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Impact of Phosphorylation of Specific Residues in the Tyrosine Autokinase, Wzc, on Its Activity in Assembly of Group 1 Capsules in *Escherichia coli*

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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_{CA} 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.

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 *Escherichia coli*, and these have been classified into groups 1 through 4 based on genetic and biosynthetic criteria (41). Group 1 CPS of *E. coli* resemble the capsules of *Klebsiella pneumoniae*, and group 1-like EPS are found in *E. coli* (colanic acid), *Erwinia amylovora* (amylovoran), and *Sinorhizobium meliloti* (succinoglycan), to name a few.

E. coli group 1 CPS are assembled via a Wzy-dependent pathway. The current model (reviewed in reference 41) is based extensively on evidence gathered from the parallel system for lipopolysaccharide (LPS) O-antigen assembly, in which repeat units are assembled on undecaprenol phosphate at the cytoplasmic face of the inner membrane by the sequential activity of glycosyltransferases. The repeat units are then believed to be flipped to the periplasmic face of the inner membrane by an unknown mechanism involving the Wzx protein. Polymerization of the lipid-linked repeat units is then carried out by Wzy, the polymerase. In *E. coli* K30, the K antigen can undergo one of two fates at this stage. High-level polymerization can occur to generate high-molecular-weight (HMW) CPS, which is then translocated to the cell surface ($K30_{CPS}$). Alternatively, one to a few repeat units of the K antigen can be ligated onto lipid A-core and expressed on the cell surface as K_{LPS} (11, 25).

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

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low-level polymerization or assembly of K_{LPS} (12, 43). There are second copies of the *wza*, *wzb*, and *wzc* genes on the chromosomes of *E. coli* 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 wza_{22min}, wzb_{22min}, and wzc_{22min}.

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 *Streptococcus pneumoniae* (18, 26), *Streptococcus agalactiae* (33, 44), and *Staphylococcus aureus* (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_{CPS} 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 *E. coli* 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_{CPS} could be obtained from incubation of a Wzc_{CPS} mutant that could not bind ATP with a Wzc_{CPS} 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_{CA} (colanic acid) protein (16). Work on the Wzc_{CA} protein in *E. coli* 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_{CA} 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_{CA} 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 an active autokinase capable of phosphorylation at the C terminus and an active phosphatase led us to propose that the cycling of Wzc_{CPS} between phosphorylated and nonphosphorylated states may be necessary for its function (43). In *E. coli* K-12 and *S. pneumoniae*, both the kinase and the phosphatase are also required for polysaccharide production (27, 38). However, in these particular systems, decreases in the phosphorylation of Wzc_{CA} (caused by overexpression of Wzb_{CA}) and CpsD (caused by removal of the site of phosphorylation) still allowed wild-type levels of polysaccharide expression. This led the authors to suggest that phosphorylated Wzc_{CA} and CpsD act as negative regulators of colanic acid and capsule production. In *Sinorhizobium meliloti*, succinoglycan is produced in HMW and low-molecular-weight (LMW) forms, similar in some respects to K_{CPS} and K_{LPS} in *E. coli*. Mutations in ExoP (Wzc homologue) result in a decrease in the amount of HMW polymer and an increase in the amount of LMW material, suggesting that polymerization itself is not prevented (5, 30). This phenotype resembles that of a wzc_{CPS} mutant, where $K30_{LPS}$ production is increased (12, 43). Data on CPS expression in group B streptococci point to a potential role for Wzc homologues in chain length determination (9). It is also interesting that the topology of the N terminus of Wzc resembles that of Wzz, a protein that influences chain length of LPS O antigens (reviewed in references 28 and 39). Current hypotheses suggest that Wzc proteins may interact with the polymerization machinery to control the level of polymerization of the EPS, but it is not yet clear what the specific roles of the Wzc proteins in CPS and EPS production are.

Although 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_{CPS} in CPS assembly in *E. coli* O9a:K30.

(These data were presented in preliminary form at the 101st General Meeting of the American Society for Microbiology [Abstr. Annu. Meet. Am. Soc. Microbiol., abstr. B-275, p.102, 2001].)

