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Perspective/Review

Biosynthesis and assembly of Group 1 capsular polysaccharides in *Escherichia coli* and related extracellular polysaccharides in other bacteria

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Abstract

Extracellular and capsular polysaccharides (EPSs and CPSs) are produced by a wide range of bacteria, including important pathogens of humans, livestock, and plants. These polymers are major surface antigens and serve a variety of roles in virulence, depending on the biology of the producing organism. In addition to their importance in disease, some EPSs also have industrial applications as gelling and emulsifying agents. An understanding of the processes involved in the synthesis and regulation of CPSs and EPSs therefore potentially contributes to an understanding of the disease state, surface expression of protective antigens, and modulation of polymer structure to give defined physical properties. *Escherichia coli* has provided important model systems for EPS and CPS biosynthesis. Here we describe current knowledge concerning assembly of the Group 1 CPSs of *E. coli* and the conservation of similar mechanisms in other bacteria.
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Keywords: Capsular polysaccharide; Extracellular polysaccharide; K-antigens; Wzy-dependent assembly; Biosynthesis; *Escherichia coli*

Contents

1. Cell surface polysaccharides in *Escherichia coli*

The cell surface of *E. coli* is a complex array of proteins and glycoconjugates. The capsular polysaccharides (CPSs) and the O-polysaccharides of the lipopolysaccharide (LPS) molecules are the major surface polysaccharides expressed at 37 °C. These polymers are serotype-specific and give rise to the K- and O-antigens, respectively. Variations in sugar composition, linkage specificity, as well as substitution with non-carbohydrate residues result in 167 different O-serogroups and more than 80 polysaccharide K-antigens in *E. coli*.[1] The primary structures of many of these antigens have been

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elucidated.[2] The O- and K-antigens provide recognized virulence determinants (reviewed in Refs. 1,3,4). Generally, the O-antigens are important for resistance to complement-mediated serum killing, whereas the capsular K-antigens are responsible for resistance against phagocytosis. However, as is usual with such generalizations, there are exceptions. *E. coli* isolates also produce two polysaccharide structures that are not serotype-specific. Enterobacterial common antigen (ECA) is produced by all isolates and is found in several forms: attached to a phosphoglyceride moiety (ECA_{PG}), linked to LPS lipid A-core (ECA_{LPS}), and as a water-soluble cyclic form ECA_{CYC}.[5] ECA may play a role in acid resistance and heat tolerance.[6,7] Colanic acid is an EPS produced by a subset of *E. coli* O:K serotypes.[8,9] Unlike K-antigenic CPSs, colanic acid does not form a discrete capsule structure on the cell surface.[10] The loose association between colanic acid and the cell surface make its purification from cell-free culture supernatants relatively easy and it has traditionally been referred to as a "slime polysaccharide".[11] Colanic acid is not usually evident at physiological temperatures unless the strain carries a regulatory defect. However, the regulatory system that controls transcription of the colanic acid biosynthesis locus can be activated by growth at lower temperatures (below 30 °C), exposure to osmotic stress,[12,13] or overexpression of specific proteins.[14] Colanic acid plays a role in biofilm formation by *E. coli* K-12 (note that K-12 refers to the genetically characterized laboratory strain and is not a K-serotype indicator)[15,16] and can help the cell withstand desiccation.[17] Thus, colanic acid may be more important for functions unrelated to virulence.

An understanding of the synthesis and expression of *E. coli* K-antigens has allowed their classification into four different groups.[18] These groups include two fundamentally different polymerization pathways and both are widespread among different bacteria. Group 1 and 4 K-antigens follow a Wzx/Wzy-dependent polymerization pathway. In overview, undecaprenol diphosphate (und-PP)-linked K-repeat units are formed at the inner leaflet of the inner membrane. These und-PP-linked intermediates are flipped across the inner membrane by a process involving the Wzx protein and then polymerized in a reaction requiring Wzy. The *E. coli* Group 1 K30 antigen provides the prototype for this pathway. Group 2 and 3 K-antigens are assembled by an ABC-transporter-dependent pathway, in which und-PP-linked polymers are formed at the inner leaflet of the membrane by processive glycosyltransfer to the non-reducing terminus of the und-PP-linked intermediate. The nascent polymer is then exported across the inner membrane by an ABC (ATP-binding cassette) transporter. Well-studied representatives of this pathway are the *E. coli* serotype K1 and K5 antigens. ECA and colanic acid are also synthesized by a Wzx/Wzy-dependent pathway, while examples using both pathways are found among *E. coli* O-antigens.[19]

While similar polymerization pathways can be used for different polysaccharides, their nature, properties and biological functions are dictated by the way they are presented on the cell surface. *E. coli* Group 1 K-antigens are expressed in the predominant capsular CPS form, but are also found as oligosaccharides comprising a small number of K-repeat units attached to a lipid A-core anchor (termed K_{LPS}).[20] K_{LPS} is a feature so far confined to *E. coli*; it has not been identified in other bacteria with Group 1 CPSs or related EPSs. The *E. coli* CPS form of K-antigen is a virulence determinant[3,21] but the biological role (if any) of K_{LPS} is unknown. While both forms share the enzymes involved in K-repeat unit synthesis and polymerization, they differ in the terminal processes involved in their translocation to the cell surface.

