Phosphorylation of Wzc, a Tyrosine Autokinase, Is Essential for Assembly of Group 1 Capsular Polysaccharides in Escherichia coli*

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Wzc proteins are tyrosine autokinases. They are found in some important bacterial pathogens of humans and livestock as well as plant-associated bacteria, and are often encoded within gene clusters determining synthesis and assembly of capsular and extracellular polysaccharides. Autophosphorylation of Wzc is essential for assembly of the serotype K30 group 1 capsule in Escherichia coli O9a:K30, although a genetically unlinked Wzc-homologue (Etk) can also participate with low efficiency. While autophosphorylation of Wzc is required for assembly of high molecular weight K30 capsular polysaccharide, it is not essential for either the synthesis of the K30 repeat units or for activity of the K30 polymerase enzyme. Paradoxically, the cognate sular polysaccharide, it is not essential for either the naturally in Gram-positive bacteria. In this format, both expressed independently to mimic the situation found intermolecular transphosphorylation of Wzccps was detected in of Wzc requires a functional Walker A motif. Intermolecular transphosphorylation of Wzccps was detected in strains expressing a combination of mutant Wzccps derivatives. The N- and C-terminal domains of Wzccps were expressed independently to mimic the situation found naturally in Gram-positive bacteria. In this format, both domains were required for phosphorylation of the Wzc C terminus, and for capsule assembly. Regulation by a post-translational phosphorylation event represents a new dimension in the assembly of bacterial cell-surface polysaccharides.

Capsular and exopolysaccharides play crucial roles in the biology of many bacteria, acting either as virulence determinants that withstand host-cell defenses, or in establishing symbiotic relationships between bacteria and plants. More than 80 different capsular structures (known as K antigens) are produced by Escherichia coli isolates and these are subdivided into four different groups based on genetic and biochemical criteria (1). Surface polysaccharides with similar features are formed by other bacterial genera. The his-linked cps loci encode enzymes for the assembly of group 1 capsular K-antigens in E. coli and Klebsiella pneumoniae. The cps loci all contain a conserved region comprising the first 4 genes (wzf, wza, wzb, and wzc) (2), indicating a shared role in CPS expression. Following the conserved genes is a serotype-specific region encoding enzymes that participate in synthesis of polysaccharide repeat units and their polymerization via a Wzy-dependent mechanism (3). The Wzy-mediated polymerization reaction is thought to result in formation of an undecaprenyl pyrophosphate-linked glycan at the periplasmic face of the plasma membrane. The nascent polymer is then translocated to the cell surface via a process that requires outer membrane complexes formed by multimers of Wza (4). These complexes resemble the “secretins” for secretion of proteins via type II and type III systems.

A minor form of the group 1 K antigen is expressed on the cell surface in the form of low molecular weight K-antigenic oligosaccharides (one or a few K-repeat units) linked to LPS lipid A-core and termed K_<LPS> (5). Surface expression of K_<LPS> follows a distinct LPS translocation pathway that does not require Wza complexes in the outer membrane (4). The capsule structure evident in electron micrographs is formed from high molecular weight capsular K antigen (i.e. CPS) that, unlike K_<LPS>, is not linked to lipid A-core (5). Despite these organizational differences, undecaprenyl pyrophosphoryl-linked intermediates provide the substrate for the Wzy polymerase required for synthesis of both CPS and K_<LPS> (3).

The Wzc proteins in E. coli and K. pneumoniae strains that produce group 1 capsular polysaccharides are essentially identical. Homologues are also found in plant-associated Gram-negative bacteria that synthesize related extracellular polysaccharides. Examples include Erwinia amylovora, Xanthomonas campestris,Ralstonia solanacearum, and Sinorhizobium mililoti (reviewed in Ref. 6). In E. coli, Wzc participates in the surface assembly of CPS but is not required for K_<LPS> synthesis (3). Deletion of the wzc homologue (exoP) in S. meliloti also eliminates high molecular polysaccharide formation without affecting the ability to synthesize repeat units (7). The Wzc proteins therefore likely play a role in high-level polymerization and it has been suggested that they belong to a larger family of “polysaccharide copolymerase” proteins that dictate chain length in surface polymers including capsules, exopolysaccharides, and LPS O antigens (8).

Investigation of the purified Wzccps homologue (Ptk) in Acin...
etobacter johnsonii by Cozzone and co-workers (9) first showed that Wzc proteins possessed tyrosine autokinase activity. Subsequent studies by the same group confirmed similar properties for purified Wzccps from E. coli K-12 (10). The wzc gene is located in the locus responsible for synthesis of the slime exopolysaccharide known as colanic acid, whose production is dependent on growth conditions (e.g. temperatures below 30 °C) (11). Phosphorylation of Ptk and Wzca is induced to proceed by a novel phosphorylation mechanism because, unlike eukaryotic kinases, Ptk and Wzca contain a Walker A box ATP-binding motif (12) that is required for phosphorylation at multiple, but presently unidentified, tyrosine residues (13). Ptk and Wzca are dephosphorylated by the cognate phosphotyrosine protein phosphatases Ptp and Wzb, respectively, and these activities have been confirmed in vitro using purified proteins (10, 14). Based on conserved gene products and preliminary biosynthesis data, colanic acid appears to be synthesized by a Wzy-dependent pathway similar to group 1 capsules (reviewed in Ref. 1). However, despite the fact that the colanic acid biosynthesis locus is widespread in E. coli, it is absent in E. coli strains with group 1 capsules (1). The Wzc homologues from the group 1 CPS and colanic acid synthesis loci are well conserved (51.9% identity; 83.1% total similarity), as are the corresponding Wzb proteins (51.0% identity; 76.2% similarity) (3). Thus they likely play similar roles in both biosynthetic systems.

Interestingly, homologues of Wzc are also found in important Gram-positive pathogens including Streptococcus pneumoniae (15, 16), Streptococcus agalactiae (17, 18), and Staphylococcus aureus (19, 20). However, in these bacteria the N- and C-terminal domains of Wzc are represented in two separate polypeptides (15, 20). The CpsC protein of S. pneumoniae is equivalent to the membrane-associated N-terminal domain of Wzc while the CpsD protein is homologous to the C-terminal Walker box-containing domain of Wzc. Recent investigations have shown that CpsD is phosphorylated at a tyrosine-rich C-terminal domain (21). The product of the cpsB gene may be involved in phosphorylation of CpsC (21), although sequence analysis shows it lacks the sequence features (and catalytic site motifs) found in other small phosphotyrosine protein phosphatases. Cognate phosphatases have not (yet) been identified in all Wzc-containing systems. Intriguingly, phosphorylation of CpsD is proposed to negatively regulate capsule biosynthesis in S. pneumoniae (21).

