto C, Candia D, Dehï G & Polissi A (2007) Characterization of lptA and lptB, two essential genes implicated in lipopolysaccharide transport to the outer membrane of *Escherichia coli*. *J Bacteriol* **189**: 244-253.

Sperandeo P, Lau FK, Carpentieri A, *et al.* (2008) Functional analysis of the protein machinery required for transport of lipopolysaccharide to the outer membrane of *Escherichia coli*. *J Bacteriol* **190**: 4460-4469.

Stevenson G, Kessler A & Reeves PR (1995) A plasmid-borne O-antigen chain length determinant and its relationship to other chain length determinants. *FEMS MicrobiolLett* **125**: 23-30.

Stevenson G, Neal B, Liu D, *et al.* (1994) Structure of the O antigen of *Escherichia coli* K-12 and the sequence of its rfb gene cluster. *J Bacteriol* **176**: 4144-4156.

Stocker BAD, Wilkinson RG & Mäkelä PH (1966) Genetic aspects of biosynthesis and structure of *Salmonella* somatic polysaccharide. *Ann N Y Acad Sci* **133**: 334-348.

Subbaiah TV & Stocker BA (1964) Rough mutants of *Salmonella* Typhimurium. I. Genetics. *Nature* **201**: 1298-1299.

Takayama K, Qureshi N & Mascagni P (1983) Complete structure of lipid A obtained from the lipopolysaccharides of the heptoseless mutant of *Salmonella* Typhimurium. *J Biol Chem* **258**: 12801-12803.

Taylor VL, Udaskin ML, Islam ST & Lam JS (2013) The D3 Bacteriophage α-Polymerase Inhibitor (Iap) Peptide Disrupts O-Antigen Biosynthesis through Mimicry of the Chain Length Regulator Wzz in Pseudomonas aeruginosa. *J Bacteriol* **195**: 4735-4741.

Tlapak-Simmons VL, Baron CA, Gotschall R, Haque D, Canfield WM & Weigel PH (2005) Hyaluronan biosynthesis by class I streptococcal hyaluronan synthases occurs at the reducing end. *J Biol Chem* **280**: 13012-13018.

Tocilj A, Munger C, Proteau A, *et al.* (2008) Bacterial polysaccharide co-polymerases share a common framework for control of polymer length. *Nat Struct Mol Biol* **15**: 130-138.

Topley WWC, Raistrick H, Wilson J, Stacey M, Challinor SW & Clark ROJ (1937) The immunising potency of antigenic components isolated from different strains of bact. Typhosum. *The Lancet* **229**: 252-256.

Ujwal R & Bowie JU (2011) Crystallizing membrane proteins using lipidic bicelles. *Methods* **55**: 337-341.

Valvano MA (2003) Export of O-specific lipopolysaccharide. *Front Biosci* **8**: s452-s471.

Verma NK, Brandt JM, Verma DJ & Lindberg AA (1991) Molecular characterization of the O-acetyl transferase gene of converting bacteriophage SF6 that adds group antigen 6 to *Shigella flexneri*. *Mol Microbiol* **5**: 71-75.

Wagner S, Bader ML, Drew D & de Gier JW (2006) Rationalizing membrane protein overexpression. *Trends Biotechnol* **24**: 364-371.

Wang L & Reeves PR (1994) Involvement of the galactosyl-1-phosphate transferase encoded by the *Salmonella enterica* rfbP gene in O-antigen subunit processing. *J Bacteriol* **176**: 4348-4356.

Weigel PH & DeAngelis PL (2007) Hyaluronan synthases: a decade-plus of novel glycosyltransferases. *J Biol Chem* **282**: 36777-36781.

Weiner IM, Higuchi T, Rothfield L, Saltmarsh-Andrew M, Osborn MJ & Horecker BL (1965) Biosynthesis of bacterial lipopolysaccharide. V. Lipid-linked intermediates in the biosynthesis of the O-antigen groups of *Salmonella* Typhimurium. *Proc Natl Acad Sci U S A* **54**: 228-235.