In this review, we will describe our current understanding of the biosynthesis of Group 1 CPSs in *E. coli* as a paradigm for a Wzx/Wzy-dependent polymerization pathway coupled to a specific translocation pathway for surface assembly. As more information becomes available, it is apparent that *E. coli* systems provide models for CPS and EPS expression in a range of gram-negative and gram-positive bacteria with different biologies. These bacteria include pathogens of humans and livestock, as well as plant-associated bacteria. Some representatives have commercial applications (e.g. xanthan gum from *Xanthomonas campestris*, the gellan family from *Sphingomonas* spp., and emulsans from *Acinetobacter* spp.).[22,23] EPS production provides a number of fascinating fundamental research problems due to the complexity in the transmembrane assembly reactions. From an applied perspective, an understanding of the processes may afford novel targets for therapeutic intervention against pathogens, and opportunities to manipulate the amount or properties of industrially important microbial polysaccharides.

2. Chromosomal organization of the region responsible for expression of *E. coli* Group 1 CPS

In *E. coli* K-12, the colanic acid biosynthesis (cps_{CA}) genes map near the chromosomal *his* (histidine biosynthesis) and *wb** (O-antigen biosynthesis) genes.[24] The Group 1 CPS locus (cps_K) occupies the same general location (Fig. 1), although there are some subtle differences in gene order, reflecting past recombination events.[25,26] Therefore, while colanic acid can be made by isolates with Group 2 capsules (Group 2 loci map elsewhere on the chromosome), expression of Group 1 capsules and colanic acid are mutually exclusive.[8,9] The Group 1 capsule locus is also conserved in location and organization in *K. pneumoniae*,[27] an indication of past

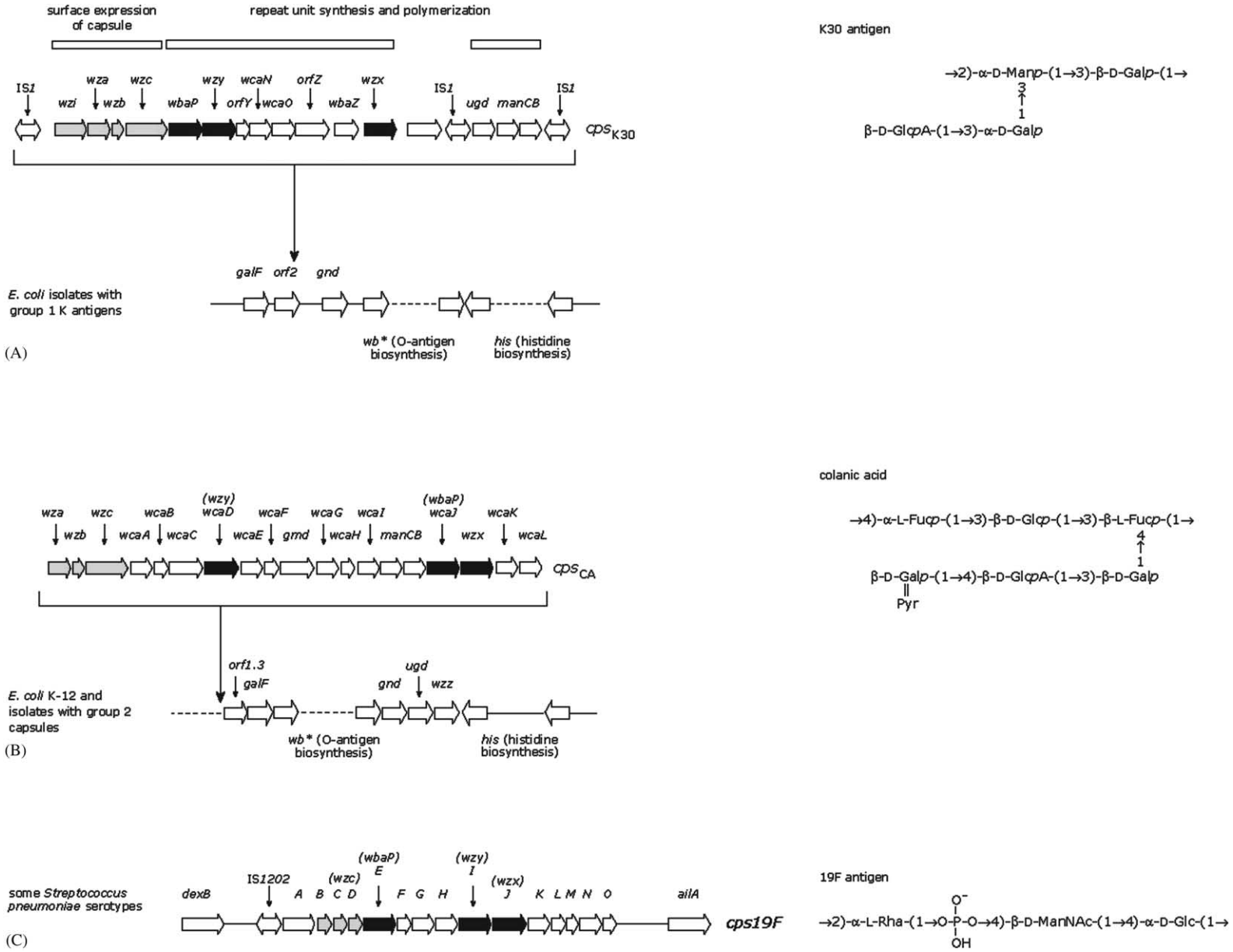

Fig. 1. Organization of chromosomal regions responsible for expression of Group 1 CPS in *E. coli* (panel A) and comparison with the loci for colanic acid synthesis from *E. coli* K-12 (panel B) and serotype 19F CPS from *Streptococcus pneumoniae* (panel C). Details of individual genes and functions are presented in the text. The genes for the characteristic families of proteins involved in Wzx-Wzy biosynthetic pathways are identified in black and genes whose products are required for high-level polymerization and surface assembly of the polymers are in gray. Note that despite the extensive similarity shared by the gene clusters for Group 1 K-antigens (e.g. cps_{K30}) and colanic acid (cps_{CA}), the insertion point for these clusters on the chromosome is different. The structures of the polymeric products are shown.