While autokinase and phosphotyrosine protein phosphatase activity has been demonstrated for some Wzc and Wzb homologues, the relationship between Wzc phosphorylation and capsular polysaccharide assembly has not been investigated in Gram-negative bacteria. Here, we show that two genetically unlinked wzc homologues on the chromosome of E. coli O9a: K30 contribute to different extents in the assembly of the K30 capsule. The phosphorylated tyrosine residues of Wzccps are located to the C-terminal 17 residues of Wzccps and assembly of the E. coli group 1 capsule is demonstrated to require both phosphorylation-competent Wzccps and active Wzbp phosphatase. The effect of phosphorylated Wzccps on capsule assembly differs from that of phosphorylated CpsD in S. pneumoniae.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions**—The bacterial strains and plasmids used in this study are listed in Table I. All strains were grown and maintained in Luria-Bertani (LB) medium at 37 °C. Where appropriate, media were supplemented with antibiotics to the following concentrations: ampicillin (Ap, 100 μg ml⁻¹), chloramphenicol (Cm, 34 μg ml⁻¹), gentamicin (Gm, 30 μg ml⁻¹), kanamycin (Kn, 50 μg ml⁻¹), and spectinomycin (Sp, 100 μg ml⁻¹). Expression of genes cloned in pET and pBAD derivatives was induced using 0.1–0.5 mM isopropyl-β-D-thiogalactopyranoside and 0.02% L-arabinose, respectively.

**Construction of Chromosomal Insertion Mutations in etk and wzb**—The etk gene is located in a gene cluster at 22 min on the E. coli K12 chromosomal map and is downstream of wzb and wzb homologues. A chromosomal wzb::aacC1 insertion mutation (strain CWG258) was reported previously (4). The spectinomycin-resistant (aadA) gene cassette is flanked by translational terminators (22) and polarity therefore eliminates expression of downstream genes (i.e. wzb and etk).

The pWQ129 suicide delivery plasmid carrying wzb::aadA was used to mutate E. coli CWG315 (wzc::aacC1) to generate CWG285 (wzccps::aadC1 wzb::aadA), by allelic exchange. To generate the chromosomal wzb::mutation, a 1.25-kilobase fragment spanning wzb::, was amplified by PCR and cloned. The nonpolar promoterless cassette was inserted into the XhoI site within wzb::. Plasmid pWQ146, a pMAK705-based suicide-delivery construct, was then used to transfer the mutation onto the chromosome of CWG343 (wzccps::aadA wzb::aph3A) by allelic exchange. Details of the approach for allelic exchange are described elsewhere (4). In all cases, correct insertions in chromosomal genes were confirmed by analysis of products of PCR reactions and by sequencing across the insertion using primers flanking the targeted region.

**PCR Amplification and Cloning of etk, wzc, and Its Deletion Derivatives**—Restriction and DNA-modifying enzymes were used according to the manufacturer’s instructions. Transformation was done by electroporation using a Gene Pulsar from Bio-Rad (23). PCR amplification was carried out using a Perkin-Elmer System 2400 thermocycler. Oligonucleotide synthesis and automated DNA sequencing services were provided by the Guelph Molecular Supercentre (University of Guelph, Ontario, Canada). The sequences of oligonucleotides are listed in Table II. All amplification reactions were performed in 50-μl reactions using Pfu polymerase (Roche Molecular Biochemicals) and conditions optimized for each primer pair. PCR products and plasmid DNA fragments were purified using a QIAQuick PCR purification kit (Qiagen) and/or Geneclean II (Bio101). To clone wzccps, the 2.5-kilobase open reading frame was amplified by PCR using primers TW6 and JD102. The PCR product was digested with EcoRI and PstI and cloned into pBAD18-Km to give plasmid pWQ130. The wzccps gene was also amplified as an N-terminal His-tagged fusion protein using primer pair TW20-TW19. These primers introduced NdeI and EcoRI restriction sites facilitating cloning in pET22a(+)(Novagen), resulting in plasmid pWQ141. The etk gene was amplified using the primer pair JD141-JD142. These primers introduced flanking MfeI and PstI restriction sites, allowing the product to be ligated to the EcoRI and PstI sites of pBAD24, resulting in plasmid pWQ131. Derivatives of wzccps with C-terminal deletions were generated by PCR, using primer TW6 and reverse primers that introduced novel termination codons. Primers TW7 (for Wzccps) and TW8 (for Wzccps) also provided appropriate restriction sites to facilitate cloning of the amplified fragments in pBAD18-Cm. The resulting plasmids were pWQ133 (expressing Wzccps and wzb) and pWQ139 (Wzccps). The DNA fragment encoding the C-terminal domain of Wzccps (Wzccps) was amplified using the primer pairs TW9-JD102 and the product was cloned in pBAD24, giving plasmid pWQ140. In all cases, the sequences of PCR-amplified genes were determined to ensure no errors were introduced during amplification.

**Localization of Wzccps in Subcellular Fractions**—Expression of Wzccps and its mutant derivatives was achieved using the pBAD arabinose-inducible expression vectors. Bacteria were grown to mid-exponential phase and expression of the Wzccps derivative was induced by adding 0.02% L-arabinose. After an induction period of 15 min to 2 h, the cells were harvested and resuspended in 100 mM Tris-HCl, pH 7.5, containing 100 mM MgCl₂ and 1 mM phenylmethylsulfonyl fluoride (buffer A). Subcellular fractionation was performed at 4 °C. The cell suspension was lysed by ultrasonication, after which unbroken bacteria and large debris were removed by centrifugation at 40,000 × g for 8 min. The cell-free lysate was centrifuged at 100,000 × g for 30 min, resulting in a cytosol-periplasm fraction (supernatant) and a pellet containing cell debris. The cell envelopes were resuspended in buffer A and further separated into inner and outer membranes by solubilization with 2% (w/v) Sarkosyl in buffer A for 30 min (24). The outer membrane is insoluble under these conditions, and was collected as a pellet at 40,000 × g for 30 min. Wzccps was identified in the cellular fractions by Strep-tag® Western blotting.

