Capsular polysaccharides (CPS) and exopolysaccharides (EPS) are important virulence determinants in many plant and animal pathogens and are also important for symbiotic interactions in some plant-associated bacteria. More than 80 types of capsular or K antigens have been identified in many gram-positive and gram-negative bacteria. Phosphorylation of tyrosine residues in the Wzc CPS C terminus is essential for HMW CPS assembly. Overexpression of Wzb CPS (phosphatase) in a wild-type background caused a 3.7-fold decrease in the amount of cell-associated K30 CPS produced, confirming the importance of Wzc CPS phosphorylation for capsule assembly. In this study, the tyrosine-rich region was dissected in an attempt to identify residues critical for Wzc CPS phosphorylation and/or capsule expression. Site-directed mutagenesis demonstrated that no single tyrosine residue in this region is sufficient for detectable phosphorylation of Wzc CPS in vivo or for HMW CPS expression. Furthermore, no single tyrosine residue is essential for phosphorylation or capsule assembly, since removal of any one tyrosine residue has no detectable effect. Altering combinations of tyrosine residues (from two to five) led to Wzc CPS derivatives that were still competent for phosphorylation but that could not support assembly of HMW CPS, showing that phosphorylation of Wzc per se is not an accurate measure of its ability to function in capsule assembly. One interpretation of these data is that the overall level of phosphorylation in this region, rather than the precise combination of residues accessible to phosphorylation, is important for the activity of Wzc CPS. Tyrosine 569, a residue shown to modulate the in vitro phosphorylation of Wzc from E. coli K-12, was also mutated. The derivative with this mutation still functioned in capsule assembly. Quantitation of K30 CPS from this mutant revealed no difference in the amount of polymer produced. Finally, dithiobis(succinimidylpropionate) cross-linking was used to confirm that Wzc CPS forms complexes in vivo, independent of the phosphorylation state of the protein.

Wzc CPS is a tyrosine autokinase essential for the assembly of a high-molecular-weight (HMW) group 1 capsular polysaccharide (CPS) in Escherichia coli. Homologues of Wzc participate in the formation of CPS and exopolysaccharides in a variety of gram-positive and gram-negative bacteria. Phosphorylation of tyrosine residues in the Wzc CPS C terminus is essential for HMW CPS assembly. Overexpression of Wzb CPS (phosphatase) in a wild-type background caused a 3.7-fold decrease in the amount of cell-associated K30 CPS produced, confirming the importance of Wzc CPS phosphorylation for capsule assembly. In this study, the tyrosine-rich region was dissected in an attempt to identify residues critical for Wzc CPS phosphorylation and/or capsule expression. Site-directed mutagenesis demonstrated that no single tyrosine residue in this region is sufficient for detectable phosphorylation of Wzc CPS in vivo or for HMW CPS expression. Furthermore, no single tyrosine residue is essential for phosphorylation or capsule assembly, since removal of any one tyrosine residue has no detectable effect. Altering combinations of tyrosine residues (from two to five) led to Wzc CPS derivatives that were still competent for phosphorylation but that could not support assembly of HMW CPS, showing that phosphorylation of Wzc per se is not an accurate measure of its ability to function in capsule assembly. One interpretation of these data is that the overall level of phosphorylation in this region, rather than the precise combination of residues accessible to phosphorylation, is important for the activity of Wzc CPS. Tyrosine 569, a residue shown to modulate the in vitro phosphorylation of Wzc from E. coli K-12, was also mutated. The derivative with this mutation still functioned in capsule assembly. Quantitation of K30 CPS from this mutant revealed no difference in the amount of polymer produced. Finally, dithiobis(succinimidylpropionate) cross-linking was used to confirm that Wzc CPS forms complexes in vivo, independent of the phosphorylation state of the protein.

In the prototype K30 strain, E69, the genes responsible for the synthesis and cell surface assembly of the K30 polymer are found in an operon located near his and rfb (42). The products of the first four genes of the K30 operon (orfX, wza, wzb, and wzc) are believed to be involved in high-level polymerization and surface expression of the K30 CPS polymer. These genes are separated from a block of downstream genes encoding enzymes for repeat unit synthesis by a transcriptional attenuator (32; A. Rahn and C. Whitfield, unpublished data). The OrfX protein has only one known homologue, found in Klebsiella K2 strains (ORF3), but its exact function is unknown (1, 2). Wza is an outer membrane lipoprotein that multimerizes to form ring-like structures resembling secretins for type II and type III protein secretion (13). HMW CPS is believed to cross the outer membrane through this complex. Wzc CPS (involved in group 1 CPS assembly) is a tyrosine autokinase, while Wzb CPS is its cognate phosphatase. The biochemical activities of these two proteins in a number of systems representing group 1 CPS and related EPS have been confirmed (8, 14, 17, 21, 30, 31, 37, 43). In E. coli K30, these proteins are essential for the assembly of a HMW capsular layer on the cell surface, though not for...
low-level polymerization or assembly of K<sub>LPS</sub> (12, 43). There are second copies of the wza, wzb, and wzc genes on the chromosomes of <i>E. coli</i> K30 and K-12 strains. The products of these genes have been shown to participate with low efficiency in K30 CPS and colanic acid production (38, 43). To distinguish these genes from those in the K30 cluster, they have been named <i>wza</i><sub>22min</sub>, <i>wzb</i><sub>22min</sub>, and <i>wzc</i>-<sub>22min</sub>.