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^{-1}) (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 amplified 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 *E. coli* 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 *Eco*RI site for cloning and a reverse primer that reintroduced the desired tyrosine codon and provided a *Pst*I site for cloning. The PCR products were cloned back into pBAD18-Km, and the resulting plasmids were transformed into *E. coli* 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_{CPS} and derivatives. Wzc_{CPS} and mutant derivatives were expressed from pBAD arabinose-inducible expression vectors. Cells were grown to mid-exponential growth phase, and expression of the Wzc_{CPS} 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 2× sodium dodecyl sulfate-polyacrylamide gel

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype, serotype, or description	Reference or source
E. coli strains		
DH5α	K-12 ϕ80d *lacZ*ΔM15 *endA1 recA1 hsdR17*($r_K^- m_K^-$) *supE44 thi-1 gyrA96 relA1* Δ(*lacZYA-argF*) *U169* F^-	Clontech
E69	Prototroph, serotype O9a:K30:H12	F. Ørskov[a]
CWG258	E69 with wza_{22min}::*aadA* insertion (polar on wzb_{22min} and wzc_{22min}); Sp^r	13
CWG285	E69 with wzc_{CPS}::*aacC1* and wza_{22min}::*aadA* insertion (polar on wzb_{22min} and wzc_{22min}); Gm^r Sp^r	43
CWG281	E69 with wza_{CPS}::*aacC1* and wza_{22min}::*aadA* insertion (polar on wzb_{22min} and wzc_{22min}); Gm^r Sp^r	13
Plasmids		
pBAD18-Km	Arabinose-inducible expression vector; Km^r	19
pBAD24	Arabinose-inducible expression vector; Ap^r	19
pWQ130	pBAD18-Km derivative containing wzc_{CPS} on an *EcoRI*/*Pst*I fragment; Km^r	43
pWQ132	pWQ130 derivative expressing Wzc_{CPS} with a Walker A mutation (Wzc^{K540R}); Km^r	43
pWQ133	pBAD18-Cm derivative expressing the C-terminal truncated Wzc_{CPS} (Wzc^{1-704}); Cm^r	43
pWQ147	pBAD24 derivative containing wzb_{CPS} on an *Eco*RI/blunt-ended fragment; Ap^r	43
pWQ212	pWQ130 derivative expressing $Wzc_{Y705-718\rightarrow F}$; Km^r	This study
pWQ213	pWQ130 derivative expressing $Wzc_{Y706-718\rightarrow F}$; Km^r	This study
pWQ214	pWQ130 derivative expressing $Wzc_{Y705/708-718\rightarrow F}$; Km^r	This study
pWQ215	pWQ130 derivative expressing $Wzc_{Y705/706/713-718\rightarrow F}$; Km^r	This study
pWQ216	pWQ130 derivative expressing $Wzc_{Y705-708/715-718\rightarrow F}$; Km^r	This study
pWQ217	pWQ130 derivative expressing $Wzc_{Y705-713/717/718\rightarrow F}$; Km^r	This study
pWQ218	pWQ130 derivative expressing $Wzc_{Y705-715/718\rightarrow F}$; Km^r	This study
pWQ219	pWQ130 derivative expressing $Wzc_{Y705-717\rightarrow F}$; Km^r	This study
pWQ220	pWQ130 derivative expressing $Wzc_{Y706-717\rightarrow F}$; Km^r	This study
pWQ221	pWQ130 derivative expressing $Wzc_{Y708-718\rightarrow F}$; Km^r	This study
pWQ222	pWQ130 derivative expressing $Wzc_{Y708-717\rightarrow F}$; Km^r	This study
pWQ223	pWQ130 derivative expressing $Wzc_{Y706-715\rightarrow F}$; Km^r	This study
pWQ224	pWQ130 derivative expressing $Wzc_{Y713-717\rightarrow F}$; Km^r	This study
pWQ225	pWQ130 derivative expressing $Wzc_{Y708-715\rightarrow F}$; Km^r	This study
pWQ226	pWQ130 derivative expressing $Wzc_{Y706-713\rightarrow F}$; Km^r	This study
pWQ227	pWQ130 derivative expressing $Wzc_{Y715/717\rightarrow F}$; Km^r	This study
pWQ228	pWQ130 derivative expressing $Wzc_{Y708/715\rightarrow F}$; Km^r	This study
pWQ229	pWQ130 derivative expressing $Wzc_{Y708/713\rightarrow F}$; Km^r	This study
pWQ230	pWQ130 derivative expressing $Wzc_{Y706/717\rightarrow F}$; Km^r	This study
pWQ231	pWQ130 derivative expressing $Wzc_{Y705\rightarrow F}$; Km^r	This study
pWQ232	pWQ130 derivative expressing $Wzc_{Y706\rightarrow F}$; Km^r	This study
pWQ233	pWQ130 derivative expressing $Wzc_{Y708\rightarrow F}$; Km^r	This study
pWQ234	pWQ130 derivative expressing $Wzc_{Y713\rightarrow F}$; Km^r	This study
pWQ235	pWQ130 derivative expressing $Wzc_{Y715\rightarrow F}$; Km^r	This study
pWQ236	pWQ130 derivative expressing $Wzc_{Y717\rightarrow F}$; Km^r	This study
pWQ237	pWQ130 derivative expressing $Wzc_{Y718\rightarrow F}$; Km^r	This study
pWQ238	pWQ130 derivative expressing $Wzc_{Y569\rightarrow F}$; Km^r	This study