**Expression and Partial Purification of GST-Wzccps**—To generate an N-terminal glutathione S-transferase (GST)-Wzccps fusion derivative, the wzccps open reading frame was amplified by PCR using primers AP05 and AP06 (Table II). After digestion of the fragment with EcoRI and SuI at sites introduced in the primers, the
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Expression and Purification of His6-Wzbcps—The wzb cps gene was amplified as a 506-bp fragment using primers JD152 and JD153 and cloned into pBAD24 to give pWQ147. For purification, a His6-Wzbcps derivative was used. The wzb cps open reading frame was amplified by PCR using primers AP03 and AP04 (Table II). After digestion of the product at the Ndel and BamHI sites introduced in the primers, the fragment was cloned in pET28a (+) giving plasmid pWQ145 and generating an N-terminal His6-tag on Wzbcps. The same approach was used to generate pWQ149 expressing the His6-tagged version of the mutated derivative WzbC13S. The protein was purified from cell-free lysates of E. coli BL21 (DE3) containing pWQ145, prepared in buffer D (50 mM Na-phosphate buffer, pH 8.0, 300 mM NaCl) containing 10 mM imidazole. After removing cellular debris by centrifugation at 30,000 × g for 30 min, the cell-free lysate was mixed with chromatography matrix on ice for 30 min, before packing in a column for washing and elution. The column was washed with 3 × 2 ml of phosphate-buffered saline containing 1% Triton X-100. Elution was performed using buffer E (50 mM Tris-HCl, pH 8.0) containing 10 mM glutathione and 1% Triton X-100. Fractions containing the GST-Wzbcps protein were pooled and dialyzed against buffer C (25 mM Tris-HCl, pH 7.0, 1 mM diethiothreitol, 5 mM MgCl2).

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**Table II**

| Primer used in this study | Sequence<sup>a</sup> | Primer features<sup>b</sup> |
|---------------------------|----------------------|-----------------------------|
| Wzc<sub>p</sub> | Wzc<sub>p</sub> |  |
| 166–721 |  |  |
| JD102 (−) | ACCATAACAGAACCACACAG | Etk; Mfi |
| JD141 (+) | TTATGAAATGATGACAACATAAAAATATG |  |
| JD142 (−) | GCCCTGCTGTTTCCTTTCT | Etk; PstI |
| TW3 (+) | AGTCCAGGCGATGGAagCAGTATTTTAGC | Wzc<sub>R</sub> |
| TW4 (−) | GCTAATGAAAGTCTTACCCTCAGTACGACT | Wzc<sub>R</sub> |
| TW6 (+) | AGACGCGCATCGTTGAGATGGC | Wzc<sub>p</sub>; Wzc<sub>1</sub>–704; Wzc<sub>1</sub>–480 |
| TW7 (−) | AACGCAGCAATCGCAGAAATAGG | Wzc<sub>R</sub> |
| TW9 (+) | CGTCAGCCTAGGAGTAGGAGAAGTTACAT | Wzc<sub>R</sub>; IbeI, stop codon |
| TW19 (+) | GATTAGCTATTAGATATTACATGACACAGG | Wzc<sub>R</sub> |
| TW20 (+) | CAGAATAAAATGATTATGTACGAGAT | EcoRI |
| TW21 (+) | AAGCTCTCTGCTACAGCCAGATAAG | Wzc<sub>R</sub> |
| JD152 (+) | GAGAACAGTACTGATATTGAGTAG | EcoRI |
| JD153 (−) | AGATTGACGCTAGCTAAATTT | Wzb<sub>R</sub> |
| AP01 (+) | CATATTGAGTATCTCAGAGGAAATACTC | Wzb<sub>R</sub> |
| AP02 (−) | GATTATGCTGTCTGAGATCATCATAAATG | Wzb<sub>R</sub> |
| AP03 (+) | AGGATATACATCTGAGCTGACAAAGA | Wzb<sub>R</sub> |
| AP04 (−) | TCTGAGAGATCCGAGATGGCTGA | Wzb<sub>R</sub> |
| AP05 (+) | GGAATCTTGCGACTCTGACATCATCT | BamHI |
| AP06 (−) | CAATAGTAAGCTCAGCAGTTTGTCGTC | Wzc<sub>R</sub> |

<sup>a</sup> Forward and reverse primers are represented by plus (+) or minus (−), respectively.

<sup>b</sup> Restriction sites are underlined and listed under primer features.

<sup>c</sup> Bases altered from those in the chromosome are in lower case.

<sup>d</sup> Use of a primer for amplification of a fragment encoding a particular polypeptide, or for site-directed mutagenesis, is indicated. Restriction sites incorporated in the primer sequences are listed.

**SDS-PAGE Analysis of Cell-surface Polysaccharides**—Cell surface polysaccharides from protease K-digested whole cell lysates were isolated as described by Hitchcock and Brown (26), and analyzed by electrophoresis on 10–20% Tricine SDS-PAGE gels from Novex (San Diego, CA). Following electrophoresis, the LPS-containing molecules were visualized by silver staining (27). For Western blotting, samples were electrophoretically transferred to 0.45-μm BioTrace NT membranes (Gelman Science) and probed with polyclonal rabbit anti-K30 serum (28), which recognizes the identical serotype K30 repeat unit found in both K30<sub>dp</sub> and K30 CPS. Rabbit polyclonal antibodies specific for the K40 antigen were described previously (29).

**Bacteriophage Sensitivity Assays**—To test for assembly of the K30 capsular layer, strains were assessed for their sensitivity to lysis by specific bacteriophages. The presence of surface-expressed K30 capsular antigen was determined using bacteriophage K30, which requires the K30 antigen as its receptor (30). Bacteriophage O9-1 is specific for the LPS O9a antigen (31). In the O9a-K30 wild-type background, the capsular layer masks the LPS O9a antigen and this strain is O9-1 resistant, but a reduction or absence of K30 capsular antigen unmask the bacteriophage O9-1 receptor.

**RESULTS**

**Two Functional wzc Homologues on the Chromosome of E. coli**

**Figure 1** shows the *wzc* gene is located in the K30 antigen biosynthesis (*cps*) locus of a prototype group 1 capsule-producing strain, *E. coli* E69 (O9a:K30) (3). When examined in Western blots using anti-K30 antigen antibodies, the cell-surface polysaccharides of the wild-type strain (*E. coli* E69) showed reactivity in both the K30<sub>dp</sub> fraction (containing primarily a single K30 repeat unit) and in high molecular weight CPS (Fig. 1). Previously, we showed that cell lysates of *E. coli* CWG315 (*wzc*<sub>p</sub>:uncC1) still contained immunoreactive K30 capsular antigen, but the amounts were significantly lower than those observed in wild-type lysates (Fig. 1). In addition, *E. coli* CWG315 was unable to assemble a capsular structure that masked the underlying LPS O9a antigen. The *wzc*<sub>p</sub> mutation in CWG315 has no discernible effect on K<sub>LPS</sub> (3). Subsequent studies by others have revealed that many *E. coli* strains contain a homologue of *wzc*<sub>p</sub>, designated *etk* (32). The *etk* gene is located in the appA (22 min) region of the sequenced *E. coli* K12 genome. The predicted Etk protein is an autokinase that is only expressed in some *E. coli* strains. It shares 74% similarity (57% identity) with *wzc*<sub>p</sub> (data not shown). Although the biological role of Etk is unknown, the 22-min locus

