Tyrosine Phosphorylation of Protein Kinase Wzc from Escherichia coli K12 Occurs through a Two-step Process

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In bacteria, several proteins have been shown to autophosphorylate on tyrosine residues, but little is known on the molecular mechanism of this modification. To get more information on this matter, we have analyzed in detail the phosphorylation of a particular autokinase, protein Wzc, from Escherichia coli K12. The analysis of the hydrophatic profile of this protein indicates that it is composed of two main domains: an N-terminal domain, including two transmembrane α-helices, and a C-terminal cytoplasmic domain. The C-terminal domain alone can undergo autophosphorylation and thus appears to harbor the protein-tyrosine kinase activity. By contrast, the N-terminal domain is not phosphorylated when incubated either alone or in the presence of the C-domain, and does not influence the extent of phosphorylation of the C-domain. The C-domain contains six different sites of phosphorylation. Among these, five are located at the C-terminal end of the molecule in the form of a tyrosine cluster (Tyr708, Tyr710, Tyr711, Tyr713, and Tyr715), and one site is located upstream, at Tyr669. The Tyr669 residue can autophosphorylate through an intramolecular process, whereas the tyrosine cluster cannot. The phosphorylation of Tyr669 results in an increased protein kinase activity of Wzc, which can, in turn, phosphorylate the five terminal tyrosines through an intermolecular process. It is concluded that protein Wzc autophosphorylates by using a cooperative two-step mechanism that involves both intra- and interphosphorylation. This mechanism may be of biological significance in the signal transduction mediated by Wzc.

Phosphorylation of protein on tyrosine has long been considered to occur exclusively in eukaryotes. In these organisms, it has been shown to play a key role in a series of fundamental biological functions (1–3). In prokaryotes, the presence of a similar phosphorylating activity was suggested, much later than in eukaryotes, by the finding of phosphotyrosine first in the proteins of Escherichia coli (4), then in the proteins of a variety of bacterial species (reviewed in Refs. 5, 6).

Recently, the existence of a protein-tyrosine kinase has actually been demonstrated, for the first time, by overproducing and purifying a particular phosphoprotein, termed Ptk, from Acinetobacter johnsonii, and by showing its capacity to autophosphorylate in vitro at the expense of ATP (7, 8). Then, other protein-tyrosine kinases, homologous to Ptk, have been characterized in Gram-negative bacteria, namely protein Wzc from E. coli K12 (9) and E. coli K30 (10), protein Etk from E. coli K30 (11), protein ExoP from Sinorhizobium meliloti (12), and protein Yco6 from Klebsiella pneumoniae (13).

Besides their capacity to function as tyrosine kinases, most of these proteins are also involved in the production and/or transport of exopolysaccharides. Because exopolysaccharides are important virulence factors, a possible relationship between tyrosine phosphorylation and bacterial pathogenicity has been proposed. This hypothesis is supported by a number of recent data (10, 11, 14).

Other proteins from Gram-negative bacteria exhibit striking similarity to Ptk and Wzc, such as protein EpsB from the phytopathogen Rastonia solanacearum (15), and protein AmsA from the other phytopathogen Erwinia amylovora (16). They also are involved in the metabolism of exopolysaccharides, but their tyrosine-kinase activity has not been clearly evidenced yet.

These various proteins share several common structural features specific to bacteria, namely the Walker A and B ATP-binding motifs (17), which are not usually found in the counterpart eukaryotic kinases, and a series of tyrosine residues clustered at the C-terminal end of the molecule. For Ptk and Wzc, the Walker A motif has been shown to be effectually employed for autophosphorylation of the protein on tyrosine, suggesting that bacteria utilize for phosphorylation a novel mechanism different from that of eukaryotes (10, 17). On the other hand, it has been suggested that the tyrosine cluster would be the target sequence for autophosphorylation (10, 18), but no accurate characterization of the concerned residues has been made and its function remains unknown. Concerning intracellular localization, these Gram-negative bacterial proteins are all anchored in the inner-membrane and, in the particular case of ExoP, membrane topology studies have indicated that it consists of two main domains: a C-terminal domain, comprising the tyrosine cluster, which is located in the cytoplasmic fraction, and an N-terminal domain, with two transmembrane α-helices, which is present in the periplasm (19).