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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 Westran polyvinylidene difluoride membranes (Schleicher & Schuell) for phosphotyrosine analysis or BioTrace NT nitrocellulose membranes (Gelman Laboratory) for Wzc expression analysis. The transfer buffer used contained 3 mM Na_2CO_3, 10 mM $NaHCO_3$, and 20% methanol. Wzc expression was detected with a Wzc_{CPS} rabbit polyclonal antiserum (43) and a goat anti-rabbit secondary antibody (Caltag, Burlingame, Calif.), while the level of phosphorylated Wzc protein was assessed with the PY20 antiphosphotyrosine monoclonal antibody (Transduction Laboratories, Lexington, Ky.) and a goat anti-mouse secondary antibody (Jackson ImmunoResearch Laboratories Inc., West Grove, Pa.). Both secondary antibodies were conjugated to alkaline phosphatase, which allowed colorimetric detection using nitroblue tetrazolium chloride and 5-bromo-4-chloro-3-indolylphosphate.

SDS-PAGE analysis of cell surface polysaccharides. Cell surface polysaccharide samples were prepared by the method of Hitchcock and Brown (20). Briefly, whole-cell lysates were prepared in 1× SDS-PAGE sample buffer lacking β-mercaptoethanol. These lysates were heated at 100°C for 30 min, proteinase K was added to 0.5 mg/ml, and the protein content was digested at 55°C overnight. These samples were then analyzed by electrophoresis on NuPAGE 4 to 12% *N*,*N*-methylenebisacrylamide–Tris gels (Invitrogen Life Technologies Inc.) and then transferred to BioTrace NT nitrocellulose membranes (Gelman Laboratory) for immunoblotting. K30 polysaccharide was detected with a rabbit anti-K30 polyclonal antiserum (11) and an alkaline phosphatase-conjugated goat anti-rabbit secondary antibody.

Bacteriophage sensitivity assays. The sensitivity of strains to lysis by bacteriophage K30 was determined in order to assess the presence or absence of K30 antigen on the cell surface. Bacteriophage K30 lyses cells bearing a full or partial K30 capsule, while the absence of K30 polymer makes strains resistant to attack by this phage (40).

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 proteinase 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 redialyzed. For both cell-free and cell-associated samples, LPS was removed by ultracentrifugation (105,000 × *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 H_2SO_4. Samples were heated to

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 A_{530} 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 Wzc_{CPS} 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 NaH_2PO_4, 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; Lomant's 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 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 Wzc_{CPS} resulted in loss of phosphorylation of Wzc_{CPS} (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 Δ*wzc* strain. In *S. pneumoniae*, *cpsCD* 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 Wzc_{CPS} 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 in the C-terminal region. To address this issue, a Wzc_{CPS} mutant in which the tyrosine residues at amino acid positions 705, 706, 708, 713, 715, 717, and 718 were each replaced by phenylalanine was constructed. This was done by sequential site-directed mutagenesis of pWQ130 (pBAD18Km-Wzc_{CPS}), and the resulting plasmid (pWQ212) was transformed into CWG285, a strain in which both chromosomal copies of the *wzc* gene have been knocked out. The mutated Wzc_{CPS} protein ($Wzc_{Y705\text{-}718\rightarrow F}$) was expressed at levels similar to those for the native Wzc_{CPS} protein, as assessed by Western blotting with antiserum against Wzc_{CPS} (Fig. 1A). However, $Wzc_{Y705\text{-}718\rightarrow F}$ was not phosphorylation competent, since no reactivity with the antiphosphotyrosine antibody PY20 was detected (Fig. 1B). The in vivo activity of this protein was assessed by determining the ability of the plasmid to restore HMW K30 CPS expression in CWG285. This is detected by Western immunoblot analysis of polysaccharide preparations probed with a polyclonal antiserum specific against the K30 capsule. $Wzc_{Y705\text{-}718\rightarrow F}$ could not restore any detectable HMW CPS ($K30_{CPS}$) production (Fig. 1C). The higher degree of polymerization of $K30_{LPS}$ (seen as a ladder of slower-migrating molecules) was evident in CWG285 with or without the plasmid.