using primers AP01 and AP02, to give pWQ148. Following PCR amplification, the reaction products were purified using the QIAquick PCR purification kit. The DNA was digested twice with *Sal*I to eliminate all nonspecific antibodies.
contains several open reading frames whose predicted products are homologous to proteins involved in polysaccharide assembly (4). The products of the last three open reading frames are part of the same transcriptional unit and encode homologues of Wza, Wzb, and Wzc (i.e., Etk). This entire region was amplified by PCR and shown to have essentially the same sequence in E. coli K12 and E. coli O9a:K30 (data not shown). Furthermore, it was demonstrated that the \( wza_{22} \) gene product functions with low efficiency in the translocation of E. coli K30 CPS (4). To assess whether the small amount of K30 CPS remaining in E. coli CWG315 (\( wzc_{22}:aadC1 \)) resulted from \( ethk \) expression, we constructed CWG285 (\( wzc_{22}:aadC1 wza_{22} \) min::aadA) in which \( wza_{22} \) min and \( ethk \) expression is eliminated by the polar \( aadA \) cassette inserted in \( wza_{22} \) min. The capsule phenotypes of these strains were assessed by Western blotting and bacteriophage-sensitivity assays.

Western blot analysis of E. coli CWG258 (\( wza_{22} \) min::aadA) using anti-K30 antigen antibodies showed no significant reduction in synthesis of the CPS compared with the wild-type (Fig. 1) and the mutant strain showed no reaction with bacteriophage O9-1, indicating the maintenance of a protective K30 capsule. In the double mutant, CWG285 (\( wzc_{22}:aadC1 wza_{22} \) min::aadA), formation of CPS was completely abolished. No reaction was detected with the capsule-specific bacteriophage K30 and the strain was sensitive to bacteriophage O9-1. Although no CPS was synthesized, CWG285 showed a significant increase in the extent of polymerization (8–10 repeat units) of K30LPS reflected in the ladder of immunoreactive material.

Plasmids carrying the \( wzc_{cpp} \) and \( ethk \) genes were used to complement the K30 capsule synthesis defect in CWG285 (\( wzc_{cpp}:aadC1 \) \( wza_{22} \) min::aadA). Expression of plasmid pWQ130-encoded Wzc\(_{cpp}\) restored synthesis of K30 CPS and reduced the degree of polymerization of K30LPS to the typical wild-type 1–2 repeat units (Fig. 1). While K30 CPS synthesis was restored in the complemented strain, the amount never achieved that seen in the wild-type strain. The reason(s) for this is unclear. While phosphorylated Wzc is clearly overexpressed in the complemented strain (see below), the precise amount of Wzc\(_{cpp}\) that is competent in CPS synthesis is unknown. This result could also reflect issues relating to stoichiometry of the components in a putative multienzyme complex. Expression of plasmid pWQ131-encoded Etk resulted in smaller amounts of CPS being formed (relative to Wzc\(_{cpp}\)) and the polymerization of K30LPS remained at the higher level. The lower activity of Etk is consistent with the absence of a detectable CPS phenotype of CWG258 (\( wza_{22} \) min::aadA). From these data and the phenotypes of CWG258 and CWG315 (above), we conclude that \( ethk \) encodes a functional homologue of Wzc\(_{cpp}\) that participates with low efficiency, relative to Wzc\(_{cpp}\), in assembly of the K30 capsule. The influence of Etk is not apparent in the presence of functional Wzc\(_{cpp}\). In all subsequent experiments, strains carrying a \( wza_{22} \) min::aadA mutation were used to simplify the analysis and isolate the effects of Wzc\(_{cpp}\) and Wzb\(_{cpp}\) without the complications of \( ethk \) and \( wzb_{22} \) min.

Subcellular Location of Phosphorylated Wzc\(_{cpp}\)—To examine Wzc\(_{cpp}\) localization in a known genetic background, it was over-expressed in E. coli DH5\(_a\). Although E. coli K-12 derivatives like DH5\(_a\) contain functional copies of \( wzc_{22} \) and \( ethk \), these are not evident in control samples. Etk may not be expressed in E. coli DH5\(_a\) (32) and there is no significant transcription of the colanic acid genes (including \( wzc_{22} \)) in E. coli K-12 at 37 °C (reviewed in Ref. 33). To determine the subcellular localization of Wzc\(_{cpp}\), cell envelopes from arabinose-induced E. coli DH5\(_a\) (pWQ130) cells were separated into the inner membrane (Sarkosyl soluble) and outer membrane (Sarkosyl insoluble) fractions. A Western blot of the membrane fractions was probed with a commercial monoclonal anti-phosphotyrosine antibody (PY20) which is known to react with homologues of Wzc\(_{cpp}\) (32). The phosphorylated Wzc\(_{cpp}\) protein (predicted molecular weight = 79,558) is localized in the inner membrane (Fig. 2), as expected from sequence features and the relationships shared among the Wzc family of tyrosine autokinases. This location was further confirmed by examining membranes fractionated by isopycnic sucrose gradient centrifugation (data not shown).

In Vitro Autophosphorylation of Wzc\(_{cpp}\) and Its Dephosphorylation by Wzb\(_{cpp}\)—To confirm the autophosphorylation of Wzc\(_{cpp}\) without ambiguity, we employed the strategy used to establish autophosphorylation of Wzc\(_{cpp}\) (10). The GST-Wzc\(_{cpp}\) fusion protein was purified by chromatography on glutathione-Sepharose 4B. Based on Coomassie Blue-stained SDS-PAGE gels, this protein preparation was >90% pure (Fig. 3, panel A). The purified protein showed autokinase activity, incorporating radioactivity from \( \gamma^{-33}P \)ATP in a time-dependent manner (Fig. 3, panel B). The cognate phosphatase (Wzb\(_{cpp}\)) was purified as a His\(_6\)-tagged derivative to >90% purity (Fig. 3, panel C). As previously shown for Wzb\(_{cpp}\) (10), Wzb\(_{cpp}\) catalyzed the dephosphorylation of Wzc\(_{cpp}\) (Fig. 3, panel C).