Weisgerber C & Jann K (1982) Glucosyldiphosphoundecaprenol, the mannose acceptor in the synthesis of the O9 antigen of *Escherichia coli*. Biosynthesis and characterization. *Eur J Biochem* **127**: 165-168.

Weisgerber C, Jann B & Jann K (1984) Biosynthesis of the 09 antigen of *Escherichia coli*. Core structure of rfe mutant as indication of assembly mechanism. *Eur J Biochem* **140**: 553-556.

West NP, Sansonetti P, Mounier J, *et al.* (2005) Optimization of virulence functions through glucosylation of Shigella LPS. *Science* **307**: 1313-1317.

Westphal O & Lüderitz O (1954) Chemische Erforschung von Lipopolysacchariden gramnegativer Bakterien. *Angewandte Chemie* **66**: 407-417.

Whitfield C (2006) Biosynthesis and assembly of capsular polysaccharides in *Escherichia coli*. *Annu Rev Biochem* **75**: 39-68.

Willis LM & Whitfield C (2013) Structure, biosynthesis, and function of bacterial capsular polysaccharides synthesized by ABC transporter-dependent pathways. *Carbohydr Res* **378**: 35-44.

Wong DK, Morris C, Lam TL, Wong WK & Hackett J (1999) Identification of O-antigen polymerase transcription and translation start signals and visualization of the protein in Salmonella enterica serovar Typhimurium. *Microbiology* **145 (Pt 9)**: 2443-2451.

Woodward R, Yi W, Li L, *et al.* (2010) In vitro bacterial polysaccharide biosynthesis: defining the functions of Wzy and Wzz. *Nat Chem Biol* **6**: 418-423.

Wright A, Dankert M, Fennessey P & Robbins PW (1967) Characterization of a polyisoprenoid compound functional in O-antigen biosynthesis. *Proceedings of the National Academy of Sciences* **57**: 1798-1803.

Wu T, McCandlish AC, Gronenberg LS, Chng SS, Silhavy TJ & Kahne D (2006) Identification of a protein complex that assembles lipopolysaccharide in the outer membrane of *Escherichia coli*. *Proc Natl Acad Sci U S A* **103**: 11754-11759.

Xu C, Liu B, Hu B, *et al.* (2011) Biochemical characterization of UDP-Gal:GlcNAc-pyrophosphate-lipid beta-1,4-Galactosyltransferase WfeD, a new enzyme from *Shigella boydii* type 14 that catalyzes the second step in O-antigen repeating-unit synthesis. *J Bacteriol* **193**: 449-459.

Zeleznick LD, Rosen SM, Saltmarsh-Andrew M, Osborn MJ & Horecker BL (1965) Biosynthesis of bacterial lipopolysaccharide, IV. Enzymatic incorporation of mannose, rhamnose and galactose in a mutant strain of *Salmonella* Typhimurium. *Proc Natl Acad Sci U S A* **53**: 207-214.

Zhang L, al-Hendy A, Toivanen P & Skurnik M (1993) Genetic organization and sequence of the rfb gene cluster of Yersinia enterocolitica serotype O:3: similarities to the dTDP-L-rhamnose biosynthesis pathway of Salmonella and to the bacterial polysaccharide transport systems. *Mol Microbiol* **9**: 309-321.