lateral gene transfer events between *E. coli* and *K. pneumoniae*.[25]

The cps_K loci for colanic acid and Group 1 CPS share a common organization (Fig. 1) in which the operon is transcribed from an upstream promoter and separated into two regions by a proposed transcriptional attenuator.[28] Transcription of the operon requires the RfaH protein that interacts with a characteristic 8 bp *ops* sequence located upstream of the operon but contained on the transcript. These elements are required for antitermination in a variety of long operons[29] and are required for transcription of the *cps* genes located downstream of the attenuator.[28] Genes downstream of the attenuator encode enzymes involved in the synthesis and low-level polymerization of the polymer repeat units. The precise genes in this region vary according to serotype (and K repeat-unit structure) but they encode glycosyltransferases, enzymes involved in the synthesis of K-antigen specific sugar nucleotide precursors, as well as the characteristic Wzy and Wzx proteins (Table 1). These gene products are required for synthesis of both capsular K-antigens and K_{LPS}. The content of this part of the locus is indistinguishable from others involved in the expression of Wzx/Wzy-dependent LPS O-antigens and some gram-positive CPS (Fig. 1) consistent with similarities in the mechanisms of synthesis.

In cps_K loci from *E. coli* and *K. pneumoniae* isolates with Group 1 K-antigens, genes upstream of the attenuator (*wzi-wza-wzb-wzc*) are highly conserved (i.e. independent of capsule structure)[25] and differentiate the CPS locus from one involved in O-antigen expression. These genes encode products essential for the high-level polymerization of capsular K-antigens and assembly of the capsule structure and are not required for K_{LPS} synthesis.[30]

Although the colanic acid biosynthesis locus shares many features with the Group 1 capsule locus, there are two important differences. First, the initial gene in the cps_K forms, *wzi*, is missing from the cps_{CA} locus. Second, colanic acid is subject to transcriptional regulation by the Rcs (Regulator of Capsule Synthesis) system.[31,32] At 37 °C, colanic acid is not normally produced in *E. coli*. Transcription of the colanic acid locus is activated in an Rcs-mediated process at lower growth temperatures, or under stress conditions.[12–14] In contrast, Group 1 capsules are formed at 37 °C and Rcs has only indirect effects on Group 1 capsule expression by influencing the level of transcription of *galF*.[28] The *galF* gene product interacts with UDP-glucose pyrophosphorylase (GalU) to elevate the amounts of UDP-glucose in the cell.[28] Since UDP-glucose leads to both UDP-galactose and UDP-glucuronic acid (precursors for several K-antigens), the Rcs effect is presumably mediated by the availability of biosynthesis precursors. The location of RcsAB box sequences, the sites of interaction with the RcsAB transcriptional activa-

Table 1
Components of the biosynthetic systems for representative Group 1 CPSs and related polysaccharides in different bacteria

Polysaccharide	Member of family:							Genbank accession	References
	WbaP	Wzx	Wzy	OMA	MPA1/PCP2	PTP	PHP		
E. coli Group 1 capsule (K30)	WbaP	Wzx	Wzy	Wza	Wzc	Wzb		AF104912	30
E. coli colanic acid	WcaJ	Wzx	WcaD	Wza	Wzc	Wzb		U38473	24
K. pneumoniae capsule (K2)	Orf14	Orf11	Orf10	Orf4	Orf6	Orf5		D21242	25,27
E. amylovora amylovoran	AmsG	AmsL	AmsC	AmsH	AmsA	AmsI		X77921	62,91
X. campestris xanthan gum	GumD	GumJ	GumE	GumB	GumC	unknown		U22511	92,93
S. meliloti succinoglycan	ExoY	ExoT	ExoQ	ExoF	ExoP	unknown		L20758, L05588	52,58,94–98
Sphingomonas S-88 sphingan	SpsB	SpsS	SpsG	SpsD	SpsE/SpsC	unknown		U51197	79,99,100
Acinetobacter lwoffii emulsan	WeeH	Wzx	Wzy	Wza	Wzc	Wzb		AJ243431	55,101
Streptococcus pneumoniae capsule (some serotypes e.g. 19F)	CpsE	CpsJ	CpsI	none	CpsC/CpsD	none	CpsB	U09239	46,56,82,102
Staphylococcus aureus capsule (e.g. serotype 5)	CapM	CapK or CapJ	CapJ or CapK	none	CapA/CapB	none[a]	CapC	U81973	71,74

[a] The chromosome contains two PTP genes[81] but it is not clear whether they are involved in CPS synthesis, since the gene cluster contains a PHP family member.