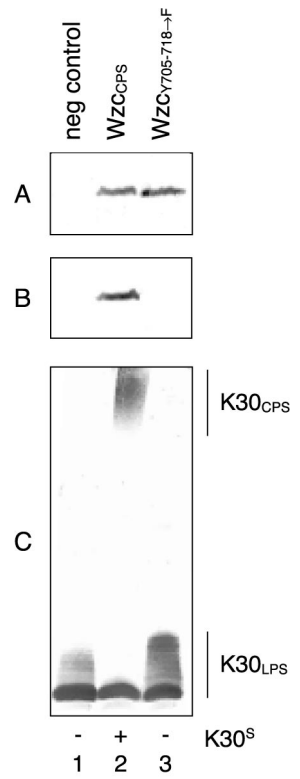

FIG. 1. Site-directed mutation of all C-terminal tyrosine residues eliminates Wzc_{CPS} phosphorylation and HMW CPS biogenesis. (A) Western blot of whole-cell protein lysates probed with polyclonal Wzc_{CPS} antiserum, showing the expression levels of Wzc_{CPS} and $Wzc_{Y705\text{-}718\rightarrow F}$ expressed from plasmids in *E. coli* CWG285. Lane 1, negative control CWG285 (no plasmid); lane 2, pWQ130 (Wzc_{CPS}); lane 3, pWQ212 ($Wzc_{Y705\text{-}718\rightarrow F}$). (B) Western blot of the same samples probed with a monoclonal antiphosphotyrosine antibody (PY20) to detect the phosphorylation levels of Wzc_{CPS} 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 K_{LPS} as reported previously (12). Each strain was tested for sensitivity to bacteriophage K30. +, sensitivity of the strain to the phage; −, resistance.

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 ($Wzc_{Y705\text{-}718\rightarrow F}$) did not enhance the base level of polymer production in CWG285 (Table 2). In contrast, introduction of pWQ130 (encoding the wild-type Wzc_{CPS}) resulted in an elevated amount of cell-associated capsular K30 antigen (Table 2). The complementation with plasmid-encoded Wzc_{CPS} derivatives never restores wild-type levels of CPS production (compare to CWG258 [Table 2]), which is consistent with previous studies (12, 43).

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

Strain(plasmid) (protein expressed)	Level of K30 CPS (ng of uronic acid/10^7 CFU)[a]	
	Cell free	Cell associated
CWG258	28 ± 5	165 ± 15
CWG258(pWQ147) (Wzb_{CPS})	33 ± 7	45 ± 5
CWG285	10 ± 4	17 ± 3
CWG285(pWQ130) (Wzc_{CPS})	8 ± 0.4	53 ± 1.6
CWG285(pWQ212) ($Wzc_{Y705-718\rightarrow F}$)	9 ± 2	17 ± 2
CWG285(pWQ238) ($Wzc_{Y569\rightarrow F}$)	9 ± 2.4	54 ± 5

[a] Values are means ± 1 standard deviation.

It is conceivable that the absence of K30 CPS in CWG285 expressing $Wzc_{Y705-718\rightarrow F}$ reflected 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. Overexpression of Wzb_{CPS} was confirmed by SDS-PAGE of cell lysates (data not shown) and did indeed cause a 3.7-fold decrease in the amount of cell-associated uronic acid but had no dramatic effect on the amount of cell-free 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.

Is any single tyrosine residue alone sufficient to maintain phosphorylation of Wzc_{CPS} and/or capsule assembly? To determine whether any single tyrosine residue in the C-terminal tyrosine-rich domain is sufficient to maintain phosphorylation of the Wzc_{CPS} protein and/or restore HMW K30 CPS, seven Wzc_{CPS} mutant proteins that each retained only one of the seven tyrosine residues were created. The anti-Wzc_{CPS} blot shows that all of these mutant Wzc_{CPS} proteins were expressed at levels comparable to that for the native Wzc_{CPS} protein (Fig. 2A). However, none of these proteins showed detectable phosphorylation by PY20 Western blotting (Fig. 2B), and none could restore the capsular phenotype to CWG285 (Fig. 2C), though each retained elevated levels of $K30_{LPS}$. These strains were also resistant to the K30 phage, confirming the absence of K30 CPS on the cell surface.

Is any one tyrosine residue essential for phosphorylation or capsule assembly? To determine if any single tyrosine residue in the C-terminal tyrosine-rich domain is essential for phosphorylation of Wzc_{CPS} or for capsule assembly, mutant Wzc_{CPS} proteins which lacked one of the seven tyrosine residues in this region were created. These proteins were expressed in CWG285, and their expression, phosphorylation, and ability to restore a capsular phenotype were determined by Western blotting. The anti-Wzc blot shows that the mutant proteins are all expressed at levels similar to that for Wzc_{CPS} (Fig. 3A), and the PY20 blot shows that all of these proteins are phosphorylated at levels comparable to that for the wild-type protein