Interestingly, several Gram-positive bacteria also contain homologues of Ptk and Wzc, including Streptococcus pneumoniae (20–22), Streptococcus agalactiae (23, 24), and Staphylococcus aureus (25, 26). However, in these bacteria, the N- and C-terminal domains are represented in two separate polypeptides encoded by two distinct genes (20, 25). Thus, the CpsC protein of S. pneumoniae is equivalent to the N-domain of Wzc, and the CpsD protein is similar to the C-domain, which is phosphorylated at a tyrosine-rich sequence (18).

In this work, we have examined in detail the process of bacterial protein autophosphorylation on tyrosine by using protein Wzc from E. coli K12 as a model. We have analyzed the region of the protein modified by phosphorylation, the effect of the rest of the molecule on this reaction, and the precise num-
ber and location of the different phosphorylation sites. In addition, the mechanism of autophosphorylation has been investigated to determine the respective account of intra- and interphosphorylation in the overall reaction.

**EXPERIMENTAL PROCEDURES**

**Bacterial Strains, Plasmids, and Growth Conditions—** *E. coli* JM109 strain (27) was used as template for PCR amplification of the *wzc* gene fragments and for generating *wzc* gene mutants. *E. coli* BL1-Blue strain (28) was used to propagate plasmids in cloning experiments. For expression experiments, either *E. coli* XLI-Blue strain, or *E. coli* BL21 (DE3) was used. Primers and plasmids used in this study are listed below in Tables I and II, respectively. All strains were grown and maintained in Luria-Bertani or 2YT medium at 37 °C. When required, media were supplemented with antibiotics at the following concentrations: ampicillin (50 μg/ml), kanamycin (25 μg/ml), and tetracycline (15 μg/ml).

**DNA Manipulation—** Plasmid isolation was carried out using a Qiagen prep purification kit (Qiagen). All restriction and DNA modifying enzymes were used as recommended by the manufacturer (Promega). All amplification reactions (PCR) were performed using *Pfu* polymerase (Promega). PCR products and plasmid DNA fragments were purified using a Qiagen gel extraction kit (Qiagen). Oligonucleotides were provided by Sigma Genosys. Transformation of *E. coli* cells was performed by the method of Dagert and Ehrlich (30). DNA sequencing was carried out by Genome-Express Corp. The nucleotide sequence of all synthesized and mutated genes was checked to ensure proper base replacement and error-free amplification. DNA sequences were analyzed by the DNAid computer program (31). BLAST searches (32) and sequence alignments (33, 34) were performed by using our laboratory site server (www.ibcp.fr).

**Construction of His<sub>6</sub> Tag Wzc Domain Expression Plasmids**—The 824-bp *wzc* (amino acids 1339–2163) and 773-bp *wzc* (amino acids 1339–2112) gene fragments, with appropriate sites at both ends, encoding the C-terminal domain of *Wzc*, respectively, with or without the tyrosine cluster, were synthesized by PCR amplification using genomic DNA from *E. coli* JM109 strain as a template and primers 4/1 and 4/2, respectively (Table I). The DNA fragment synthesized was restricted by *Bam*HI and *Hind*III and ligated into pQE30 vector opened with the same enzymes. The resulting plasmids were termed pQE30-1356 and pQE30-1275. The amplified fragments were restricted by *Bam*HI and *Hind*III and ligated into pQE30 vector opened with the same enzymes. The resulting plasmids were termed pQE30-14569F-L6 with the same enzymes. The resulting plasmids were termed pQE30-14569F-L6 with the same enzymes. The resulting plasmids were termed pQE30-1356 and pQE30-1275 (see Table II).

**Construction of Wild and Mutated GST-Wzc Cytoplasmic Domain Expression Plasmids**—To construct plasmids expressing the cytoplasmic domain of *Wzc* fused with GST, PCR amplification was carried out using, on the one hand, pQE30-41 or pQE30-41K (see Table II) as templates with the primer pair 4/1bis and, on the other hand, pQE30-42 or pQE30-42K (see Table II) as templates with the primer pair 4/2bis.