Figure legends

Figure 1. Maturation and export of O-antigens to the cell surface. **A)** Wzy-dependent pathway: The lipid and oligosaccharide core components of lipid A are synthesized by the Lpx pathway and by the sequential action of glycosyl transferases, respectively (I). Lipid A is flipped into the periplasm by an ABC-transporter MsbA (II). O-units are synthesized *via* glycosyltransferases in a step-wise fashion on the undecaprenyl diphosphate lipid anchor (1) and are transported into the periplasm by Wzx flippase (2). In the periplasm, the O-units undergo polymerization by Wzy polymerase (3) to yield mature O-antigens of certain length. The length of the O-antigen polymer is controlled by Wzz. The O-antigens are transferred to the lipid A outer core by WaaL ligase (4). The LPS-Oag complex is extracted from the inner membrane by LptBFG complex (5) and is transported to the outer membrane via a transenvelope passage formed by LptC and LptA proteins (6). At the outer membrane, LPS-Oag is recognized by LptE-LptD proteins, which complete its transfer to the surface; **B)** ABC-transporter dependent O-antigen biosynthesis: O-antigens are assembled in the cytosol by corresponding glycosyltransferases (1) and are transferred to the periplasm via an ABC-transporter (2). Mature O-antigens are ligated to the outer core of the lipid A via WaaL ligase (3). The polymers are transported to the cell surface as described in A.

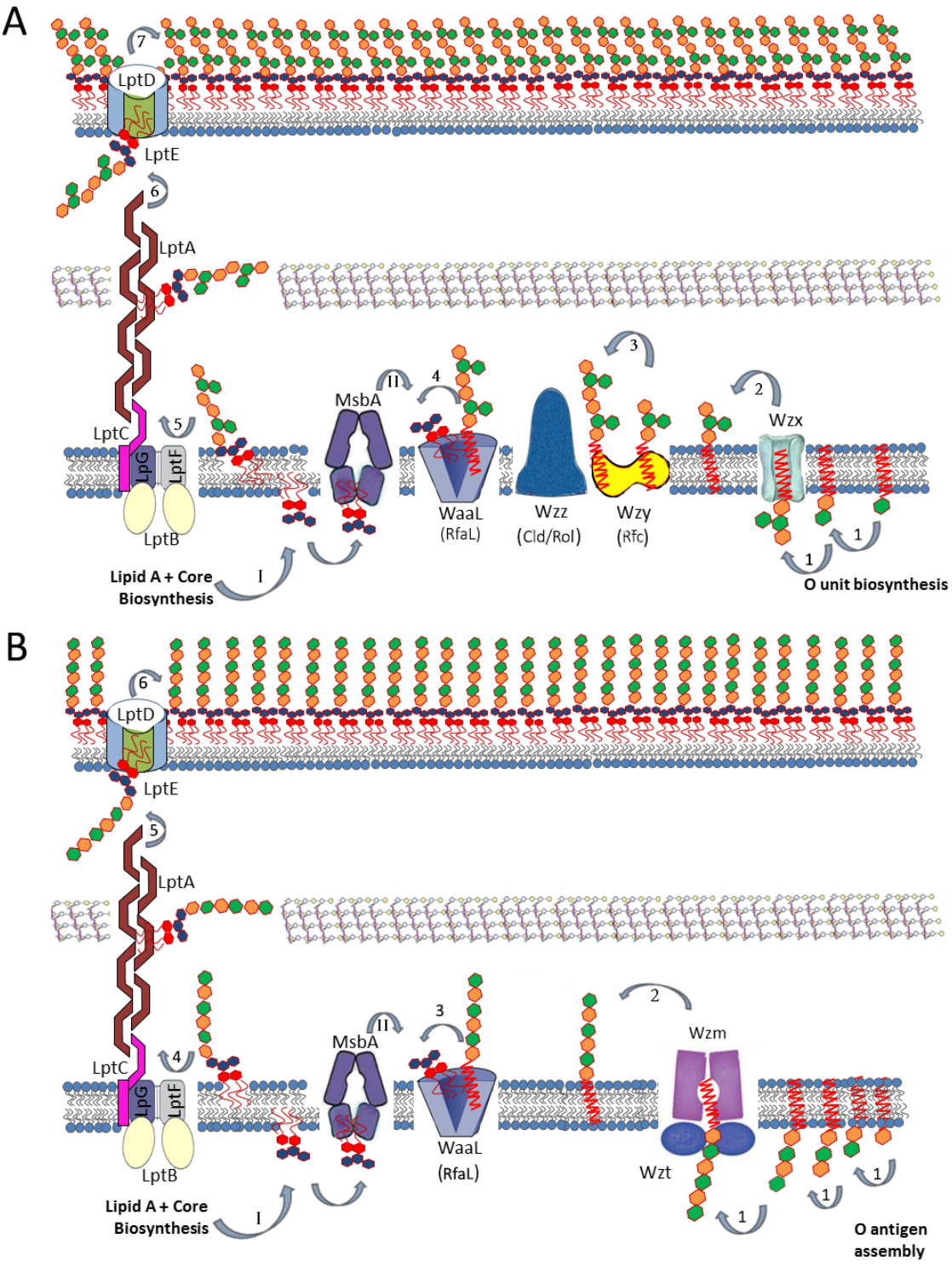
Figure 2. Outline of the cytosolic O-antigen synthesis initiation reaction involving a transfer of an activated nucleotide sugar donor to the undecaprenyl phosphate acceptor catalyzed by either **A)** a polyisoprenyl-phosphate N-acetylhexosamine-1-phosphate transferase (PNPT) (WecA) mediating the transfer of N-acetyl-glucosamine-phosphate to the undecaprenyl-phosphate yielding undecaprenyl diphosphate-N-acetyl-glucosamine or **B)**