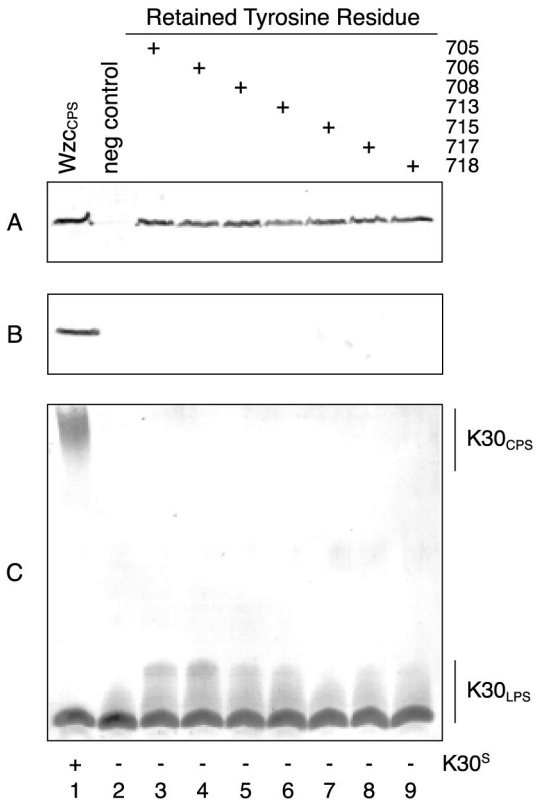

FIG. 2. No single tyrosine residue in the C-terminal region is sufficient for phosphorylation of Wzc_{CPS} or for HMW CPS expression. Wzc_{CPS} and its derivatives were expressed in *E. coli* CWG285. The retained tyrosine residue is indicated above each lane. (A) Western blot of whole-cell protein lysates probed with polyclonal Wzc_{CPS} antiserum. Lane 1, pWQ130; lane 2, negative control CWG285 (no plasmid); lane 3, pWQ213; lane 4, pWQ214; lane 5, pWQ215; lane 6, pWQ216; lane 7, pWQ217; lane 8, pWQ218; lane 9, pWQ219. (B) Western blot of the same samples probed with the PY20 antiphosphotyrosine antibody. (C) Western blot of cell surface polysaccharides probed with polyclonal K30 antiserum. The sensitivity (+) or resistance (−) of each strain to bacteriophage K30 was assessed.

(Fig. 3B). Furthermore, all of these proteins could restore the capsular phenotype to the Δ*wzc* strain and reduce the amount of $K30_{LPS}$ 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? Finally, 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 ($Wzc_{Y706/717\rightarrow F}$, $Wzc_{Y708/713\rightarrow F}$, $Wzc_{Y708/715\rightarrow F}$, and $Wzc_{Y715/717\rightarrow F}$,) 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, $Wzc_{Y706/717\rightarrow F}$ (Fig. 4C, lane 3), $Wzc_{Y708/715\rightarrow F}$ (Fig. 4C, lane

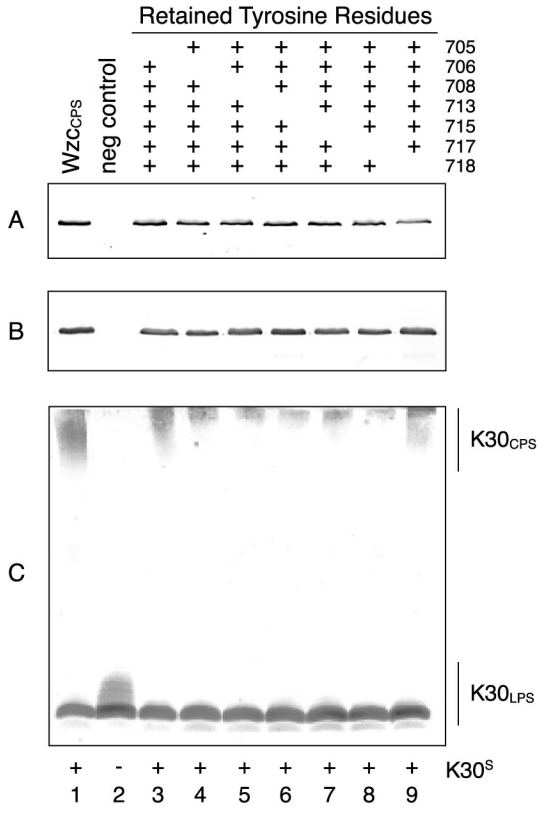

FIG. 3. No single tyrosine residue in the C-terminal region is essential for phosphorylation of Wzc_{CPS} or for HMW CPS expression. (A) Western blot of whole-cell protein lysates probed with polyclonal Wzc_{CPS} antiserum, showing the expression levels of Wzc_{CPS} and its derivatives in *E. coli* CWG285. The tyrosine residues retained are indicated above each lane. Lane 1, pWQ130; lane 2, negative control CWG285 (no plasmid); lane 3, pWQ231; lane 4, pWQ232; lane 5, pWQ233; lane 6, pWQ234; lane 7, pWQ235; lane 8, pWQ236; lane 9, pWQ237. (B) Western blot of the same samples probed with the PY20 antiphosphotyrosine antibody. (C) Western blot of the cell surface polysaccharides probed with polyclonal K30 antiserum. Each strain was tested for its sensitivity (+) or resistance (−) to bacteriophage K30.