**Site-directed Mutagenesis**—Site-directed mutagenesis was carried out by using either the Transformer site-directed mutagenesis kit from the same enzymes. By using the different primers F1L4, F2L3, F3L2, F4L1, and F5 (see Table I) in combination with primer 4, PCR amplification using pQE30-41Y569F-L6 as a template was carried out. The five different DNA fragments synthesized were restricted by *Bam*HI and *Hind*III and ligated into pQE30 vector opened with the same enzymes. The resulting plasmids were termed pQE30-41Y569F-L6, pQE30-41Y569F-L1, pQE30-41Y569F-L11, pQE30-41Y569F-L12, and pQE30-41Y569F-L1356 (see Table II). To generate a plasmid overproducing His<sub>6</sub>-Wzc-(Ser<sup>447</sup>-Lys<sup>279</sup>) with only tyrosine 705 conserved, a PCR amplification was carried out using pQE30-41Y569F as a template and the primer pair 4/155 as template (see Table II). The DNA synthesized was restricted by *Bam*HI and *Hind*III and ligated into pQE30 vector opened with the same enzymes. The resulting plasmids were termed pQE30-41Y569F-L5. GST overexpression was monitored by densitometry using a LI-COR Personal DensiTrak System, with wild type-GST-Wzc as a control (36). Fractions containing purified His<sub>6</sub>-tagged proteins were pooled and dialyzed 2×2 h at 4 °C against a lysis buffer A (50 mM sodium phosphate, pH 7.4, 150 mM NaCl, 100 mM imidazole, 10% glycerol, 5 mM β-mercaptoethanol, and 1 mM RNaseA at a final concentration of 5 μg/ml each) and stored at −20 °C in the same buffer adjusted to 20% glycerol.

**Protein-tyrosine Phosphorylation in E. coli**

The resulting supernatant was centrifuged at 3000 × g for 10 min, washed in 10 ml of buffer A (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 10 mM β-mercaptoethanol), and centrifuged again in the same conditions. The cell pellet was resuspended in buffer B containing deoxyribonuclease I (DNase I) and ribonuclease A (RNase A) at a final concentration of 5 μg/ml each. Cells were disrupted in a French pressure cell at 16,000 lb/in<sup>2</sup> (p.s.i.). The resulting supernatant was centrifuged at 4 °C for 30 min at 30,000 × g. The supernatant was added to Ni<sup>2+</sup>-NTA-agarose matrix and batch binding was allowed to proceed for 1 h at 4 °C under gentle shaking. The lysate/Ni<sup>2+</sup>−NTA-agarose mixture was loaded on a column and was washed with buffer B (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 100 mM imidazole, 10% glycerol, 10 mM β-mercaptoethanol), and eluted fractions were analyzed by SDS-PAGE (36). Fractions containing purified His<sub>6</sub>-tagged proteins were pooled and dialyzed 2×2 h at 4 °C against a lysis buffer A (50 mM sodium phosphate, pH 7.4, 150 mMNaCl, 10% glycerol, 5 mM MgCl<sub>2</sub>, 3 mM dithiothreitol) and stored at −20 °C in the same buffer adjusted to 20% glycerol.

**Overproduction and Purification of His<sub>6</sub> Tag Fusion Wzc N-terminal Fragment—** *E. coli* BL21 (DE3) cells were transformed with pQE30 vector derivatives pQE30-NTwzc1275 and pQE30-NTwzc1356 (see Table II), expressing *Wzc* N-terminal fragment His<sub>6</sub>-Wzc-(Thr<sup>2</sup>-Gly<sup>425</sup>) and His<sub>6</sub>-Wzc-(Thr<sup>2</sup>-Gly<sup>452</sup>), respectively. Cells from these strains were subjected to 100 ml of BL medium supplemented with ampicillin and tetracycline, and was incubated at 37 °C under shaking until the A<sub>600</sub> reached 0.5. IPTG was then added at a final concentration of 0.5 mM, and growth was continued for 3 h at 37 °C under shaking. Cells were harvested by centrifugation at 3000 × g for 10 min, washed in 10 ml of buffer A (50 mM sodium phosphate, pH 8.0, 300 mM NaCl, 10 mM imidazole, 10% glycerol, 10 mM β-mercaptoethanol). The supernatant was added to Ni<sup>2+</sup>-NTA-agarose matrix and batch binding was allowed to proceed for 1 h at 4 °C under gentle shaking. The lysate/Ni<sup>2+</sup>−NTA-agarose mixture was loaded on a column and was washed with buffer B (50 mM sodium phosphate, pH 7.4, 150 mM NaCl, 10% glycerol, 5 mM MgCl<sub>2</sub>, 3 mM dithiothreitol) and stored at −20 °C in the same buffer adjusted to 20% glycerol.
Phosphorylation of Wzc Occurs Specifically in the C-terminal Domain—Wzc is a 79344-Da protein of 720 amino acids bound to the inner membrane of *E. coli* K12 (9, 10). To get more information on its topography within the cell, we analyzed the hydrophobic profile of its amino acid sequence according to the predictive method of Kyte and Doolittle (37). Two main domains in the protein (Fig. 1) could thus be detected: on the one hand, in the N-terminal part, a domain containing two transmembrane α-helices, termed TM1 and TM2, which include amino acids Trp32 to Ala52 and Leu426 to Leu445, respectively, and appear to flank the periplasmic region of the protein; on the other hand, a C-terminal domain extending from Ser447 to Lys720, which seems to correspond to the cytoplasmic region of the protein. The latter domain harbors in particular a Walker A ATP-binding motif, from Gly533 to Val543 and a Walker B motif, from Asp637 to Asp642 (9). Such predicted organization of Wzc into two domains is in agreement with the previous data obtained with protein ExoP from *S. meliloti* showing that this Wzc homologue contains two transmembrane α-helices and a large C-terminal cytoplasmic domain (19). Concerning the distribution of the 18 tyrosine residues contained in total in Wzc, 7 of them are present in the N-domain (from Met7 to Arg446), and the other 11 are in the C-domain, namely in the form of a cluster of 6 residues, at the C-terminal end, between Ala704 and Lys720.