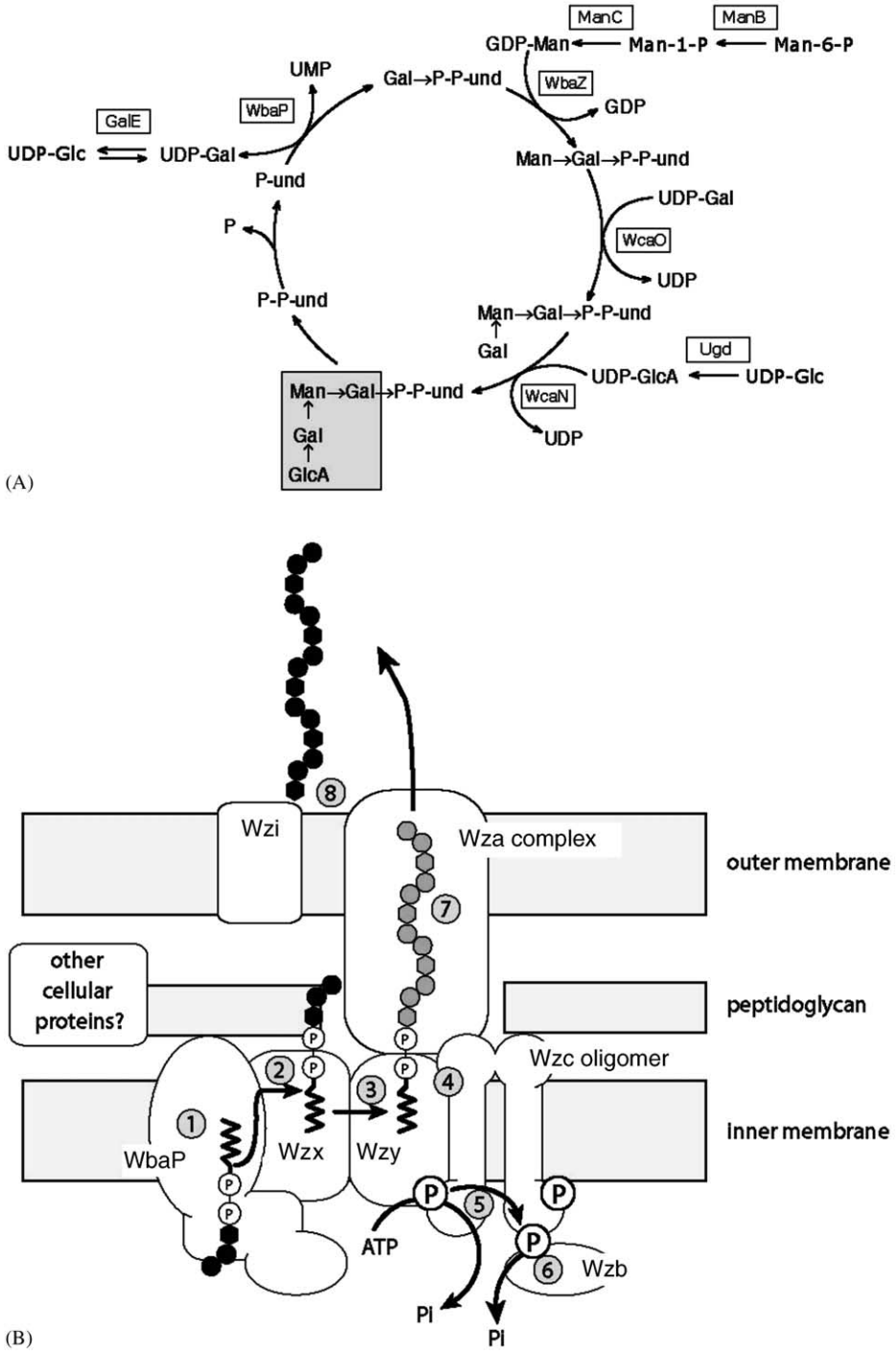

Fig. 2. Biosynthesis of the Group 1 CPS in *E. coli* K30. Panel A shows the proposed pathway for synthesis of lipid-linked K30 repeat units. The assignment of glycosyltransferases catalyzing individual steps is the product of sequence data and preliminary biochemical investigations.[30] Glycosyltransferases and enzymes involved in the synthesis of sugar nucleotide precursors are identified in boxes. GalE is UDP-galactose-4-epimerase; ManBC are phosphomannomutase and GDP-mannose pyrophosphorylase, respectively; and Ugd is UDP-glucose dehydrogenase. The carrier lipid is presumed to be undecaprenyl phosphate (und-P), consistent with the many other systems that have been investigated. Panel B shows a cartoon depicting a hypothetical biosynthetic complex carrying out a coordinated sequence of reactions. (1) The glycosyltransferase (WbaP, WbaZ, WcaO and WcaN) reactions given in panel A synthesize lipid-linked repeat units at the cytoplasmic face of the inner membrane. (2) The und-PP-linked repeat units are flipped across the inner membrane by a process involving Wzx. (3) The repeat units are polymerized through a reaction requiring Wzy. (4) Wzc function is essential for high-level polymerization. (5) Wzc function is mediated by autophosphorylation followed by transphosphorylation between proteins in an oligomeric form. (6) Dephosphorylation of Wzc by the Wzb phosphatase is also crucial for CPS synthesis. (7) Export of polymer to the surface requires the outer membrane Wza complex, perhaps playing the role of an export channel. (8) The nascent CPS is assembled on the cell surface and Wzi is required for efficient encapsulation. Details of each biosynthetic step are described in the text. It is uncertain whether other housekeeping functions also participate in the overall process.

tors,[28,33] dictate the difference in the influence of Rcs on expression of the cps_{CA} and cps_{K} loci. The *Klebsiella* version of the cps_{K} locus has an additional and unique level of transcriptional control imparted by the RmpA protein, encoded by a gene on a large virulence plasmid.[34]

The Wzx/Wzy-dependent pathway is also used for polymerization of some gram-positive CPSs, including representatives of *Streptococcus pneumoniae* and *Staphylococcus aureus* (Table 1). Consequently, the general organization of the locus shares many similarities with Group 1 *cps* loci. The initial genes in the *S. pneumoniae* cluster encode a novel regulator (*cpsA*)[35] and genes whose products may play comparable roles to Wzc and Wzb in gram-negatives (see below). Genes downstream encode the characteristic serotype-specific sugar nucleotide synthetases, glycosyltransferases, Wzx and Wzy (Table 1). Clearly the major distinction from gram-negative bacteria is that the gram-positive systems do not require the translocation mechanisms to move CPS/EPS across a periplasm and outer membrane.