5), and $Wzc_{Y715/717\rightarrow F}$ (Fig. 4C, lane 6) show larger amounts of immunoreactive K30 antigen than $Wzc_{Y708/713\rightarrow F}$ (Fig. 4C, lane 4). Furthermore, the K30 phage does not produce clear plaques when $Wzc_{Y708/713\rightarrow F}$ is expressed. For the three strains that express a Wzc_{CPS} protein lacking three of the terminal tyrosine residues, only $Wzc_{Y713\text{-}717\rightarrow F}$ (Fig. 4C, lane 9) showed a detectable $K30_{CPS}$ 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). $Wzc_{Y708\text{-}715\rightarrow F}$ showed only turbid plaques when subjected to the K30 phage, while $Wzc_{Y706\text{-}713\rightarrow F}$ was not sensitive to the K30 phage at all. It is again of interest to note the variable amounts of immunoreactive $K30_{CPS}$ material produced by these derivatives. Two Wzc_{CPS} 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 $Wzc_{Y708\text{-}718\rightarrow F}$ (Fig. 4B, lane 14) and $Wzc_{Y706\text{-}717\rightarrow F}$ (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 Wzc_{CA} 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 Wzc_{CA} 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 Wzc_{CPS} 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 Wzc_{CPS} was changed to phenylalanine by site-directed mutagenesis. The resulting $Wzc_{Y569\rightarrow F}$ 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 $Wzc_{Y569\rightarrow F}$ appeared to be reduced slightly compared to that of wild-type Wzc_{CPS}. 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 Wzc_{CPS} 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 Wzc_{CPS} and $Wzc_{Y569\rightarrow F}$. There was no apparent difference in the amounts of cell-free K30 CPS produced by Wzc_{CPS} and $Wzc_{Y569\rightarrow F}$ or in the amounts of cell-associated CPS produced by these strains (Table 2).

In Wzc_{CPS}, 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 Wzc_{CA}. 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 Wzc_{CPS} 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 Wzc_{CPS} 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-Wzc_{CPS} 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-