From these observations, it seemed interesting to determine in what region(s) of the molecule the phosphorylation reaction would occur when Wzc undergoes autophosphorylation. For this, the C-terminal fragment of the protein, from Ser447 to Lys720 (approximate molecular mass of 31 kDa), was overproduced by using pQE30 vectors. It was expressed with a His$_6$-tag, purified to homogeneity, incubated with $\gamma$-32P-ATP, and analyzed by SDS-PAGE and autoradiography. Fig. 2 (lane 1) shows that this His$_6$-Wzc-(Ser$^{447}$-Lys$^{720}$) fragment was significantly labeled *in vitro*, which indicates that it contains both an intrinsic protein kinase activity and specific phosphorylation sites.

A similar experiment was performed in the case of the N-terminal domain. Two types of pQE30 derivative vectors were constructed to express either the polypeptide fragment from Thr$^2$ to Gly$^{125}$ containing the N-domain with the transmembrane α-helix TM1, or the fragment from Thr$^2$ to Gly$^{452}$ containing both TM1 and TM2 α-helices. The idea was to check the possible effect of the second α-helix on the phosphorylation of the N-domain. These two fragments, obtained with a His$_6$-tag, respectively, His$_6$-Wzc-(Thr$^2$-Gly$^{452}$) and His$_6$-Wzc-(Thr$^2$-Gly$^{452}$), were purified and incubated separately in the presence of radioautopic ATP, then analyzed by SDS-PAGE and autoradiography.

### RESULTS

**Phosphorylation of Wzc Occurs Specifically in the C-terminal Domain**—Wzc is a 79344-Da protein of 720 amino acids bound to the inner membrane of *E. coli* K12 (9, 10). To get more information on its topography within the cell, we analyzed the hydrophobic profile of its amino acid sequence according to the predictive method of Kyte and Doolittle (37). Two main domains in the protein (Fig. 1) could thus be detected: on the one hand, in the N-terminal part, a domain containing two transmembrane α-helices, termed TM1 and TM2, which include amino acids Trp32 to Ala52 and Leu426 to Leu445, respectively, and appear to flank the periplasmic region of the protein; on the other hand, a C-terminal domain extending from Ser447 to Lys720, which seems to correspond to the cytoplasmic region of the protein. The latter domain harbors in particular a Walker A ATP-binding motif, from Gly533 to Val543 and a Walker B motif, from Asp637 to Asp642 (9). Such predicted organization of Wzc into two domains is in agreement with the previous data obtained with protein ExoP from *S. meliloti* showing that this Wzc homologue contains two transmembrane α-helices and a large C-terminal cytoplasmic domain (19). Concerning the distribution of the 18 tyrosine residues contained in total in Wzc, 7 of them are present in the N-domain (from Met7 to Arg446), and the other 11 are in the C-domain, namely in the form of a cluster of 6 residues, at the C-terminal end, between Ala704 and Lys720.