Phosphorylation of Wzc\(_{cpp}\) Is Essential for Capsule Synthesis—Sequence alignments revealed that Wzc\(_{cpp}\) (like its homologues) contains a Walker A or kinase-1a motif, commonly found in kinases (Fig. 4). The sequence Ala\(^{534}\)Ser-Pro-Ser-Ala-Gly-Lys-Thr\(^{541}\) fits the general consensus sequence motif (AG(X,GK)ST). Two putative Walker B or kinase-2 motifs (hh-

**Fig. 1.** Two functional Wzc homologues contribute to formation of the E. coli K30 capsule. Panel A shows a silver-stained Tricine-PAGE analysis of cell-surface polysaccharides and panel B, the corresponding Western blot probed with polyclonal K30 antiserum. Lane 1, wild-type E. coli E69 (O9a:K30); lane 2, CWG258 (\( wza_{22} \) min::aadA); lane 3, CWG315 (\( wzc_{22}:aadC1 \)); lane 4, CWG315 complemented by plasmid pWQ130 (\( Wzc_{cpp} \)); lane 5, CWG285 (\( wzc_{cpp}:aadC1 wza_{22} \) min::aadA); lane 6, CWG285 complemented by plasmid pWQ131 (Etk\(_{cpp} \)); and lane 7, CWG285 complemented by plasmid pWQ130 (Wzc\(_{cpp}\)). Each construct was tested for susceptibility to bacteriophage K30 that requires K30 CPS as its receptor. The receptor for bacteriophage O9-1 is serotype O9a LPS; this bacteriophage can only lyse bacteria where the receptor is unmasked by reduction or elimination of the K30 capsule.
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Fig. 2. Localization of overexpressed Wzccps in an E. coli DH5α-background. A, Coomasie Blue-stained SDS-PAGE; B, corresponding Western blot, probed with monoclonal anti-phosphotyrosine antibody (PY20). MW, molecular weight markers; lane 1, whole cell lysate; lane 2, cell envelope fraction; lane 3, Sarkosyl-solubilized inner membrane fraction; and lane 4, Sarkosyl-insoluble outer membrane fraction.

Fig. 3. In vitro autokinase activity of GST-Wzccps and its dephosphorylation by Wzbcps. Panel A shows soluble fusion proteins, GST-Wzc::His6-Wzb::His6-Wzb purified by affinity chromatography procedures. The GST-Wzc::His6 protein shows an apparent molecular weight of 105,000 (predicted Mr = 106,267; native Wzc::His6, Mr = 79,558). The apparent molecular weight of His6-Wzb::His6-Wzb is 18,000 (predicted Mr = 18,796; native Wzb::His6, Mr = 16,604). Panel B is an autoradiogram showing autophosphorylation of GST-Wzc using [γ-32P]ATP as substrate. The only phosphorylated protein comigrated with Wzc::His6 and only the relevant part of the gel is shown. Panel C shows the dephosphorylation of 32P-labeled GST-Wzc::His6-Wzb::His6-Wzb by purified His6-Wzb. Panel D shows an equivalent dephosphorylation experiment using the catalytically inactive His6-Wzb::His6-Wzb.

hhD; where h represents a hydrophobic amino acid) were detected. The optimal spatial distances between Walker A and B motifs are approximately 61 or 145 amino acid residues (34). While neither of the two Walker B motif candidates (VLFID562 and LIID642) provides such optimal spacing, the aspartic acid residue within the sequence VIID651 of the A. johnsonii Ptk protein (which corresponds to the LIID642 motif in Wzc::His6) was found to be essential for ATP binding (13).

Tyrosine phosphorylation of Ptk requires a functional Walker A box for ATP binding and hydrolysis (13). To confirm that Wzc::His6 autophosphorylation follows a similar mechanism, PCR-based site-directed mutagenesis was used to generate WzcK540R (Fig. 4). Residues equivalent to lysine 540 in the Walker A kinase-1a-motif are believed to be required for ATP hydrolysis in the phosphotransfer reaction (34, 35). The WzcK540R derivative was detected with anti-Wzc::His6 antibodies (Fig. 5A) and was expressed at levels equivalent to the wild-type Wzc::His6 protein. When probed with monoclonal PY20 anti-phosphotyrosine antibody, only the wild-type protein was phosphorylated (Fig. 5B). The function of WzcK540R in capsule assembly was also completely abolished. No K30 capsular antigen could be detected in Western blots using polyclonal anti-K30 serum (Fig. 5C) or in phage sensitivity assays (data not shown) and the increased level of K5 CPS polymerization was not altered by this derivative. These data show that a functional Walker box, capable of ATP binding, is necessary for Wzc::His6 function in capsular assembly.

C-terminal Tyrosine Residues Provide the Sites of Phosphorylation and Are Required for Wzc::His6 to Function in Capsule Formation—Previous studies suggested that Ptk, and presumably its homologues, are phosphorylated at multiple tyrosine residues (9) but the site of phosphorylation was not determined. Sequence alignments including Wzc::His6 homologues from Gram-negative and Gram-positive bacteria revealed several highly conserved C-terminal tyrosine residues (Fig. 4) and while this work was in progress phosphorylation of CpsD was shown to occur in the C-terminal tyrosine-rich domain (21). Seven of the last 17 amino acid residues of Wzc::His6 are tyrosine residues. To address the relevance of this tyrosine-enriched domain, the 17 C-terminal amino acids were removed in a truncated version of Wzc::His6 designated Wzc1–704 (Fig. 3). Wzc1–704 was reactive with anti-Wzc::His6 antibody (Fig. 5A) and localized to the inner membrane (data not shown) but the derivative could no longer undergo tyrosine phosphorylation (Fig. 5B). Furthermore, the Wzc1–704 protein was no longer able to complement the CPS-deficient phenotype in strain CWG285 (wzc::aacC1 wzb::mini-aph3A) (Fig. 5C), consistent with the proposal that phosphorylation of these tyrosine residues is required for normal Wzc::His6 function.

To determine whether the truncated Wzc1–704 derivative still retains the ability to bind ATP, it was coexpressed in CWG285 with the WzcK540R protein. This resulted in transphosphorylation of WzcK540R (Fig. 5B) and generation of Wzc activity that restored the assembly of CPS (Fig. 5C).