a polyisoprenyl-phosphate hexose-1-phosphate transferase (PHPT) WbaP catalyzing the transfer of galactosyl-phosphate to the undecaprenyl phosphate and yielding undecaprenyl-diphosphate-galactose.

Figure 3. Electron micrograph of *Escherichia coli* published by Shands in 1965, where the O-antigen was labeled with a ferritin-coupled antibody to demonstrate the cellular localization of the O-antigen polysaccharide chains (reproduced with permission from the publisher).

Figure 4. The timeline of the key discoveries underlying the understanding of the O antigen biosynthesis.

Figure 1


A
7
LptD
LptE
6
LptA
LptC
5
LpG
LptF
LptB
II
MsbA
4
WaaL
(RfaL)
3
Wzz
(Cld/Rol)
Wzy
(Rfc)
2
Wzx
1
1
Lipid A + Core
Biosynthesis
I
O unit biosynthesis
B
6
LptD
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LptA
LptC
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LptB
II
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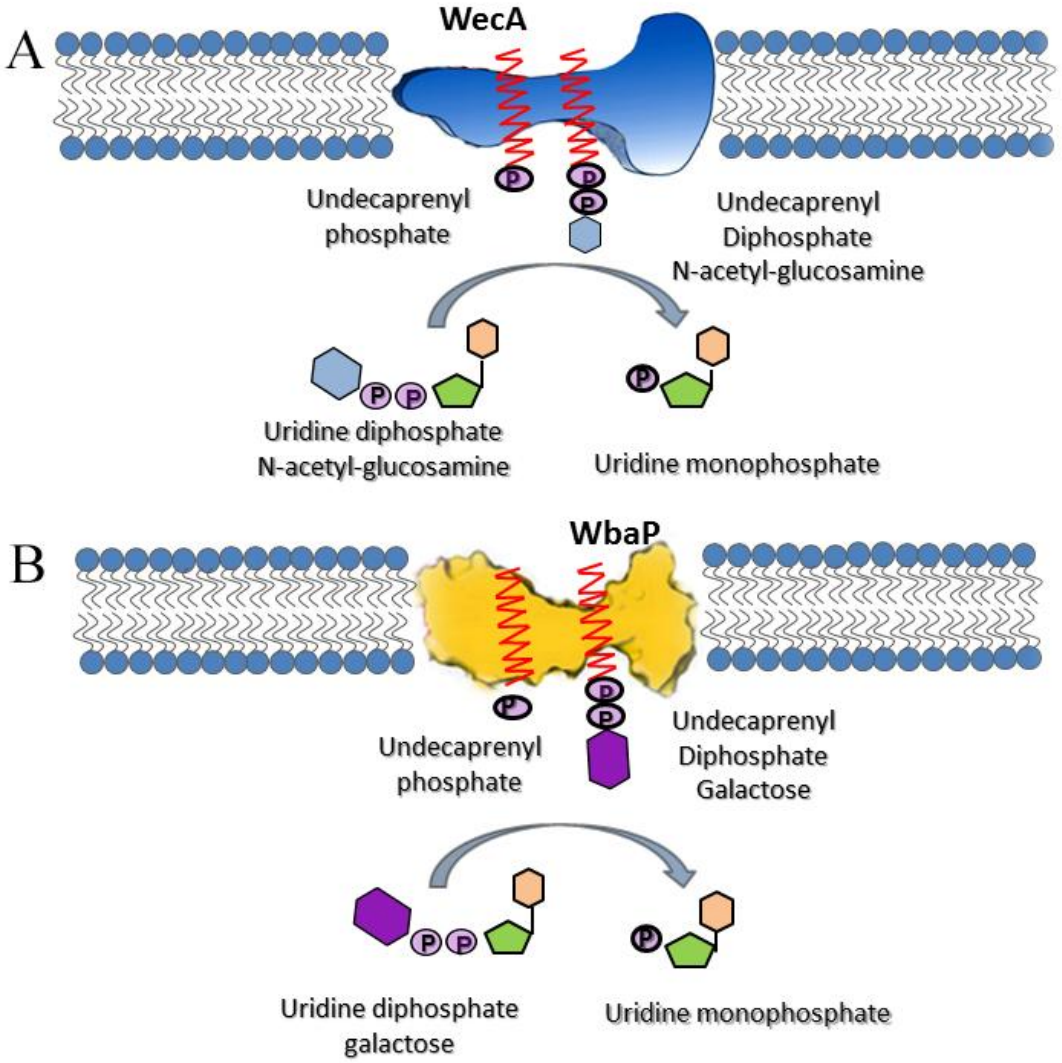
Lipid A + Core
Biosynthesis
I
O antigen
assembly

Figure 2


A
WecA
Undecaprenyl
phosphate
Undecaprenyl
Diphosphate
N-acetyl-glucosamine
Uridine diphosphate
N-acetyl-glucosamine
Uridine monophosphate
B
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phosphate
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Diphosphate
Galactose
Uridine diphosphate
galactose
Uridine monophosphate