3. Wzy-dependent polymerization

Polymer synthesis is a complex process involving a series of reactions occurring in different cellular compartments (Fig. 2). Synthesis begins with nucleotide diphosphosugar precursors that are formed in the cytoplasm (Fig. 2A). The individual repeat units are assembled on a carrier lipid (undecaprenyl phosphate; und-P) by the sequential activities of glycosyltransferase enzymes active at the cytoplasmic face of the inner membrane.[30,36] According to the current biosynthetic model, lipid-linked repeat units are then transferred across the inner membrane and polymerized at the periplasmic face. The ultimate fate of lipid-linked polymerized material depends on whether it is destined for CPS/EPS, or a molecule containing lipid A-core, such as O-antigens or K_{LPS}. There are distinct surface expression (translocation) pathways for each.

The name ''Wzy-dependent'' is derived from the putative polymerase, Wzy. A wide variety of bacterial surface polymers are formed by Wzy-dependent processes, based on biochemical analysis and sequence similarities. These include CPSs and EPSs in both gram-negative and gram-positive bacteria (Table 1), as well as LPS O-antigens (see for example the O-antigens of *Yersinia* in this volume).[37] Synthesis of polymers by this pathway is initiated by transfer of either hexose-1-P or *N*-acetamidohexose-1-P to und-P.[19] For CPS and EPS, a member of a family of UDP-hexose:undecaprenylphosphate hexose-1-P transferase enzymes transfers either Glc-1-P or Gal-1-P to the carrier lipid. The sequences of the family members (WbaP homologues; Table 1) predict at least one transmembrane segment, as might be expected for enzymes that interact with a membrane-bound lipid acceptor. The subsequent glycosyltransferases all appear to be peripheral membrane proteins and act sequentially to assemble the completed lipid-linked repeat unit. The pathway for assembly of the repeat unit of the *E. coli* K30 antigen is shown in Fig. 2A).

The role of Wzy was first described in classic experiments involving the assembly of the *Salmonella* serogroups B, D and E O-antigens.[19] Undecaprenol-PP-linked repeat units provide the substrates for the polymerase in a process where the nascent und-PP-linked polymer grows at the reducing terminus, by addition of one repeat unit at a time. Mutations in *wzy* prevent polymerization but do not eliminate synthesis of individual repeat units. In the case of the *E. coli* K30 antigen, a specific *wzy* mutation eliminates polymerization of the K30 CPS (and formation of the capsular structure) and the K_{LPS} form is confined to molecules with a single K-antigen repeat unit, indicating they share a common polymerization machinery.[30] Many studies have reported the phenotypes of mutations in *wzy* homologues and these are all consistent with the assignment of Wzy as a polymerase. However, Wzy proteins are integral membrane proteins that typically have 10–13 transmembrane segments and overexpression has proven difficult,[38,39] limiting attempts to understand the detailed mechanism of Wzy function at a biochemical level.

The Wzy-dependent polymerase reaction occurs at the periplasmic face of the plasma membrane,[40] and the assembly pathway therefore requires an enzyme that transfers lipid-linked repeat units across the inner membrane (Fig. 2B). Preliminary data obtained with an experimental hybrid O-antigen system suggests that the *wzx* gene product may play a role in this export process.[41] This has been confirmed in additional systems[42] and a Wzx homologue from ECA biosynthesis has been shown to mediate transmembrane flipping of a water-soluble isoprenyl-PP-GlcNAc derivative in vesicles.[43] Like Wzy, Wzx homologues also have multiple predicted transmembrane segments and sequence similarities place them in a family of putative ''polysaccharide exporter proteins'' recently designated PST(1) for the capsule assembly homologues and PST(2) for those involved in O-antigen synthesis.[44] As with Wzy, unequivocal biochemical proof is lacking for the precise catalytic activity of the Wzx enzyme. It also remains to be established whether Wzx is the only protein involved in the transfer process. Indeed, there is preliminary data suggesting that the N-terminal domain of WbaP (the initiating Gal-1-P transferase) plays some role in export of some *Salmonella* O-antigens.[45] Sequences available for the biosynthesis gene clusters for Group 1 K-antigens and colanic acid indicate that all contain homologues of *wbaP*, *wzx* and *wzy* (Table 1),

consistent with a common biosynthetic process. The same is true of gene clusters whose products are Wzy-dependent O-antigens or EPSs, although it is frequently difficult to distinguish between the genes for the two integral proteins, Wzx and Wzy based on simple sequence alignments. In some cases, application of iterative approaches like PSI-BLAST allows a higher degree of confidence in the assignments. This has been done for assignments in some *S. pneumoniae cps* loci.[46]