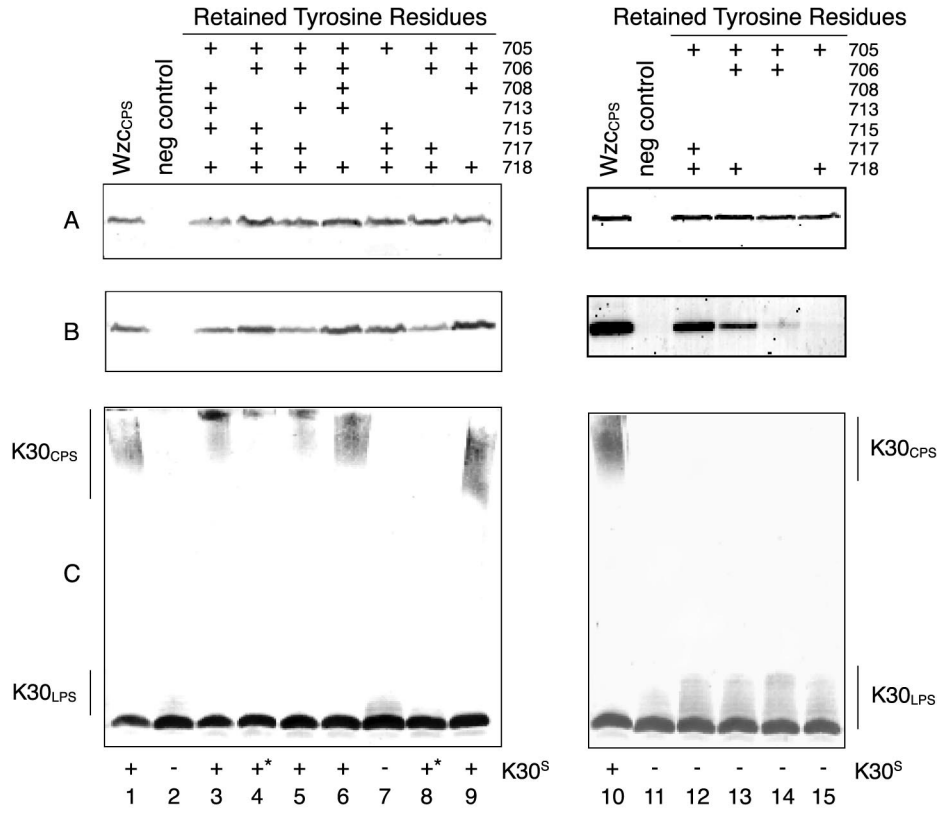

FIG. 4. Effect of the removal of combinations of two to five tyrosine residues from the C-terminal region. Western blots of whole-cell protein lysates of Wzc_{CPS} and mutant derivatives expressed in CWG285 were probed with polyclonal Wzc_{CPS} antiserum (A) and PY20 antiphosphotyrosine antibodies (B). Retained tyrosine residues are indicated above each lane. Lane 1, pWQ130; lane 2, negative control CWG285 (no plasmid); lane 3, pWQ230; lane 4, pWQ229; lane 5, pWQ228; lane 6, pWQ227; lane 7, pWQ226; lane 8, pWQ225; lane 9, pWQ224; lane 10, pWQ130; lane 11, CWG285; lane 12, pWQ223; lane 13, pWQ222; lane 14, pWQ221; lane 15, pWQ220. Lanes 10 to 15 of panel B were developed for a longer period of time to allow detection of weakly phosphorylated proteins. (C) Western blot of the cell surface polysaccharides produced by these strains probed with polyclonal K30 antiserum. The sensitivity of each strain to bacteriophage K30 was assessed. Asterisks indicate the formation of turbid plaques.

ative lacking both chromosomal copies of *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 *E. coli* DH5α expressing Wzc_{CPS} encoded by pWQ130 were performed. An *E. coli* K-12 derivative (DH5α) was used in these experiments to eliminate any possible complicating associations of Wzc with other components of the capsule translocation apparatus in E69 derivatives. *E. coli* DH5α contains the *wza*, *wzb*, and *wzc* genes in the colanic acid biosynthesis cluster, but these are not expressed in *E. coli* K-12 at the growth temperatures used in this study (15). Western immunoblot analysis using PY20 showed that Wzc_{CPS} 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 Wzc_{CPS} polyclonal antiserum, but the lower reactivity of the antibody generated a weaker signal (data not shown). Further studies were then undertaken to investigate whether Wzc_{CPS} 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 Wzc_{CPS} and/or assembly of HMW K30 CPS. DSP cross-linking was carried out in DH5α expressing various Wzc_{CPS} constructs in *trans*. When a Wzc_{CPS} Walker A box mutant (Wzc^{K540R}; pWQ132) was expressed in DH5α, a cross-linking profile similar to that obtained with Wzc_{CPS} was observed (Fig. 6B). The same was seen when either the truncated Wzc_{CPS} protein lacking the C-terminal tyrosine-rich domain (Wzc^{1-704}; pWQ133) or the site-directed mutant lacking all the C-terminal tyrosine residues ($Wzc_{Y705-718\rightarrow F}$) was expressed in DH5α (data not shown).

DISCUSSION

In this study, the importance of phosphorylation of Wzc_{CPS} 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 Wzc_{CPS} protein in order to determine which residues, if any, were essential for the phosphorylation of Wzc_{CPS} and for the surface expression of HMW CPS in *E. coli* O9a:K30.

Previously we showed that the C-terminal tyrosine-rich region of Wzc_{CPS} from *E. coli* O9a:K30 is the site of phosphor-

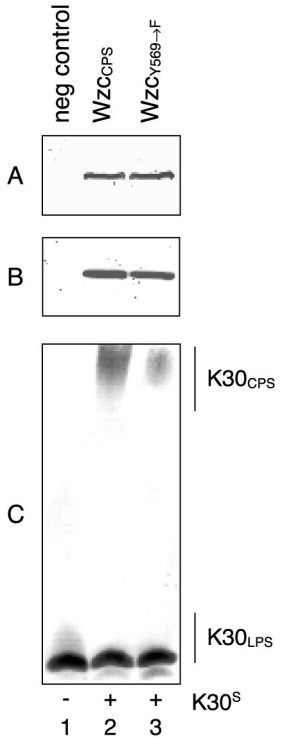

FIG. 5. Site-directed mutagenesis of Y569 does not eliminate phosphorylation of Wzc_{CPS} or expression of HMW K30 CPS. Wzc_{CPS} and $Wzc_{Y569\rightarrow F}$ were expressed from plasmids in *E. coli* CWG285. (A) Western blot of whole-cell protein lysates probed with polyclonal Wzc_{CPS} antiserum. (B) Western blot of these same samples probed with PY20 phosphotyrosine antibodies. Lane 1, CWG285 (no plasmid); lane 2, pWQ130 (Wzc_{CPS}); lane 3, pWQ238 ($Wzc_{Y569\rightarrow F}$). (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).

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 Δ*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_{CPS} to function in capsule assembly (43). Similar findings were reported from studies on Wzc_{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_{CPS} might have altered the folding of the protein and thus altered its phosphorylation and capsule assembly functions. However, the $Wzc_{Y705\text{-}718\rightarrow F}$ 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_{LPS} assembly. The importance of the phosphorylation of Wzc_{CPS} in CPS production was further confirmed by findings that overexpression of Wzb_{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_{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).