Activity of the Phosphotyrosine-protein Phosphatase, Wzb::His6 Is Required for Capsule Assembly—Since Wzb homologues are known to dephosphorylate their cognate Wzc proteins (10, 14) and the phosphotyrosine-protein phosphatase activity for Wzb::His6 was confirmed as described above, we constructed CWG343 (wza::mini-aph3A wzb::mini-aph3A) to examine the effect of Wzb::His6 dephosphorylation on CPS assembly. The presence or absence of Wzb::His6 has no significant effect on the amount of Wzc polypeptide produced (data not shown), or on the amount of phosphorylated Wzc::His6 (Fig. 6). However, CWG343 retains only trace amounts of K30 CPS synthesis, detectable as immunoreactive material in Western blots (Fig. 6) and by sensitivity to the K30 CPS-specific bacteriophage K30 (data not shown). Introduction of pWQ147 (Wzb::His6) restored synthesis of K30 CPS indicating that the defect in CWG343 was attributable only to the single nonpolar insertion in wzb::His6. Coomasie Blue-stained SDS-PAGE gels of CWG343/pWQ147 whole cell protein lysates showed the presence of an overexpressed protein with the apparent molecular weight (16,604 predicted) ex-
One possible interpretation of these data is that the phenotype of CWG343 is due to a loss of protein-protein interactions, rather than a simple loss of phosphotyrosine protein phosphatase activity. To address this question, we constructed pWQ148 and pWQ149 in which Wzb CPS has a C13S mutation. The phosphotyrosine-protein phosphatase signature motif (H/V)C(X5)R(S/T) contains a nucleophilic cysteine residue (Cys13 in Wzb CPS) that forms a phosphocysteine intermediate during catalysis. This residue is essential for catalysis (37, 38) and has been shown to be required for the activity of Wzb CPS (14). As expected, the Wzb C13S showed no activity in vitro against phosphorylated Wzc CPS (Fig. 3, panel D). Although the mutant protein was made in and readily detectable in CWG343 cell lysates (data not shown), it was unable to restore CPS synthesis.

The transmembrane (TM) helices and tyrosine-containing C-terminal domain are indicated.

Fig. 4. Structural features of Wzc CPS. A, C-terminal sequence alignment of selected Wzc CPS homologues from Gram-positive and Gram-negative bacteria. The GenBank™ accession for each homologue is indicated. The arrows indicate the 7 tyrosine residues in Wzc CPS from E. coli O9a:K30. Identical residues are indicated by asterisks (*). Residues with high (:) or low (.) levels of similarity are also identified. B, schematic organization of Wzc CPS and its mutant derivatives. The location and sequence of the Walker A ATP-binding motif of wild-type Wzc CPS and the mutant Wzc<sup>K540R</sup> are shown. The strategy for separating the N- (Wzc<sup>1-480</sup>) and C-terminal (Wzc<sup>486-721</sup>) domains was based on the "two-part" Wzc CPS homologues in Gram-positive bacteria.

FIG. 5. Phosphorylation of Wzc CPS is essential for capsule synthesis. Panel A shows a Western blot of whole cell protein lysates developed using anti-Wzc CPS antibodies, showing expression of Wzc CPS and its mutant derivatives expressed from plasmids in E. coli CWG285 (wzc<sup>cpp</sup>:aacC1 wza<sup>22</sup> min<sup>22</sup> :aadA); lane 1, CWG285 (no plasmid); lane 2, pWQ130 (Wzc<sup>cpp</sup>); lane 3, pWQ132 (Wzc<sup>cpp</sup>:aacC1); lane 5, pWQ132 plus pWQ133. Panel B shows a Western blot of the same samples probed with monoclonal anti-phosphotyrosine antibody (PY20) to detect the phosphorylation of the Wzc CPS derivatives. Panel C shows a Western blot of the cell-surface polysaccharides from the same constructs probed with polyclonal K30 antiserum.

FIG. 6. Assembly of CPS also requires a functional Wzb protein. Panel A shows a Western blot of whole cell protein lysates probed with PY20 anti-phosphotyrosine antibodies, and panel B shows cell-surface polysaccharides from the same constructs in a Western blot probed with K30 antiserum. Lane 1, E. coli CWG258 (wza<sup>22</sup> min<sup>22</sup> :aadA); lane 2, CWG285 (wzc<sup>cpp</sup>:aacC1 wza<sup>22</sup> min<sup>22</sup> :aadA); lane 3, CWG343 (wza<sup>22</sup> min<sup>22</sup> :aadA wzb<sup>cpp</sup>:aph<sup>3</sup>A); lane 4, CWG343 containing pWQ148 (Wzb<sup>13S</sup>); lane 5, CWG343 containing pWQ147 (Wzb<sup>cpp</sup>).
whether different domains of Wzc CPS would also be functional.

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A Functional Wzc CPS Comprising Independently Expressed N- and C-terminal Domains—Wzc CPS homologues in Gram-positive bacteria are encoded as two distinct proteins with the corresponding genes generally found next to one another in the same cluster. For example, in S. pneumoniae CpsC resembles the N-terminal membrane-anchored part of Wzc CPS. The adjacent gene (cpsD) encodes a predicted polypeptide equivalent to the C-terminal hydrophilic domain containing the ATP-binding motif and the conserved tyrosine residues (15). Both polypeptides are required for autophosphorylation (21). To determine whether different domains of Wzc CPS would also be functional as separate polypeptides, initiation and termination codons were introduced and fragments expressing the N and C termini of Wzc CPS were cloned independently. The break point (between amino acid residues 481 and 494) was dictated by sequence alignments with known Wzc CPS homologues in Gram-positive bacteria (data not shown). A termination codon was introduced after Lys480 generating the N-terminal polypeptide Wzc1-480. The Gly486 codon was replaced with an ATG start codon to express the soluble C-terminal polypeptide Wzc486-721. The Wzc1-480 and Wzc486-721 derivatives were cloned behind the arabinose-inducible promoter in pBAD18-Cm and pBAD24, generating plasmids pWQ139 and pWQ140, respectively.

The Wzc CPS N- and C-terminal domains (Wzc1-480 and Wzc486-721) were expressed and located in the inner membrane and cytosol, respectively (data not shown). While the truncated Wzc1-480 N-terminal fragment was readily detected in Western blots with anti-Wzc CPS antibodies (Fig. 7A), the Wzc486-721 C-terminal fragment was expressed at only low levels and could only be detected in heavily overloaded SDS-PAGE gels (data not shown). When expressed alone in strain E. coli CWG285, neither part of Wzc CPS could function in capsule formation (Fig. 7C). However, when both proteins were expressed simultaneously in CWG285, CPS synthesis was restored. The function in capsule formation was correlated with phosphorylation of the Wzc486-721 C-terminal domain, as this only occurred when both parts of Wzc CPS were coexpressed (Fig. 7B). The amount of CPS was lower than wild-type and a masking K30 capsule was not formed based on the sensitivity of the transformant to bacteriophage O9-1. The low efficiency in the two-part construct may be related to low level expression of soluble Wzc486-721 and its phosphorylation in a CWG285 background. It is also conceivable that the stoichiometry of the two Wzc CPS domains is important and this cannot be controlled in the two-plasmid system.