4. The Wzc protein and high-level polymerization of Group 1 CPS and related EPS

For Wzy-dependent LPS O-antigens, polymerization is terminated by transfer of the polymer (or oligosaccharide) from the lipid intermediate to lipid A-core acceptor by the ligase enzyme, WaaL.[19] The products are LPS molecules with characteristically heterogeneous O-antigen chain lengths readily revealed in sodium dodecyl sulfate polyacrylamide gel electrophoresis analysis of LPS preparations. For long chain O-antigens, an additional component, Wzz (O-antigen chain length determinant; Cld or Rol) controls the extent of polymerization in a process that requires Wzy and WaaL.[19,47] The *wzz* gene is found in *E. coli* K-12 and isolates with Group 2 K-antigens (Fig. 1). If the biosynthesis of *E. coli* Group 1 K_{LPS} basically follows the same assembly route, why is the chain length limited to a short oligosaccharide comprising one or a few repeat units? This is explained by the finding that *E. coli* strains with Group 1 CPSs lack chromosomal *wzz* genes[48,49] (Fig. 1) and the polymerization of K_{LPS} is therefore unregulated unless a plasmid containing cloned *wzz* is introduced.[48] Group 1 CPS are co-expressed with a limited range of LPS O-antigens, predominantly serogroups O8 and O9, and these are synthesized by ABC-2-transporter-dependent processes.[50] These assembly mechanisms operate without the involvement of Wzx, Wzy, or Wzz homologues.[19] The chain length of Group 1 CPS is determined by a different (non Wzz-dependent) method,[30,48] presumably because ligation to lipid A-core is not required for its expression on the cell surface.[20,48] This raises the question of how CPS chain length is controlled.

One potential candidate for a component filling an equivalent role to Wzz in Group 1 CPSs and related EPSs is Wzc. Wzc proteins belong to the MPA1 (cytoplasmic membrane periplasmic auxiliary) family,[44] also called PCP (polysaccharide co-polymerase) proteins[51] whose members are involved in a growing number of Group 1 CPS and Group 1-like CPS and EPS systems. Sequence predictions[47] and analysis of protein fusions[52,53] show Wzc homologues possess 2 transmembrane helices. Sequence data predicts the large periplasmic loop to have propensity to form coiled-coil structures.[51] This topology is reminiscent of Wzz, which led to both proteins being grouped together within the PCP family.[51] However, Wzc (PCP-2) is distinguished from Wzz (PCP-1) by its possession of a C-terminal cytoplasmic domain harboring ATP-binding motifs (Walker A and B) as well as a tyrosine-rich C-terminus. Wzc and its homologues are tyrosine autokinases and they are dephosphorylated by the cognate phosphatases, Wzb in *E. coli* and many gram-negative bacteria and CpsB in some gram-positive organisms, such as *S. pneumoniae*.

Although the details vary, in all systems examined to date, both the kinase and the phosphatase activities are essential for the assembly of the respective CPS or EPS. Unlike *wzz* mutants in O-antigen biosynthesis where the effect on polymerization control is evident, the phenotypes of mutants lacking Wzc, or harboring Wzc derivatives with phosphorylation defects, are not simply interpreted. In *E. coli* K30 and *Acinetobacter lwoffii*, phosphorylation of Wzc is essential for the assembly of a capsular layer on the cell surface and for emulsan activity, respectively.[30,54,55] However, *wzb* and *wzc* mutations in *E. coli* K30 both have a similar acapsular phenotype. This led to the hypothesis that in Group 1 CPS systems, Wzc needs to cycle between phosphorylated and non-phosphorylated forms.[54] In contrast, in *E. coli* K-12 and *S. pneumoniae* strain Rx1-19F, it has been proposed that phosphorylated Wzc acts as a negative regulator of colanic acid and CPS production, respectively.[56,57]

The data implicating Wzc in altered chain length is not extensive, due to the difficulties in analyzing chain length distributions in CPSs and EPSs. In systems like *E. coli* K30 CPS synthesis, the phenotype of a *wzc* mutant could be explained either by altered chain length determination, or an overall reduction in synthetic activity.[30,54] However, in *Sinorhizobium meliloti*, mutations in ExoP (Wzc homologue) led to an increase in low molecular mass succinoglycan and a decrease in the amount of HMW polymer.[52,58,59] Mutation of the Wzc homologue in Group B Streptococci also resulted in a decrease in the molecular mass of the polymer produced.[35]

Analysis of Wzc functions in *E. coli* is complicated by the presence on the chromosome of a second copy of the *wza*, *wzb* and *wzc* genes. The genes are found in a locus corresponding to 22 minutes on the *E. coli* linkage map. To distinguish these genes from those in the K30 CPS and colanic acid gene clusters, they are referred to as wza_{22min}, wzb_{22min} (also designated *etp*[60]) and wzc_{22min} (or *etk*[60]).[30,54] The wzc_{22min} gene is present in a number of *E. coli* strains with different biological properties (EPEC, ETEC, EHEC, EAEC, EIEC, and K-12), but the protein was only expressed by EPEC, ETEC and EHEC strains, suggesting a role in virulence.[60] The kinase and phosphatase activities of these proteins have

been confirmed, and they have been shown to participate with low efficiency in K30 CPS/colanic acid production.[54,57] However, a recent study revealed an additional complication in the form of the involvement of wzb_{22min} and wzc_{22min} in the regulation of heat shock response in *E. coli* via altered phosphorylation states of RpoH and the RseA antisigma factor.[61]

The role of phosphorylation of Wzc homologues has been studied through overexpression of Wzb in some systems. Overexpression of the phosphatase would be expected to greatly decrease the amount of phosphorylated Wzc in the cell. Overexpression of AmsI (Wzb) in *E. amylovora* caused a strong reduction in EPS synthesis.[62] Furthermore, in *E. coli* K30, overexpression of Wzb_{K30} caused a 3.7-fold reduction in the amount of cell-associated K30 CPS produced.[63] In contrast, overexpression of Wzb_{CA} in *E. coli* K-12 led to a mucoid phenotype and wild-type levels of colanic acid.[57] These results could be interpreted as reflecting an opposing role for Wzc ~ P in the respective systems. However, the potential involvement of wzb_{22min} and wzc_{22min} in other regulatory circuits[61] suggest that, in isolation, overexpression data should be considered with caution.