Figure 3

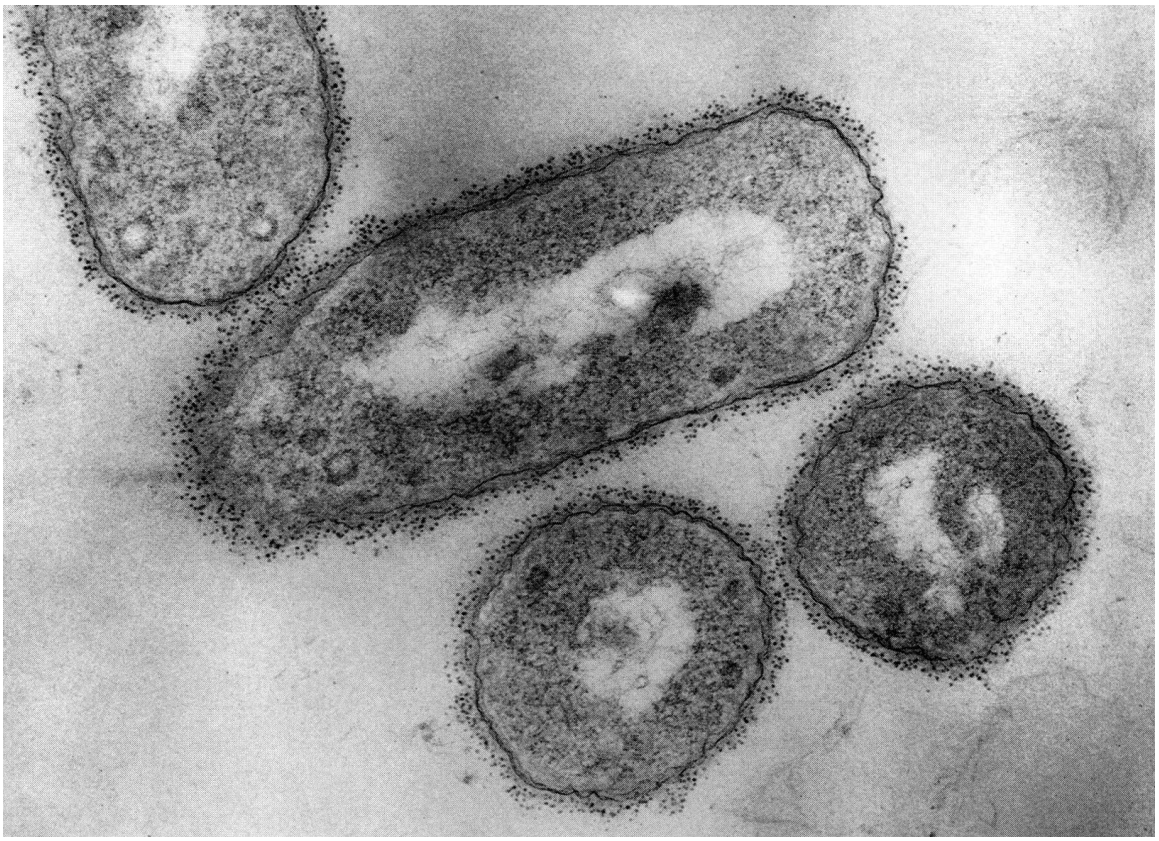