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The comparative analysis of various proteins known to autophosphorylate on tyrosine shows that they share a number of common structural features, namely a series of tyrosine residues, termed “tyrosine cluster,” located in their C-terminal end (Fig. 3). From observations based on substitution or deletion experiments, a few reports have previously indicated that this cluster would be the target of the phosphorylation reaction in Gram-negative (10) as well as in Gram-positive (18) bacteria, even though the nature and number of phosphorylation sites were not characterized. We re-examined this concept by measuring phosphorylation in a C-terminal fragment of Wzc deleted from its tyrosine cluster. By using a pQE30 derivative vector, a His-tagged polypeptide missing the 6 terminal tyrosine residues, Tyr705 to Tyr710 (Fig. 1), was synthesized and purified to homogeneity. This His6-Wzc-(Ser447-Ala704) construct was incubated with [γ-32P]ATP and analyzed by SDS-PAGE and autoradiography as already described. As shown in Fig. 4 (lane 1), a significant amount of radioactivity was then incorporated in a 30-kDa molecule corresponding to His6-Wzc-(Ser447-Ala704), thus indicating that some phosphorylation occurred in the C-domain of Wzc outside the terminal tyrosine cluster. Upstream of the 6-residue tyrosine cluster, the C-domain of Wzc contains 5 different tyrosine residues, respectively at positions 467, 491, 569, 636, and 668 (Fig. 1). The next question was therefore to determine which tyrosine(s) among these five could be phosphorylated and account for the radioactive labeling of His6-Wzc-(Ser447-Ala704). To answer, site-directed mutagenesis experiments were carried out by using the pQE30–42 vector (Table II) as a template. Each of the 5 relevant tyrosines was substituted individually for phenylalanine in His6-Wzc-(Ser447-Ala704) to generate five different vectors: pQE30–42Y467F, pQE30–42Y491F, pQE30–42Y636F, pQE30–42Y668F, and pQE30–42Y698F (Table II). Each mutant polypeptide was overproduced, purified to homogeneity, incubated with radioactive ATP, and analyzed by gel electrophoresis and autoradiography. In all cases, except one, the mutant fragment appeared to keep incorporating radioactivity to the same extent as the non-mutant control, showing that the 4 corresponding tyrosine residues (467, 491, 636, and 668) are not involved in the phosphorylation reaction (Fig. 4). The only exception concerned the His6-Wzc-(Ser447-Ala704) polypeptide substituted for phenylalanine at position 569 (Fig. 4, lane 4). This result demonstrated, for the first time, that a tyrosine residue located outside the C-terminal tyrosine cluster in a phosphorylatable protein represents an active phosphorylation site for endogenous protein-tyrosine kinase activity.

**The C-terminal Tyrosine Cluster of Wzc Harbors Five Phosphorylation Sites**—Experiments were undertaken to determine which tyrosines are phosphorylated in the C-terminal tyrosine cluster of Wzc. Because tyrosine 569 was previously identified as a phosphorylation site, site-directed mutagenesis was carried out with pQE30–41 (Table II) as a template to generate the pQE30–41Y569F vector expressing His6-Wzc-(Ser447-Lys720) with the substitution Y569F. This procedure was chosen with the aim of eliminating the phosphorylation background due to Tyr569 so as to allow specific analysis of the phosphorylation sites of the tyrosine cluster. First, we constructed a vector encoding a phosphorylation switch-off form of Wzc-(Ser447-Lys720), i.e. with all six tyrosines Tyr705, Tyr708, Tyr710, Tyr711, Tyr713, and Tyr715 substituted to phenylalanine in addition to the Y569F substitution. This vector was obtained by PCR mutagenesis and termed pQE30–41Y569F-L6 (Table II). Then,
site-directed mutagenesis was performed to reverse, one by one, the Y3F substitutions in the tyrosine cluster, and to restore in each case one of the six different tyrosine residues. By doing so, six different vectors expressing each His6-Wzc-(Ser447-Lys720)-Y569F with only one phosphorylatable tyrosine remaining in the C-terminal cluster were obtained (Table II). The corresponding mutant forms of His6-Wzc-(Ser447-Lys720) were overproduced, purified to homogeneity, and assayed individually for radioactive ATP incorporation. The non-mutated form His6-Wzc-(Ser447-Lys720) containing Tyr569 and the 6 tyrosine residues of the cluster, the mutated form His6-Wzc-(Ser447-Lys720)-Y569F containing the 6 terminal tyrosines, and the substituted form His6-Wzc-(Ser447-Lys720)-Y668F-L6 with no tyrosine residue were analyzed in parallel. Extensive labeling was observed when the tyrosine cluster was present (lane 2), especially when Tyr569 was not mutated (lane 1). By contrast, no phosphorylation occurred in His6-Wzc-(Ser447-Lys720)-Y569-L6 (lane 3), which provided evidence that, besides Tyr569, the phosphorylation sites for the protein-tyrosine kinase activity of Wzc are all located in the C-terminal tyrosine cluster. The analysis of the six different mutant peptides containing each only one terminal restored tyrosine (Fig. 5, lanes 4–9) showed, in addition, that they all were able to undergo phosphorylation except the mutant containing tyrosine 705 (lane 4). This finding indicated that, in the C-terminal cluster of Wzc, the 5 tyrosine residues at positions 708, 710, 711, 713, and 715 are phosphorylation sites, whereas tyrosine at position 705 is not.