5. Biochemical properties of Wzc and its homologues

Wzc autophosphorylates at multiple tyrosine residues using ATP as a substrate. The biochemical activities of several Wzc proteins have been confirmed in systems representing Group 1 CPS and related EPS from *E. coli*,[54,57,60,64] *K. pneumoniae*,[65] *Erwinia amylovora*,[60] *Acinetobacter johnsonii*,[66,67] *Acinetobacter lwoffii*,[55] *Sinorhizobium meliloti*,[59] and *Streptococcus pneumoniae*.[56,68] Phosphorylation of Wzc occurs at the C-terminal tyrosine-rich region.[54–56,59,60,69] Studies of Wzc from *E. coli* K30 revealed that no single tyrosine is essential to Wzc phosphorylation or activity in CPS assembly, nor is any one single tyrosine residue capable of supporting activity of the protein.[63] In *E. coli* K-12, phosphorylation of five of the six terminal tyrosine residues of Wzc_{CA} was possible in vitro.[69] Interestingly, competence of Wzc for phosphorylation does not necessarily equate with high-molecular mass CPS expression in *E. coli* K30.[63] Replacement of combinations of tyrosine residues revealed the need for at least four intact tyrosine residues in the C-terminus for function of the protein in CPS assembly, even though phosphorylation was detected in proteins retaining only two terminal tyrosine residues. This study suggests that the level of phosphorylation in this region may be important, rather than phosphorylation of specific residues.

The ability of Wzc molecules to participate in transphosphorylation reactions was suggested by the finding that phosphorylation of Wzc_{K30} and capsule assembly occurred in *E. coli wzc* mutants transformed simultaneously with plasmids expressing two mutant forms of Wzc; one could not bind ATP while the other could bind ATP but lacked the C-terminal site of phosphorylation.[54] This transphosphorylation function has been confirmed and extended by a study of the Wzc_{CA} (colanic acid) protein.[69] In Wzc_{CA}, an additional tyrosine residue outside the C-terminal tyrosine-rich domain (Y569) was modified by autophosphorylation only, while the C-terminal domain was accessible to transphosphorylation by other Wzc_{CA} molecules.[69] The authors proposed a two-step process (autophosphorylation–transphosphorylation) for the phosphorylation of Wzc_{CA}, where phosphorylation of Y569 modulates the level of phosphorylation of the tyrosine-rich region in this system. However, a corresponding $Y \rightarrow F$ mutation in Wzc_{K30} did not affect the amount of Group 1 CPS produced[63] and the role of Y569 in colanic acid production in *E. coli* K-12 in vivo is currently unknown. Transphosphorylation can be mediated by protein:protein interactions and phosphorylation-independent oligomerization of Wzc has been demonstrated by in vivo cross-linking methods in *E. coli* K30[63] and K-12.[53] Oligomerization of Wzc_{CA} was detected when the C-terminal domain alone was expressed, though not when the N-terminal domain alone was expressed. This is in contrast to the oligomerization of *Shigella flexneri* Wzz (O-antigen chain-length determinant), where oligomerization is mediated by the N-terminal portion of the protein.[70]

In gram-positive bacteria such as *Staphylococcus aureus*,[71–74] *Streptococcus pneumoniae*,[75,76] and *S. agalactiae*,[77,78] the Wzc homologues exist as two separate polypeptides, one equivalent to the Wzc N-terminal region bearing two transmembrane domains (CpsC in streptococci, or CapA in staphylococci)[46] and a second polypeptide equivalent to the Wzc C-terminal region (CpsD or CapB) with the ATP binding motifs and tyrosine-rich domain.[46,56,68,74,75] Autophosphorylation[56,68] and transphosphorylation[68] have been described in some representatives. There is no obvious functional significance in the "2-part Wzc" because an engineered 2-part mimic constructed in *E. coli* K30 was functional for phosphorylation and CPS assembly[54] and sequence data suggests some gram-negative bacteria (e.g. *Sphingomonas* strain S-88[79]) naturally have a 2-part Wzc. In the case of CpsCD in *S. pneumoniae*,[56,68] and in *E. coli* K30,[54] phosphorylation of the C-terminal domain is dependent on co-expression with the N-terminal transmembrane component. In contrast, C-terminal domains of ExoP from *S. meliloti*[59] and *E. coli* K-12[57,69] are competent for phosphorylation in isolation. It is unclear whether these contradictory results reflect authentic differences in the properties of the relevant domains, if they result from differing sensitivities of the methods used to examine this phosphoryla-

tion, or if they reflect the involvement of additional cellular factors in vivo.

Phosphate residues can be removed from Wzc proteins by Wzb, a cytoplasmic phosphotyrosine protein phosphatase that resembles low MW acid phosphatases of eukaryotes.[54,55,57,61–65,80,81] In some bacteria, sequences homologous to *wzb* are not detected in the exopolysaccharide biosynthesis loci. In the case of *S. pneumoniae*, the dephosphorylating activity is provided by CpsB, a member of Mn^{2+}-dependent phosphatases belonging to the PHP (polymerase and histidinol phosphatase) family of phosphoesterases[68,82] and it has been suggested that CpsB has an additional function in modulating the autophosphorylating activity of CpsCD.[68] Wzb homologues are not confined to gram-negative bacteria. For example, the *S. aureus* genome sequence reveals two Wzb homologues.[81] The precise biological role of these homologues (and any role in capsule assembly) is not yet resolved.