Interest in the function of Wzb and Wzc homologues arises from the limited distribution of tyrosine phosphorylation in prokaryotes (reviewed in references 3 and 36) and the involvement of equivalent autokinase and phosphatase proteins in the assembly of capsules in gram-positive bacteria such as <i>S. pneumoniae</i> (18, 26), <i>S. agalactiae</i> (33, 44), and <i>S. aureus</i> (24, 35). In these systems, the Wzc homologue consists of two separate polypeptides. One protein contains the two transmembrane domains and the periplasmic loop (corresponding to the Wzc N terminus), while the other protein corresponds to the C terminus and contains the Walker A ATP-binding motif as well as the C-terminal tyrosine-rich domain (18, 35).

Previous studies from our laboratory have shown that the last 17 amino acids of Wzc<sub>CPS</sub> include the site(s) of phosphorylation of the protein and that phosphorylation at this site is essential for assembly of HMW K30 CPS (43). This C-terminal region is tyrosine rich, with seven tyrosine residues in the last 17 amino acids. Similar features were reported for a number of Wzc homologues (16, 27, 37). In <i>E. coli</i> K-12, five of the six C-terminal tyrosine residues can be phosphorylated in vitro (16). However, it is not clear at this point whether all of the Wzc C-terminal tyrosine residues or one or more specific tyrosine residues are accessible for phosphorylation in vivo. The ability of Wzc molecules to participate in transphosphorylation reactions was suggested by the finding that phosphorylated Wzc<sub>CPS</sub> could be obtained from incubation of a Wzc<sub>CPS</sub> mutant that could not bind ATP with a Wzc<sub>CPS</sub> mutant that could bind ATP but that lacked the C-terminal site of phosphorylation (43). This transphosphorylation function was later confirmed and extended by a study of the Wzc<sub>CA</sub> (colanic acid) protein (16). Work on the Wzc<sub>CA</sub> protein in <i>E. coli</i> K-12 has revealed that a tyrosine residue outside of the C-terminal tyrosine-rich domain (Y569) was modified by autophosphorylation only, while the C-terminal domain was accessible to transphosphorylation by other Wzc<sub>CA</sub> molecules (16). Furthermore, it was shown in the same study that phosphorylation of the C-terminal region was greatly enhanced in the presence of Y569. The authors proposed a two-step process (autophosphorylation and transphosphorylation) for the phosphorylation of Wzc<sub>CA</sub> and suggested that Y569 may modulate the level of phosphorylation of the C-terminal tyrosine residues. However, the biological role of this modulation has not been established.

The essential requirement in group 1 capsule assembly for the biology of the organism has not been addressed. The purpose of this research was to study the importance of phosphorylation of specific tyrosine residues in the C-terminal domain of Wzc<sub>CPS</sub> in CPS assembly in <i>E. coli</i> O9a:K30.

Althoah several studies have begun to decipher the impact of phosphorylation of particular residues on the in vitro activity of the autokinase, the importance of this phosphorylation in the biology of the organism has not been addressed. The purpose of this research was to study the importance of phosphorylation of specific tyrosine residues in the C-terminal domain of Wzc<sub>CPS</sub> in CPS assembly in <i>E. coli</i> O9a:K30.


**MATERIALS AND METHODS**

**Bacterial strains, plasmids, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. Strains were grown at 37°C in Luria-Bertani (LB) medium (Invitrogen Life Technologies, Burlington, Ontario, Canada) supplemented with kanamycin (50 μg ml<sup>-1</sup>) (Sigma, St. Louis, Mo.) where appropriate.

**Site-directed mutagenesis.** Mutagenesis was carried out by using a modification of the QuiKChange site-directed mutagenesis kit from Stratagene (La Jolla, Calif.). Complementary oligonucleotides containing the desired base changes were designed. Primer sequences and details of the mutagenesis strategy (template and primer pair combinations) are available from the authors upon request. The plasmids were amplifid in 50-μl reaction mixtures with Pwo polymerase (Roche Molecular Biochemicals, Laval, Quebec, Canada) in a Perkin-Elmer GeneAmp PCR System 2400 thermocycler. The products were cleaned with a column by using a QIAquick PCR purification kit (Qiagen, Mississauga, Ontario, Canada), and the template DNA was digested with Dpn I (used according to the manufacturer's instructions). DNA was precipitated and used to transform <i>E. coli</i> DH5α by electroporation with a Gene Pulser from Bio-Rad (Hercules, Calif.) (6).

For the creation of pWQ214 to pWQ219, the wzc gene from pWQ212 was amplified with a forward primer that introduced an EcoRI site for cloning and a reverse primer that reintroduced the desired tyrosine codon and provided a PstI site for cloning. The PCR products were cloned back into pBAD18-Km, and the resulting plasmids were transformed into <i>E. coli</i> DH5α.

**Sequencing.** Site-directed mutations were confirmed and plasmid sequences were verified by sequencing at the Guelph Molecular Supercentre (University of Guelph, Guelph, Ontario, Canada).

**Expression of Wzc<sub>CPS</sub> and derivatives.** Wzc<sub>CPS</sub> and mutant derivatives were expressed from pBAD arabinose-inducible expression vectors. Cells were grown to mid-exponential growth phase, and expression of the Wzc<sub>CPS</sub> derivative was induced by addition of 0.02% L-arabinose. After induction, cells were grown for 2.5 h and then harvested. Cell pellets corresponding to a cell optical density at 600 nm of 1.0 were resuspended in 2x sodium dodecyl sulfate-polyacrylamide gel
electrophoresis (SDS-PAGE) sample buffer and heated at 100°C for 10 min. These cell lysates were then analyzed by SDS-PAGE and Western blotting.