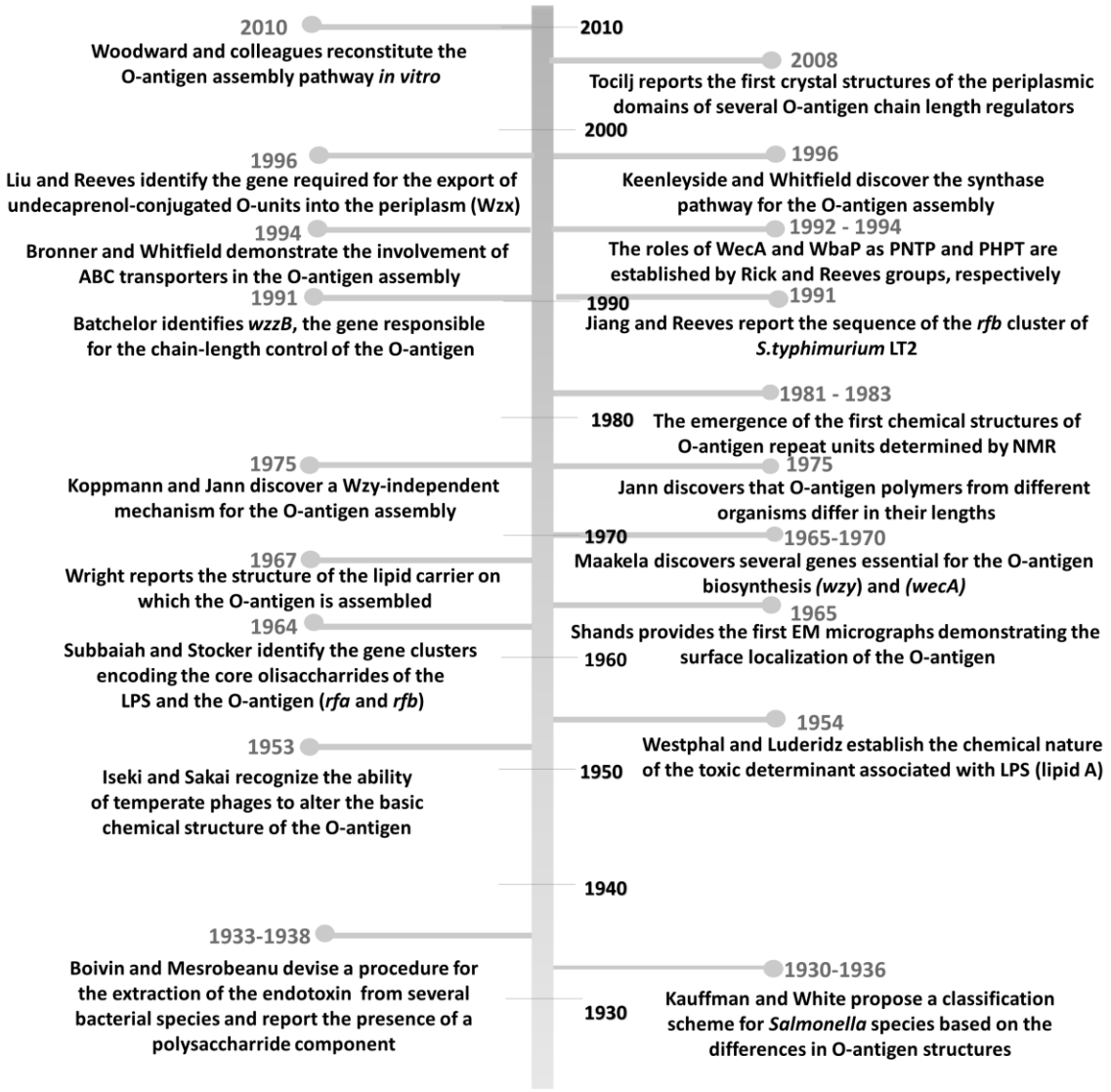