6. Translocation and cell-surface assembly of Group 1 CPS and related polymers

While some aspects of polymerization pathways are reasonably well documented, the terminal steps in capsule assembly are largely still open questions. These processes include release of nascent polymer from the lipid intermediate, and translocation of the polymer through the periplasm and across the outer membrane. Electron microscopy studies showed that assembly of Group 1 capsule in a conditional mutant of *E. coli* K29 occurs at specific sites where the plasma and outer membranes appear to come into close apposition.[83] The interpretation of these "zones of adhesion" has been controversial, but there are increasing examples in the literature of cell envelope-spanning multienzyme complexes for import and export processes. A coordinated CPS assembly complex (Fig. 2B) would provide a physical and functional connection between the cell surface and the polymerization machinery in the inner membrane and would overcome the practical problem of transferring high-molecular-mass capsular polymers ($M_r > 100{,}000$) to the surface. Multienzyme complexes have been reported for *E. coli* Group 2 CPS assembly.[84] It has been suggested that one role of und-P may be in stabilizing such putative complexes.[85]

One component of the capsule translocation/surface-assembly machinery is Wza. Like Wzc and Wzb, Wza is highly conserved among different Group 1 K serotypes of *E. coli* and *K. pneumoniae*, indicating these proteins play a generic role in CPS assembly, and are not influenced by specific features in individual polymers.[25,30] If *wza* is mutated, *E. coli* K30 strains are able to polymerize the K30 antigen in K_{LPS} and translocate these molecules to the surface via an LPS-specific pathway, but they are unable to assemble a capsular layer on the cell surface.[86] Wza is an outer membrane lipoprotein with a β-barrel structure. Structural features of Wza place it in the OMA (Outer Membrane Auxiliary) family of proteins.[44] The Wza protein forms heat and detergent-stable multimeric ring-like structures[86] that resemble the "secretins" associated with filamentous phage assembly, and protein translocation through type II and type III systems, in a range of gram-negative bacteria.[87] Secretins exist as large channels formed by multimeric complexes of ten or more monomers. The capsule secretin has an apparent internal diameter of 3 nm but channel-forming activity has not yet been demonstrated.

Members of the OMA family are encoded by genes in the loci for Group 2 CPS in a variety of bacteria,[44] indicating their function is independent of the mechanism of synthesis. Mutation of CtrA, an OMA homologue from *Neisseria meningitidis* (whose CPS biosynthesis resembles that of *E. coli* Group 2 K-antigens[18]) results in a capsule-deficient phenotype.[88]

Given their size and potential impact on outer membrane integrity, such channels are presumed to be "gated". ATP-binding proteins have been suggested to play a role in gating some protein export secretins[89] and it is conceivable that Wzc plays an additional role in capsule assembly along these lines. Recent preliminary data suggests that Wza and Wzc may be part of a larger complex in *E. coli* K30 (J. Nesper, A. Paiment and C. Whitfield, unpublished data). It is striking that mutations in Wza have an acapsular phenotype but do not accumulate polymerized material within the cell (in the periplasm) in any detectable amount. These mutants still have wild-type levels of other CPS biosynthesis proteins in their membranes and measurable glycosyltransferase activity. Such results provide further indirect support for an assembly and export complex in which an inability to export the final product leads to reduced synthesis via some form of feedback mechanism.

For CPS, the final product remains associated with the cell surface, although the exact mode of linkage for Group 1 CPS is still unknown. In *E. coli* Group 1 CPS, the Wzi protein is a heat-modifiable monomeric β-barrel protein that plays a role in the final stages of CPS assembly.[90] The exact role of Wzi is still unknown but mutants in *wzi* show a significant reduction in surface-associated CPS and a corresponding increase in cell-free material. This phenotype is consistent with Wzi playing a role (direct or indirect) in surface attachment and it is striking that *wzi* is confined to those systems where the polymer product is tightly associated with the cell surface in a discrete capsular structure and is absent in those where the polymer is loosely associated EPS, such as colanic acid.

7. Conclusions

Significant inroads have been made into understanding the synthesis and genetics of bacterial capsules at a descriptive level. In particular, rapid sequencing techniques and genome projects have dramatically increased the numbers of known genes and loci. Many of the steps in capsule synthesis are now known, primarily from mutant phenotypes. Biochemical analyses have been initiated for some enzymes but an understanding of the enzyme mechanisms underlying most of these reactions is still elusive. For the Group 1 CPS and related EPS, the most important open questions surround the mechanism of flipping und-PP-linked intermediates across the inner membrane, the enzymatic activity and regulation of Wzy and any other proteins (e.g. Wzc?) involved in polymerization, and finally the mechanism of translocation. While current studies have identified many of the players involved in CPS/EPS assembly, it is likely that other cellular housekeeping systems also play critical roles. It will be important to map the interactions between these and the CPS/EPS-specific components in a hypothetical multiprotein complex for synthesis and translocation. As should be evident from the preceding text, there are common strategies and components used for assembly of capsules in bacteria with different physiologies and ecological niches. Resolution of the details of biosynthesis for one system should therefore rapidly lead to an understanding of others.

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