**SDS-PAGE and Western immunoblot analysis.** Whole-cell lysates were analyzed by SDS-PAGE (23) with 7.5% or 10% polyacrylamide resolving gels. Proteins were transferred to Westan polyvinylidene difluoride membranes (Schleicher & Schuell) for phosphotyrosine analysis or BioTrace NT nitrocellulose membranes (Gelman Laboratory) for Wzc expression analysis. The transfer buffer used Schleicher & Schuell) for phosphotyrosine analysis or BioTrace NT nitrocellulose membranes.

**TABLE 1. Bacterial strains and plasmids used in this study**

<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype, serotype, or description</th>
<th>Reference or source</th>
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</thead>
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<td>K-12 Δ80d lacZΔM15 endA1 recA1 hsdR17(λamu mK') supE44 thi-1 gyrA96 relA1 (ΔlacZYA-argF) U1169 F’</td>
<td>Clontech</td>
</tr>
<tr>
<td>E69</td>
<td>Prototroph, serotype O9a:K30:H12</td>
<td>F. Ørskov</td>
</tr>
<tr>
<td>CWG258</td>
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*International Escherichia and Klebsiella Center, Statens Seruminstitut, Copenhagen, Denmark.*

CPS purification and quantitation. Cell-free and cell-associated CPS were purified as described previously (22). Briefly, cells were harvested from colonies grown for 18 h on LB-kanamycin (or LB-ampicillin) containing 0.02% l-arabinose. The colonies were scraped into phosphate-buffered saline, and viable counts were determined from this suspension. Cells were harvested by centrifugation, leaving cell-free polysaccharide in the supernatant. The supernatant was treated with DNase (Roche), RNase A (Roche), and protease K (Sigma) and dialyzed against water. The pellet was extracted with phenol, allowing recovery of cell-associated CPS in the aqueous phase. The aqueous phase was dialyzed against water, treated with enzyme as described for the cell-free CPS, and redialized. For both cell-free and cell-associated samples, LPS was removed by ultracentrifugation (10,000 × g, 1 h, 15°C).

Purified CPS samples were analyzed for uronic acid content by the modified carbazole assay of Bitter and Muir (7). Briefly, 0.5 ml of an appropriate dilution of the sample was added to 3 ml of 0.025 M sodium tetraborate (BDH Chemicals Ltd., Poole, United Kingdom) in concentrated H₂SO₄. Samples were heated to 100°C for 10 min. This suspension was then analyzed by ultracentrifugation (105,000 × g, 1 h, 15°C), allowing recovery of cell-associated CPS in the aqueous phase. The aqueous phase was dialyzed against water, treated with enzyme as described for the cell-free CPS, and redialized. For both cell-free and cell-associated samples, LPS was removed by ultracentrifugation (10,000 × g, 1 h, 15°C).
100°C for 10 min and then cooled to room temperature. Next, 0.1 ml of 0.125% (wt/vol) carbazole (Sigma) in absolute ethanol was added to the samples, and the samples were heated to 100°C for an additional 15 min. After the samples were cooled, the A6440 was determined. A standard curve of glucuronolactone was generated for determination of the amount of uronic acid in the samples.

**DSP cross-linking.** Fifty-milliliter cultures were grown to mid-exponential growth phase, and the expression of wild-type and mutant WzcCPS proteins in pBAD-based constructs was induced by addition of 0.02% l-arabinose. Growth was continued for an additional 2 h, and cells were harvested and washed with buffer A (150 mM NaCl in 20 mM NaH2PO4, pH 7.5). Cell pellets were resuspended in 5 ml of buffer A, and samples were collected prior to cross-linking. Cross-linking was carried out for 30 min by the addition of 1 mM dithiobis(succinimidylpropionate) (DSP; Lomart, Reagent; Pierce, Rockford, Ill.) from a 25 mM stock prepared in anhydrous dimethyl sulfoxide (Aldrich, Oakville, Ontario, Canada). The reaction was quenched for 15 min by the addition of Tris-HCl (pH 7.5) to a final concentration of 100 mM. Cells were harvested and resuspended in 20 mM Tris-HCl (pH 7.5) and lysed by sonication. Sonication is required for efficient cell lysis of cross-linked samples. Samples of cross-linked material were prepared by addition of NuPAGE 4% lithium dodecyl sulfate sample buffer (Invitrogen Life Technologies Inc.) to the lysates. In one set of samples, the cross-links were cleaved by addition of dithiothreitol (DTT) to a final concentration of 50 mM. Samples were heated to 100°C before being run on SDS–7.5% PAGE gels and transferred onto polyvinylidene difluoride membranes for antration of 50 mM. Samples were heated to 100°C before being run on SDS-PAGE gels and transferred onto nitrocellulose membranes for anti-Wzc antibodies. The reaction was quenched for 15 min by the addition of Tris-HCl (pH 7.5) to a final concentration of 100 mM. Cells were harvested and resuspended in 20 mM Tris-HCl (pH 7.5) and lysed by sonication. Sonication is required for efficient cell lysis of cross-linked samples. Samples of cross-linked material were prepared by addition of NuPAGE 4% lithium dodecyl sulfate sample buffer (Invitrogen Life Technologies Inc.) to the lysates. In one set of samples, the cross-links were cleaved by addition of dithiothreitol (DTT) to a final concentration of 50 mM. Samples were heated to 100°C before being run on SDS–7.5% PAGE gels and transferred onto polyvinylidene difluoride membranes for antiphosphotyrosine (PY20) blots or onto nitrocellulose membranes for anti-Wzc blots.