Figure 4


2010
Woodward and colleagues reconstitute the O-antigen assembly pathway *in vitro*
2010
2008
Tocilj reports the first crystal structures of the periplasmic domains of several O-antigen chain length regulators
2000
1996
Liu and Reeves identify the gene required for the export of undecaprenol-conjugated O-units into the periplasm (Wzx)
1996
Keenleyside and Whitfield discover the synthase pathway for the O-antigen assembly
1994
Bronner and Whitfield demonstrate the involvement of ABC transporters in the O-antigen assembly
1992 - 1994
The roles of WecA and WbaP as PNTP and PHPT are established by Rick and Reeves groups, respectively
1991
Batchelor identifies *wzzB*, the gene responsible for the chain-length control of the O-antigen
1990
1991
Jiang and Reeves report the sequence of the *rfb* cluster of *S.typhimurium* LT2
1981 - 1983
1980
The emergence of the first chemical structures of O-antigen repeat units determined by NMR
1975
Koppmann and Jann discover a Wzy-independent mechanism for the O-antigen assembly
1975
Jann discovers that O-antigen polymers from different organisms differ in their lengths
1970
1965-1970
1967
Wright reports the structure of the lipid carrier on which the O-antigen is assembled
Maakela discovers several genes essential for the O-antigen biosynthesis *(wzy)* and *(wecA)*
1965
1964
Subbaiah and Stocker identify the gene clusters encoding the core olisaccharrides of the LPS and the O-antigen (*rfa* and *rfb*)
Shands provides the first EM micrographs demonstrating the surface localization of the O-antigen
1960
1954
1953
Iseki and Sakai recognize the ability of temperate phages to alter the basic chemical structure of the O-antigen
Westphal and Luderidz establish the chemical nature of the toxic determinant associated with LPS (lipid A)
1950
1940
1933-1938
Boivin and Mesrobeanu devise a procedure for the extraction of the endotoxin from several bacterial species and report the presence of a polysaccharride component
1930-1936
1930
Kauffman and White propose a classification scheme for *Salmonella* species based on the differences in O-antigen structures