The N Terminus of Wzc CPS Cannot Serve as a Functional Replacement for the LPS O-Antigen Chain-length Regulator, Wzz—The N-terminal domains of Gram-negative Wzc proteins contain two putative transmembrane helices flanking a periplasmic loop. This topology resembles that of Wzz, the LPS O antigen chain-length determinant and the two classes of proteins share sequence similarity (6, 8, 39). Wzz proteins lack the additional C-terminal sequences that, in Wzc, contain the ATP-binding motif and tyrosine-rich domain. Wzz determines the distribution of O-antigen chain lengths and generates a modal pattern in LPS size distribution, evident as clusters of bands in SDS-PAGE. To determine whether the N terminus of Wzc CPS can alter chain length modality as does Wzz, we used the E. coli K40 antigen as a reporter system. The K40 antigen is a group 4 capsule (1) in which the majority of the polysaccharide is linked to lipid A as an LPS O antigen. Wzz controls the modality of the K40 LPS (29) and the wzz::aacC1 mutant (CWG290) shows a loss of a modal cluster of K40 LPS bands in SDS-PAGE gels and accumulation of lower molecular weight lipid oligosaccharides of E. coli O8:K40 and its derivatives. The blot was probed with anti-K40 antibodies. Lanes 1 and 2 show the wild-type strain (2757) and its wzz::aacC1 derivative (CWG280). Lane 3 shows CWG280 transformed with pWQ139 (encoding Wzc1-480) grown in the presence of 0.2% glucose to repress the pBAD promoter. Lanes 4 and 5 show the same strain after induction with 0.02 and 0.2% arabinose, respectively. Lane 6 shows CWG290 transformed with pWQ30 (Wzc CPS) to illustrate restoration of modality. The sample in lane 7 is from CWG290 transformed with pWQ139 (Wzc CPS) induced with 0.2% arabinose.
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Tyrosine autokinase activity has been documented in Ptk from A. johnsonii (41), WzcE from E. coli K-12 (10), Etk from E. coli (32), AmsA from E. amylovora (32), and now Wzc$_{epsa}$ from E. coli isolates with group 1 capsules. However, the exact function of these proteins and the role of their phosphorylation in the biology of their respective microorganisms have not been established. The involvement of a Walker A motif in autokinase activity in these enzymes suggests that the kinase catalytic mechanism differs from that in eukaryotic protein kinases (13). Phosphorylation of Ptk requires ATP binding and hydrolysis (13) and a similar activity in Wzc$_{epsa}$ was confirmed experimentally by site-directed mutation of the invariant Lys$^{640}$ of the Walker A box. This residue is believed to be involved in ATP binding and hydrolysis (34, 35). The Wzc$^{K540R}$ mutation prevented phosphorylation of Wzc and completely abolished its function in capsule assembly, providing the first indication that ATP binding is essential, and that the phosphorylated form of Wzc$_{epsa}$ is the one that is functional in CPS expression. The truncated Wzc$_{epsa}$ derivative (Wzc$^{1–704}$) identified the highly conserved C-terminal tyrosine-rich domain residues as the site of phosphorylation and confirmed the essential requirement for Wzc$_{epsa}$ phosphorylation in group 1 CPS synthesis. While the CPS phenotypes arising from an ATP-binding defect and a lack of the phosphorylation site are identical, we cannot rule out the possibility that they have different detrimental effects on the CPS synthesis process. Although the role of phosphorylation of Wzc$_{epsa}$ has not been directly tested in other Gram-negative CPS synthesis systems, production of the exopolysaccharide succinoglycan is drastically reduced in S. meliloti strains expressing a derivative of ExoP (Wzc) lacking the C terminus (42). By analogy to Wzc, this region of ExoP contains the phosphorylated tyrosine residues. As with the E. coli group 1 CPS system, the exoP-deletion strain is still able to synthesize lipid-linked glycan repeat units (7).

The effect of Wzc phosphorylation on group 1 CPS expression differs from that seen in the representative Gram-positive system from S. pneumoniae (21), where domains resembling the N and C termini of Gram-negative Wzc are found in separate polypeptides encoded by adjacent genes. Proteins sharing sequence similarity and features with the C-terminal domain of Wzc$_{epsa}$ include CpsD in S. pneumoniae (15) and S. agalactiae (17, 18), CapB from S. aureus (19), EpsD from Streptococcus thermophilus (43), and EpsB from Lactococcus lactis (44). CpsC, CapA, EpsC, and EpsA encode the corresponding membrane-associated domains, respectively. In S. pneumoniae CpsD, a Walker box mutation renders the protein unable to autophosphorylate and generates bacteria that synthesize only trace amounts of CPS (21). The size of the remaining CPS product was not reported. In contrast, the phosphorylation of the tyrosine residues per se was not required for CPS synthesis in S. pneumoniae. In fact, replacement of the C-terminal tyrosines with phenylalanine leads to a mucoid phenotype, presumed to reflect an increase in CPS expression. The differences in phenotype in the E. coli and S. pneumoniae systems are not simply due to unexpected effects arising from the use of a C-terminal deletion derivative for the E. coli analyses. The ability of Wzc$^{K540R}$ and Wzc$^{1–704}$ to undergo intermolecular transphosphorylation and restore CPS synthesis is consistent with the notion that the phenotype arising from Wzc$^{1–704}$ is due to a loss of phosphorylation. Preliminary experiments using site-directed mutagenesis to change tyrosine residues to phenylalanines demonstrate that phosphorylation occurs at multiple tyrosines in Wzc$_{epsa}$, and work is in progress to determine which tyrosine residues are important. However, a mutant in which all the tyrosines are mutated lacks phosphorylation and confers a CPS phenotype identical to that of Wzc$^{1–704}$ (2). Thus,
the *S. pneumoniae* system differs from the *E. coli* group 1 CPS in two respects: CpsD mutants defective in ATP binding/hydrolysis and tyrosine phosphorylation have different effects on CPS phenotype, and phosphorylated tyrosine may act as a negative effector in *S. pneumoniae*. The role of phosphotyrosine-protein phosphatase activity in *S. pneumoniae* is currently unclear. Although the product of an adjacent gene (*cpsB*) is implicated in dephosphorylation of CpsD (21), the putative CpsB protein lacks the motif (and active site cysteine) typical of phosphotyrosine-protein phosphatase. While CpsB might reflect a new type of phosphatase, such an activity has not yet been tested.