**RESULTS**

**Site-directed mutation of all the C-terminal tyrosine residues.** Our previous research showed that truncation of the last 17 amino acids of the C-terminal tyrosine-rich region of WzcCPS resulted in loss of phosphorylation of WzcCPS (assessed by reactivity with PY20 antiphosphotyrosine antibodies) and loss of function in the assembly of HMW CPS (43). This phenotype is identical to that seen in a Δwzc strain. In S. pneumoniae, cpsCDKF are also essential for capsule assembly. However, in this system, phosphorylation of CpsD acts as a negative regulator of CPS production (27). One possible interpretation of the different result for E. coli O9a:K30 is that the WzcCPS truncation alters protein folding or inner membrane interactions with other proteins. In this case, the phenotype would not be attributable solely to the removal of the seven tyrosine residues but may also involve other conformational changes. This is a characteristic feature of O9a:K30, the amount of K30 LPS (seen as a ladder of slower-migrating molecules) was evident in CWG285 with or without the plasmid.

This is a characteristic feature of wzc mutants in E. coli O9a:K30 (12, 43). The absence of an HMW polymer was confirmed by the resistance of this strain to bacteriophage K30, which requires the K30 antigen as its receptor.

It has been proposed that, in E. coli K-12 (38) and S. pneumoniae (27), phosphorylated Wzc and CpsD are negative regulators of polymer production, and a tyrosine-null mutant leads to a mucoid phenotype in S. pneumoniae (27). To support the Western blotting data for E. coli O9a:K30, the amount of polysaccharide produced by CWG285(pWQ212) was determined. By chemical analysis, introduction of pWQ212 (Wzc2767507-718→F) did not enhance the base level of polymer production in CWG285 (Table 2). In contrast, introduction of pWQ130 (encoding the wild-type WzcCPS) resulted in an elevated amount of cell-associated capsular K30 antigen (Table 2). The complementation with plasmid-encoded WzcCPS derivatives never restores wild-type levels of CPS production (compare to CWG258 [Table 2]), which is consistent with previous studies (12, 43).

**FIG. 1.** Site-directed mutation of all C-terminal tyrosine residues eliminates WzcCPS phosphorylation and HMW CPS biogenesis. (A) Western blot of whole-cell protein lysates probed with polyclonal WzcCPS antiserum, showing the expression levels of WzcCPS and Wzc2767507-718→F. Expressed from plasmids in E. coli CWG285. Lane 1, negative control CWG285 (no plasmid); lane 2, pWQ130 (WzcCPS); lane 3, pWQ212 (Wzc2767507-718→F). (B) Western blot of the same samples probed with a monoclonal antiphosphotyrosine antibody (PY20) to detect the phosphorylation levels of WzcCPS and its derivatives. (C) Western blot of the cell surface polysaccharides from the same strains probed with polyclonal K30 antiserum. Note that strains lacking HMW CPS have increased amounts of K1PS as reported previously (12). Each strain was tested for sensitivity to bacteriophage K30. +, sensitivity of the strain to the phage; −, resistance.
It is conceivable that the absence of K30 CPS in CWG285 expressing WzcY705-718 indicated a loss of protein function (e.g., due to improper folding) caused by the replacement of seven tyrosine residues with seven phenylalanine residues rather than a direct effect of tyrosine phosphorylation. To confirm the importance of Wzc CPS phosphorylation for CPS expression, Wzb CPS was overexpressed in CWG258 and the amounts of cell-free and cell-associated K30 polymer produced by this strain were determined. Overexpression of Wzb would be expected to greatly decrease the amount of phosphorylated Wzc, providing a different means to examine the role of Wzc phosphorylation.

Mutants lacking two tyrosine residues (Wzc Y706/717) were still resistant to the K30 phage, confirming the importance of Wzc CPS phosphorylation for CPS expression. Wzb CPS was overexpressed in CWG258 and the amounts of cell-free and cell-associated K30 polymer produced (Table 2). These experiments were performed in a background (CWG258) lacking the second copies of wza, wzb, and wzc (at 22 min on the E. coli K-12 chromosome) in order to simplify their interpretation.

**TABLE 2. Quantitation of cell-free and cell-associated K30 CPS**

<table>
<thead>
<tr>
<th>Strain(plasmid) (protein expressed)</th>
<th>Level of K30 CPS (ng of uronic acid/10^7 CFU)*</th>
</tr>
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<tr>
<td></td>
<td>Cell free</td>
</tr>
<tr>
<td>CWG258</td>
<td>28 ± 5</td>
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<tr>
<td>CWG258(pWQ147) (WzbCPS)</td>
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<tr>
<td>CWG285</td>
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<td>CWG285(pWQ130) (WzcCPS)</td>
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<tr>
<td>CWG285(pWQ212) (WzcY705-718→F)</td>
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</tr>
<tr>
<td>CWG285(pWQ238) (WzcY699→F)</td>
<td>9 ± 2.4</td>
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</tbody>
</table>

* Values are means ± 1 standard deviation.