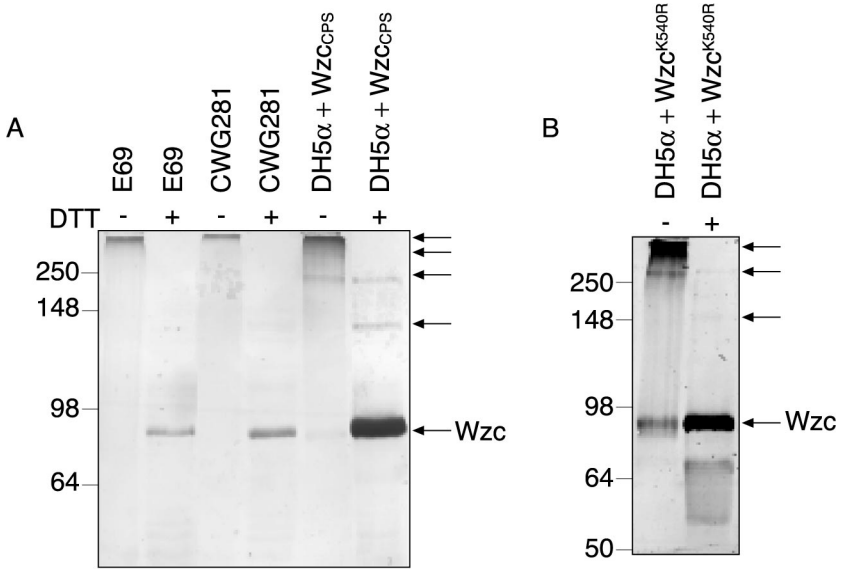

FIG. 6. DSP cross-linking of Wzc_{CPS} derivatives in *E. coli* E69, CWG281, and DH5α. (A) Western blot of DSP-cross-linked whole-cell protein lysates probed with a monoclonal antiphosphotyrosine antibody (PY20). +, use of DTT for cleavage of cross-links. (B) Western blot of DSP cross-linked whole-cell protein lysates probed with a polyclonal Wzc_{CPS} antiserum. Arrows, positions of monomeric Wzc and complexes containing Wzc.

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 Wzc_{CPS} 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 Wzc_{CPS} protein entirely. Neither scenario occurred, as any single tyrosine residue could be replaced without the loss of phosphorylation of Wzc_{CPS} 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 Wzc_{CPS}. These altered Wzc_{CPS} proteins could also provide some information about the minimal number of phosphorylation sites required for the biological activity of Wzc_{CPS}. The loss of three or four tyrosine residues in this region was enough to eliminate capsule assembly, though phosphorylation of these Wzc_{CPS} 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 Wzc_{CPS} 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 $K30_{CPS}$ by Western immunoblotting revealed apparent differences in the amounts of CPS produced by strains expressing certain mutant Wzc_{CPS} 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 $K30_{CPS}$ by SDS-PAGE did not reveal any difference in the sizes of the polymers produced when different Wzc_{CPS} 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 Wzc_{CPS} proteins in CWG285. The K30 capsular material 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 Wzc_{CPS} proteins.

Grangeasse and coworkers (16) studied the importance of tyrosine residues throughout the entire cytoplasmic domain for the activity of the Wzc_{CA} protein in vitro. Purified protein was incubated with [γ-^{32}P]ATP, and the levels of phosphate incorporation in Wzc_{CA} 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 Wzc_{CA} 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 Wzc_{CA} and the Wzc_{CPS} 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 ($Wzc^{1\text{-}704}$) (43) and in $Wzc_{Y705\text{-}718\rightarrow F}$, 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 Wzc_{CPS}. This suggests that phosphorylation of Wzc_{CPS} 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 Wzc_{CPS} molecules can participate in a transphosphorylation

reaction. No phosphorylation was detected when Wzc^{K540R}, which bears a Walker A mutation that prevents ATP binding and/or hydrolysis, or Wzc^{1-704}, 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 Wzc^{1-704} was able to bind and hydrolyze ATP and that it could transfer phosphate to the C-terminal region of Wzc^{K540R} (43). This first evidence of a transphosphorylation reaction between Wzc molecules was supported by subsequent detailed in vitro work done with Wzc_{CA} (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 Wzc^{1-704} (truncation of the Y-rich region), Wzc^{K540R} (Walker A mutation), and $Wzc_{Y705-718\rightarrow F}$ (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 Wzc_{CPS}. These data suggest that changes in the level of phosphorylation of the C-terminal tyrosine-rich region do not affect the interactions of Wzc_{CPS} 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.

ACKNOWLEDGMENTS

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ADDENDUM 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 Wzc_{CPS}, Wzc_{CA} was also known to oligomerize in the presence or absence of ATP-binding sites or phosphorylation sites.

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