Given the overall similarity in the components for Wzy-dependent CPS biosynthesis in Gram-positive and Gram-negative bacteria, the differential effect of the phosphorylation-deficient Wzccps and CpsD derivatives was surprising. The two domains of Wzccps could be expressed as independent polypeptides to mimic the Gram-positive situation and these constructs were able to function in both phosphorylation of the C-terminal domain and in capsule assembly. However, there are some significant structural differences between CpsCD and Wzccps. The extra-cytoplasmic membrane loop of CpsC and its homologues are usually 270–300 residues smaller than the corresponding domain in Wzccps (6, 8). Another interesting feature distinguishing the Gram-negative and *S. pneumoniae* systems is the fact that the tyrosines in CpsD are arranged in a (YGX)4 motif. Whether these latter features play a role in the apparent differences in the requirement for Wzccps/CpsD phosphorylation in CPS synthesis remains to be established.

Phosphorylated Ptk and Wzccps are substrates for the phosphotyrosine-protein phosphatases, Ptp and Wzb, and these enzymes were found to be functionally interchangeable (10). Wzccps is also dephosphorylated by Wzb_ppt. Unexpectedly, a Wzb_ppt mutant also lacks K30 CPS biosynthesis. To rule out the possibility that the CPS phenotype in CWG343 (wzb:aacC1) reflected a loss of important protein-protein interactions rather than the phosphotyrosine-protein phosphatase activity per se, we used a catalytically inactive mutant (Wzb_C13S). The inability of this mutant to complement the defect in CWG343 strongly supports the notion that phosphatase activity is essential. The most likely explanation for the CPS phenotype of the wzb_ppt mutant is an inability to dephosphorylate Wzccps. This is based on activity of Wzb_ppt against Wzccps, the fact that Wzccps is the only known tyrosine-phosphorylated protein in the CPS biosynthesis system, and the fact that these *E. coli* strains have no other detectable tyrosine-phosphorylated proteins other than Etk. The requirement for both autokinase and phosphatase activity in CPS biosynthesis raises the interesting possibility that cycling between phosphorylated and dephosphorylated Wzccps is involved in CPS assembly. However, attempts to demonstrate cycling in vitro were not successful (data not shown). Either the activities are confined to one-time events, or an essential component or condition needed for cycling is absent in the in vitro system. The level of autokinase activity is insufficient to address this question in vitro.

Although the results presented here provide the first indication of the requirement for phosphorylated Wzccps in assembly of the K30 capsule, its exact role has yet to be resolved. The resemblance of Wzc homologues from Gram-negative and Gram-positive bacteria suggests they may act at conserved stages in the assembly process and the most logical candidate is polymerization. Significantly, the Wzc homologues in both Gram-positive and Gram-negative bacteria are involved in the assembly of polysaccharides that appear to be polymerized by Wzy-dependent polymerization systems. The Wzccps protein is a member of the cytoplasmic membrane-periplasmic auxiliary protein 1 family (39). More recently, these proteins have been suggested to be part of a larger family including Wzz, a protein associated with chain length determination of Wzy-dependent LPS O antigens. An alternative name, polysaccharide copolymerase was proposed (8). The membrane-periplasmic auxiliary/polyasaccharide copolymerase proteins are associated with loci for capsule and exopolysaccharide expression in Gram-positive and Gram-negative bacteria and they share similarities in the transmembrane topology. Wzc proteins are distinguished from Wzz by an extended C terminus that contains the ATP-binding motif and phosphotyrosines, suggesting a more complex functional role for Wzz.

Wzy polymerase proteins catalyze glycosidic bond formation and are therefore specific for a given polymer repeat-unit structure. There is little primary sequence relatedness shared by Wzy homologues but their hydrophathy profiles are similar. A given Wzz protein can interact with polymerization systems for different LPS O antigen repeat units (see, for example, Refs. 28 and 45–47). This suggests few (if any) limits are imposed on Wzz function by either Wzy primary structure or by the repeat unit structure of the polysaccharide product. However, the chain-length modalities are certainly sensitive to differences in Wzy primary sequences (45–47). In contrast to the sequence variation in Wzz within different *E. coli* serogroups, both *E. coli* and *K. pneumoniae* have cps gene clusters encoding essentially identical Wzccps proteins (2). Wzz regulation is confined to lipid A core-linked polymers and while it can generate modality in KLP, it does not influence assembly of the *E. coli* group 1 CPS (28). The natural lack of modality in KLP in the wild-type strains results from the absence of wzz on the chromosome of *E. coli* isolates with group 1 capsules (28). Conversely, we show here that Wzccps cannot restore modality in the absence of Wzz. While Wzc1–489 does not influence O-chain length in precisely the same manner as Wzz, it does alter the profile of O-antigen-substituted LPS molecules in SDS-PAGE, suggesting it can interact with and modulate an O-antigen polymerization system providing that the C-terminal domain is absent.

It should be noted that enzymatic activity directly involving polymerization has not been proven for either Wzz or Wzc and their role could be indirect. In *E. coli*, polymerization reactions for K30LPS and K30 CPS share the same Wzy-polymerase enzyme (3) and K30LPS molecules comprising 8–10 repeat units of K30 antigen are formed in strains devoid of Wzccps and Etk. Thus, Wzccps is not an essential partner for Wzy activity per se. However, Wzccps might be essential for a specific subset of substrates, or enzyme complexes devoted to assembly of the high molecular weight capsular K30 antigen. For example, Wzccps may regulate the flow of substrates into high molecular weight K30 CPS, accounting for the increased polymerization of K30LPS evident in the absence of CPS synthesis. Wzz proteins could also potentially influence the synthesis of CPS by affecting formation of a capsule assembly complex. Both Wzz and Wzc proteins have periplasmic domains that are predicted to form coiled-coils (8), a feature important for protein-protein interactions. In the case of Wzz, oligomerization has been demonstrated by cross-linking methods (47). The demonstration that coexpression of Wzccps and Wzc1–489 allows intermolecular transphosphorylation and restoration of capsule expression strongly suggests that Wzccps proteins can also interact with one another. If Wzccps plays a role in assembling a functional complex for assembly of the K30 CPS, the presence of polymerized KLP can only be explained by separate biosynthetic complexes for the polymerization of K30 CPS (Wzy-dependent) and K30LPS (Wzz-independent). The phenotype of *E. coli* CWG285 (wzc:aacC1 wza22 min::aadA) would then be explained by the absence of functional CPS polymerization
complexes. The availability of additional Wzy enzyme and its lipid-linked substrates for K\textsubscript{LPS} complexes would be reflected in the higher level of K\textsubscript{LPS} polymerization. This issue provides the direction for future studies.

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Phosphorylation of Wzc, a Tyrosine Autokinase, Is Essential for Assembly of Group 1 Capsular Polysaccharides in *Escherichia coli*

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