Finally, mutant proteins that lacked various combinations of tyrosine residues capable of supporting phosphorylation of Wzc CPS and/or capsule assembly (Fig. 3B). Furthermore, all of these proteins could restore the capsular phenotype to the ∆wzc strain and reduce the amount of K30LPS assembled (Fig. 3C). These strains were also sensitive to infection by the K30 phage.

### Are specific combinations of tyrosine residues capable of supporting phosphorylation of Wzc CPS and/or capsule expression?

Initially, mutant proteins that lacked various combinations of two, three, four, and five of the seven C-terminal tyrosine residues were generated. All of the proteins generated were expressed at levels similar to that for Wzc CPS (Fig. 4A). Mutants lacking two tyrosine residues (WzcY706/717→F, WzcY708/713→F, WzcY708/712→F, and WzcY715/717→F) were still phosphorylated, as seen by PY20 immunoblotting (Fig. 4B, lanes 3 to 6, respectively), and these strains still assembled the HMW K30 polymer, as shown by the anti-K30 immunoblot and the phage sensitivity data (Fig. 4C, lanes 3 to 6). However, the relative amounts of polymer produced by these strains as assessed by immunoblotting appear to vary depending on the combination of tyrosine residues remaining. Specifically, WzcY706/717→F (Fig. 4C, lane 3), WzcY708/715→F (Fig. 4C, lane 10).
and WzcY_{715/717}\rightarrow F (Fig. 4C, lane 6) show larger amounts of immunoreactive K30 antigen than WzcY_{708/713}\rightarrow F (Fig. 4C, lane 4). Furthermore, the K30 phage does not produce clear plaques when WzcY_{708/713}\rightarrow F is expressed. For the three strains that express a WzcCPS protein lacking three of the terminal tyrosine residues, only WzcY_{713,717}\rightarrow F (Fig. 4C, lane 9) showed a detectable K30CPS polymer by immunoblotting (Fig. 4C, lanes 7 to 9), although Wzc derivatives from all three strains are expressed and phosphorylated (Fig. 4A and B, lanes 7 to 9). WzcY_{708,715}\rightarrow F showed only turbid plaques when subjected to the K30 phage, while WzcY_{706,713}\rightarrow F was not sensitive to the K30 phage at all. It is again of interest to note the variable amounts of immunoreactive K30CPS material produced by these derivatives. Two WzcCPS proteins that lacked four of the seven terminal tyrosine residues and two others that lacked five of these seven residues were constructed. All of these proteins were expressed at similar levels (Fig. 4A, lanes 12 to 15) and were phosphorylated (Fig. 4B, lanes 12 to 15), although WzcY_{708,718}\rightarrow F (Fig. 4B, lane 14) and WzcY_{706,717}\rightarrow F (Fig. 4B, lane 15) showed a greatly reduced level of phosphorylation. All of these proteins were unable to restore HMW CPS expression in CWG285, as shown by immunoblotting and resistance to the K30 phage (Fig. 4C, lanes 12 to 15).

**Site-directed mutation of Y569, a tyrosine residue outside of the C-terminal domain.** A recent study on the phosphorylation of WzcCA from *E. coli* K-12 has shown that a tyrosine residue outside of the C-terminal region (Y569) is phosphorylated in vitro and that the presence of this residue increases the in vitro phosphorylation state of WzcCA 45-fold (16). It was also shown that this residue could be autophosphorylated by using ATP as a substrate but that it could not accept phosphate residues from the C-terminal region in a transphosphorylation reaction (16). The corresponding residue in WzcCPS is also tyrosine 569, and the two proteins align well in this region (16, 43). To address the potential role of Y569 in the assembly of HMW CPS, residue Y569 in WzcCPS was changed to phenylalanine by site-directed mutagenesis. The resulting WzcY_{569}\rightarrow F protein was expressed at levels similar to those for the wild-type protein (Fig. 5A) and was still phosphorylated, as assessed by PY20 immunoblotting (Fig. 5B). The phosphorylation level of WzcY_{569}\rightarrow F appeared to be reduced slightly compared to that of wild-type WzcCPS. Analysis of K30 capsule assembly by immunoblotting revealed that HMW capsular material was still assembled when this protein was expressed in CWG285 (Fig. 5C), and the phage sensitivity data revealed no difference from wild-type WzcCPS expressed in CWG285. To determine whether there is any subtle difference in the amount of K30 polymer produced by this derivative, cell-free and cell-associated CPS were purified from CWG285 expressing WzcCPS and WzcY_{569}\rightarrow F. There was no apparent difference in the amounts of cell-free K30 CPS produced by WzcCPS and WzcY_{569}\rightarrow F or in the amounts of cell-associated CPS produced by these strains (Table 2).

In WzcCPS, an additional tyrosine residue (Y606) is present in this region. This residue was also mutated in case it was, instead, the residue corresponding to Y569 in WzcCA. The function of the resulting mutant protein was identical to that of the wild-type protein (data not shown).

**Do mutations in the C-terminal tyrosine-rich region affect the ability of WzcCPS proteins to interact?** Previous research has shown that Wzc proteins have the ability to participate in transphosphorylation reactions (16, 43). This provides evidence that Wzc proteins can interact both in vivo and in vitro. Chemical cross-linking of whole cells by using DSP was performed to investigate the possible interactions of WzcCPS proteins. Cross-linking in E69, which expresses the protein from the chromosomal copies of *wzc*, was first examined. Cells were cross-linked and lysed, and the lysates were examined by Western immunoblotting with both the anti-WzcCPS polyclonal antiserum and the PY20 phosphotyrosine antibodies. After cross-linking, Wzc was detected in a single HMW complex that barely migrated into the gel (Fig. 6A). Upon reduction, monomeric Wzc was detected. This complex may represent the association of Wzc into oligomers or reflect interactions with other components of the K30 biosynthesis, assembly, and translocation machinery. To examine whether smaller complexes could be formed in the absence of the outer membrane protein Wza, which has been shown to assemble into large multimeric structures, cross-linking in CWG281, an E69 deriv-
ative lacking both chromosomal copies of \textit{wza}, was carried out. In this strain, Wzc CPS is expressed from a chromosomal copy only. The results were the same as those for E69, with a single large complex containing Wzc detected by immunoblotting after cross-linking (Fig. 6A). To isolate the effects of cross-linking on the multimerization state of Wzc alone, further experiments with whole cells of \textit{E. coli} \textit{DH5}/\textit{H9251} expressing WzcCPS encoded by pWQ130 were performed. An \textit{E. coli} \textit{K-12} derivative (\textit{DH5}/\textit{H9251}) was used in these experiments to eliminate any possible complicating associations of Wzc with other components of the capsule translocation apparatus in E69 derivatives. \textit{E. coli} \textit{DH5}/\textit{H9251} contains the \textit{wza}, \textit{wzb}, and \textit{wzc} genes in the colanic acid biosynthesis cluster, but these are not expressed in \textit{E. coli} \textit{K-12} at the growth temperatures used in this study (15).

Western immunoblot analysis using PY20 showed that WzcCPS was present in at least two HMW complexes of greater than 150 kDa (Fig. 6A). Cleavage by DTT resulted in detection of monomeric Wzc and of two complexes, one smaller than 150 kDa and another 250 kDa (Fig. 6A). The same result was obtained when the immunoblot was probed with WzcCPS polyclonal antiserum, but the lower reactivity of the antibody generated a weaker signal (data not shown). Further studies were then undertaken to investigate whether WzcCPS derivatives bearing changes in the C-terminal tyrosine-rich region can still interact. Loss of this interaction is one possible explanation for why some of the mutations impair the phosphorylation of WzcCPS and/or assembly of HMW K30 CPS. DSP cross-linking was carried out in \textit{DH5}/\textit{H9251} expressing various WzcCPS constructs in \textit{trans}. When a WzcCPS Walker A box mutant (Wzc \textit{K540R}; pWQ132) was expressed in \textit{DH5}/\textit{H9251}, a cross-linking profile similar to that obtained with WzcCPS was observed (Fig. 6B). The same was seen when either the truncated WzcCPS protein lacking the C-terminal tyrosine-rich domain (Wzc \textit{1-704}; pWQ133) or the site-directed mutant lacking all the C-terminal tyrosine residues (Wzc\textit{Y705-718F}) was expressed in \textit{DH5}/\textit{H9251} (data not shown).

**DISCUSSION**

In this study, the importance of phosphorylation of WzcCPS at various tyrosine residues on the in vivo function of the protein was investigated. Our main objective was to dissect the tyrosine-rich region of the WzcCPS protein in order to determine which residues, if any, were essential for the phosphorylation of WzcCPS and for the surface expression of HMW CPS in \textit{E. coli} O9:aK30.

Previously we showed that the C-terminal tyrosine-rich region of WzcCPS from \textit{E. coli} O9:aK30 is the site of phosphor-
ylation of the protein and that the removal of these tyrosine residues by truncation results in a protein whose phosphorylation cannot be detected by an antiphosphotyrosine antibody. Furthermore, this protein could not restore synthesis of HMW CPS in a \( \Delta wzc \) strain. It was therefore suggested that phosphorylation at one or more of the tyrosine residues in the C-terminal region was important for the ability of \( Wzc_{\text{CPS}} \) to function in capsule assembly (43). Similar findings were reported from studies on \( Wzc_{\text{CA}} \) and ExoP, which showed that the C-terminal domains of these proteins were phosphorylation competent (16, 30, 38).

One concern in interpreting data from truncated mutants is that the removal of the last 17 amino acids of \( Wzc_{\text{CPS}} \) might have altered the folding of the protein and thus altered its phosphorylation and capsule assembly functions. However, the \( Wzc_{\text{Y705-718-F}} \) mutant lacking only the seven C-terminal tyrosine residues showed the same phenotype as the truncated version of the protein. This supported the proposal that phosphorylation of one or more of the C-terminal tyrosine residues is essential for HMW CPS expression in group 1 \( E. coli \) strains, though not essential for \( K_{\text{LPS}} \) assembly. The importance of the phosphorylation of \( Wzc_{\text{CPS}} \) in CPS production was further confirmed by findings that overexpression of \( Wzb_{\text{CPS}} \) caused a 3.7-fold reduction in the amount of cell-associated uronic acid produced. This observation provides additional support for the hypothesis that \( Wzc_{K30} \) must undergo cycles of phosphorylation and dephosphorylation for \( K30 \) CPS to be expressed. This finding is in contrast to the situation in \( E. coli \) K-12 and \( S. pneumoniae \), where phosphorylated \( Wzc_{\text{CA}} \) and CpsD are believed to act as negative regulators of polysaccharide production (27, 38). It is unclear whether differences in the \( E. coli \) K30 antigen and colanic acid situations reflect the fact that one is primarily capsular (K30) and the other is a cell-free EPS (colanic acid).

![FIG. 5. Site-directed mutagenesis of Y569 does not eliminate phosphorylation of \( Wzc_{\text{CPS}} \) or expression of HMW K30 CPS. \( Wzc_{\text{CPS}} \) and \( Wzc_{\text{Y569-F}} \) were expressed from plasmids in \( E. coli \) CWG285. (A) Western blot of whole-cell protein lysates probed with polyclonal \( Wzc_{\text{CPS}} \) antiserum. (B) Western blot of these same samples probed with PY20 phosphotyrosine antibodies. Lane 1, CWG285 (no plasmid); lane 2, pWQ130 (\( Wzc_{\text{CPS}} \)); lane 3, pWQ238 (\( Wzc_{\text{Y569-F}} \)). (C) Western blot of the cell surface polysaccharides from the same strains probed with polyclonal K30 antiserum. The sensitivity of each strain to bacteriophage K30 was assessed (+, sensitive; −, resistant).](http://jb.asm.org/)

![FIG. 6. DSP cross-linking of \( Wzc_{\text{CPS}} \) derivatives in \( E. coli \) E69, CWG281, and DH5\( \alpha \). (A) Western blot of DSP-cross-linked whole-cell protein lysates probed with a monoclonal antiphosphotyrosine antibody (PY20). +, use of DTT for cleavage of cross-links. (B) Western blot of DSP cross-linked whole-cell protein lysates probed with a polyclonal \( Wzc_{\text{CPS}} \) antiserum. Arrows, positions of monomeric \( Wzc \) and complexes containing \( Wzc \).](http://jb.asm.org/)
The effect of the replacement of six of the seven tyrosine residues in the C-terminal tyrosine-rich region was then studied. None of these proteins were detectably phosphorylated by immunoblot analysis, and the absence of detectable phosphorylation correlates with the loss of the biological activity of the protein, i.e., the loss of HMW CPS expression. It remains to be established whether the absolute level of phosphorylation in these mutants is zero or whether the sensitivity of the PY20 antibody is an issue. However, it is important to note that phosphorylation is readily detected by the PY20 antibody in some constructs harboring only two tyrosine residues. This does not detract from the conclusion that phosphorylation at more than one residue in this region is essential for capsule assembly.

The removal of a single tyrosine residue in this region could have revealed whether any one residue was essential to the phosphorylation of WzcCPS or to capsule assembly. Furthermore, if a specific sequence of phosphorylation events was required, removal of the first tyrosine residue in a fixed reaction cascade should also have eliminated phosphorylation of the WzcCPS protein entirely. Neither scenario occurred, as any single tyrosine residue could be replaced without the loss of phosphorylation of WzcCPS or capsule assembly. From the data currently available it appears unlikely that phosphorylation in this region needs to occur in any given order. However, we cannot rule out cooperative effects among phosphorylated tyrosines by using this approach. Such effects have been suggested by Niemeyer and Becker (30) in their studies of ExoP.

Mutants that lacked two to five of the tyrosine residues in the C-terminal domain were then created to determine whether any specific combination of tyrosine residues was important for the activity of WzcCPS. These altered WzcCPS proteins could also provide some information about the minimal number of phosphorylation sites required for the biological activity of WzcCPS. The loss of three or four tyrosine residues in this region was enough to eliminate capsule assembly, though phosphorylation of these WzcCPS derivatives was still detected by immunoblotting. The requirement of at least four tyrosine residues in the C-terminal region for HMW CPS assembly raises the possibility that specific combinations of these tyrosine residues are more important than others. However, the available data do not allow the determination of exactly which combination(s) of residues is most important for WzcCPS activity. Given the number of permutations involving seven residues, the generation of mutants representing every possible combination of tyrosine alterations was impractical. Furthermore, as indicated above, it is conceivable that phosphorylation of tyrosine residues in this region occurs cooperatively and that certain residues in this region may not be as accessible to phosphorylation. Based on the phenotypes of wzc and wzb mutants, we previously proposed that the level of phosphorylation in this region appears to be important (43). This is supported by the fact that Wzc phosphorylation and HMW CPS assembly can be uncoupled, for example, when only two or three tyrosine residues remain in this region. It is therefore important in functional studies of Wzc to distinguish between derivatives that are competent for phosphorylation and those that are capable of supporting capsule assembly.

Studies of ExoP from Sinorhizobium meliloti have shown that deletion of the C terminus of the protein or replacement of certain tyrosine residues causes a reduction in the amount of EPS produced (5, 30). In contrast, studies of CpsD have shown that there is no change in the amount of CPS produced when the C-terminal tyrosine residues are replaced, though the colonies appear to be more mucoid (27). In this study, analysis of K30CPS by Western immunoblotting revealed apparent differences in the amounts of CPS produced by strains expressing certain mutant WzcCPS derivatives. In S. agalactiae, the Wzc homologues CpsC and CpsD were shown to affect capsule chain length. In the absence of CpsC or CpsD, CPS chain length was reduced (9). Examination of K30CPS by SDS-PAGE did not reveal any difference in the sizes of the polymers produced when different WzcCPS derivatives were expressed. However, it is possible that SDS-PAGE profiles do not reflect the entire picture, as some molecules may be too large to enter the resolving gel. Attempts were made to determine whether K30 CPS chain length was affected by the expression of mutant WzcCPS proteins in CWG285. The K30 capsular protein produced by a representative group of these mutants was purified, and sizing was performed by gel filtration chromatography using a column with a fractionation range for globular proteins of 5,000 to 5 × 10^6 Da. All of the K CPS samples tested eluted at the void volume, with no additional peaks representing smaller CPS material (data not shown). From these limited data, there are certainly no K30 CPS molecules with significantly smaller molecular weights formed by a biosynthesis machinery containing mutant WzcCPS proteins.

Grangeasse and coworkers (16) studied the importance of tyrosine residues throughout the entire cytoplasmic domain for the activity of the WzcCA protein in vitro. Purified protein was incubated with [γ-32P]ATP, and the levels of phosphate incorporation in WzcCA were detected by autoradiography and scintillation counting. They showed that one tyrosine residue located between the Walker A and B ATP-binding motifs (Y569) greatly enhanced the level of phosphorylation of the WzcCA protein in vitro (45-fold). The authors suggested that phosphorylation of this site with ATP could provide a means of modulating the level of phosphorylation of the C-terminal domain of Wzc. Alignment of the cytoplasmic region of the WzcCA and the WzcCPS proteins shows that this Y569 residue and the regions around it are well conserved. It was therefore of interest to determine what the biological consequence of a Y569→F mutation would be in the K30 system. In the C-terminally truncated form of Wzc (Wzc1-704) (43) and in WzcV705-718→F, no in vivo phosphorylation was detected with the phosphotyrosine antibody, which can detect as few as two phosphorylated tyrosine residues in the C-terminal region. The replacement of Y569 with phenylalanine resulted in an apparent reduction in the amount of phosphorylation of the protein, as determined by antiphosphotyrosine immunoblotting. However, this protein could restore HMW CPS production in CWG285 to amounts very similar to those produced by WzcCPS. This suggests that phosphorylation of WzcCPS at position Y569 is not essential for in vivo phosphorylation of the C-terminal domain of the protein or for assembly of HMW CPS. This does not, however, preclude the involvement of this residue in modulating the phosphorylation of the C-terminal domain, as suggested by Grangeasse and coworkers (16).

Previous results from our laboratory have shown that WzcCPS molecules can participate in a transphosphorylation...
reaction. No phosphorylation was detected when WzcK540R, which bears a Walker A mutation that prevents ATP binding and/or hydrolysis, or Wzc1-704 which has a functional Walker A motif but which lacks the C-terminal tyrosine-rich region, was expressed alone in CWG285. Furthermore, neither protein was functional in capsule expression. However, when they were coexpressed, phosphorylated Wzc was detected and capsule expression was restored. This showed that Wzc1-704 was able to bind and hydrolyze ATP and that it could transfer phosphate to the C-terminal region of WzcK540R (43). This first evidence of a transphosphorylation reaction between Wzc molecules was supported by subsequent detailed in vitro work done with WzcCA (16). Chemical cross-linking studies were therefore performed to determine whether Wzc proteins could form complexes and to assess the possible role of phosphorylation in such interactions. Studies were carried out with E. coli DH5α in order to better study the interactions between Wzc molecules without the interference of other components of the capsule translocation apparatus, which potentially exist in a complex involving Wzc. Wild-type Wzc was found in several HMW complexes after DSP cross-linking. Cleavage of the cross-link eliminated most of these complexes and restored a large amount of monomeric Wzc. The patterns of complexes formed by Wzc1-704 (truncation of the Y-rich region), WzcK540R (Walker A mutation), and WzcY705-718→F (all C-terminal Ys changed to Fs) were examined. All of these proteins are phosphorylation null and do not function in capsule assembly. However, the cross-linking profiles of strains expressing these proteins were indistinguishable from the profiles of those expressing wild-type WzcCPS. These data suggest that changes in the level of phosphorylation of the C-terminal tyrosine-rich region do not affect the interactions of WzcCPS proteins. The present study emphasizes the importance of Wzc phosphorylation in capsule assembly but does not shed light on the precise role played by Wzc in this biological process. One possibility is that Wzc coordinates other components in the assembly complex in a manner analogous to the proposed role of Wzz in the biosynthesis of O antigens (4, 29). A similar role is hypothesized for the ATP-binding protein PulE and its homologues in type II protein secretion (reviewed in reference 34). It is not unreasonable to assume that interactions between Wzc molecules or between Wzc and other proteins occur mainly through the periplasmic loop. This region has one or more predicted coiled-coil domains (28), and such elements are implicated in protein-protein interactions. In fact, the N-terminal region of Wzz was sufficient for the oligomerization of this protein (10). Perhaps changes in the phosphate load in the cytoplasmic region affect the ability of Wzc to interact with other members of the capsule assembly and translocation machinery. Further research is now required to determine whether the functional form of Wzc is as a monomer, dimer, or multimer and to dissect its possible interactions with other proteins.

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ADDITION IN PROOF

While this paper was in press, Doublet and coworkers (P. Doublet, C. Grangeasse, B. Obadia, E. Vaganay, and A. J. Cozzone, J. Biol. Chem. 277:37339–37348, 2002) reported studies on Wzc from the E. coli K-12 colanic acid biosynthesis system. Like WzzCPS, WzzCA was also known to oligomerize in the presence or absence of ATP-binding sites or phosphorylation sites.

REFERENCES


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