Wzc Is Subject to Both Intra- and Interphosphorylation—Because the phosphorylation sites of Wzc appeared to be located in two distinct parts of the Wzc molecule, one at Tyr569 and the others in the 5 neighboring tyrosines of the C-terminal end, an attempt was made to assess the existence of a functional relationship between these sites during the phosphorylation process. This possibility was supported primarily by the fact that the extent of phosphorylation of a complete C-terminal fragment His6-Wzc-(Ser447-Lys720) (Fig. 2, lane 1) is about 700-fold higher than the phosphorylation signal produced by a fragment like His6-Wzc-(Ser447-Ala704)-Y569F-L6 (lane 3), which provided evidence that, besides Tyr569, the phosphorylation sites for the protein-tyrosine kinase activity of Wzc are all located in the C-terminal tyrosine cluster. The analysis of the six different mutant peptides containing each only one terminal restored tyrosine (Fig. 5, lanes 4–9) showed, in addition, that they all were able to undergo phosphorylation except the mutant containing tyrosine 705 (lane 4). This finding indicated that, in the C-terminal cluster of Wzc, the 5 tyrosine residues at positions 708, 710, 711, 713, and 715 are phosphorylation sites, whereas tyrosine at position 705 is not.

Protein-tyrosine Phosphorylation in E. coli

**Fig. 3.** Comparative analysis of the predicted cytoplasmic C-terminal domains of *E. coli* K12 Wzc homologues. Alignment of *S. pneumoniae* Cps19F, *S. aureus* Cap5B, *B. subtilis* YwqD, *E. coli* K30 Wzc, *E. coli* K12 Wzc, *E. amylovora* AmsA, *K. pneumoniae* Yco6, *A. johnsonii* Ptk, *R. solanacearum* EpsB, and *S. meliloti* ExoP, was performed by using the program ClustalW (34). Dashes indicate gaps introduced in the alignment process. Tyrosine residues and Walker A ATP-binding motif are boxed and indicated by asterisks in Wzc. GenBank accession numbers are U09239, U81973, Z92952, AF104912, U38473, X77921, D21242, P38134, Y15162, U17898, and L20758, respectively.

**Fig. 4.** Characterization of the phosphorylation sites located outside the C-terminal tyrosine cluster of Wzc. The C-terminal domain of *E. coli* K12 Wzc, deleted from the tyrosine cluster, His6-Wzc-(Ser447-Ala704), was overproduced, purified on Ni2+-immobilized matrix, and assayed for in vitro phosphorylation with [γ-32P]ATP (lane 1). Five site-directed mutagenesis experiments were carried out to generate a single-tyrosine substitution to phenylalanine in positions Y467F, Y491F, Y569F, Y636F, and Y668F, respectively, on His6-Wzc-(Ser447-Ala704). The mutated Wzc C-terminal domains, His6-Wzc-(Ser447-Ala704)-Y467F (lane 2), His6-Wzc-(Ser447-Ala704)-Y491F (lane 3), His6-Wzc-(Ser447-Ala704)-Y569F (lane 4), His6-Wzc-(Ser447-Ala704)-Y636F (lane 5), and His6-Wzc-(Ser447-Ala704)-Y668F (lane 6) were overproduced, purified, incubated with [γ-32P]ATP, and then analyzed by SDS-PAGE and autoradiography.
TABLE II  
Plasmids used in this study

| Plasmid            | Description                                                                 | Reference or source |
|--------------------|-----------------------------------------------------------------------------|---------------------|
| pREP4-groESL       | Vector expressing the two chaperone proteins GroEL and GroEL                | (29)                |
| pQE30              | Expression vector generating GST fusion proteins, AmpR                       | Purchased from Qiagen|
| pGEX-KT            | Expression vector generating GST fusion proteins, AmpR                       | This study          |
| pQE30-41K          | Encoding Wzc cytoplasmic fragment from Ser447 to Lys720, His₆ Wzc-(Ser447-Lys705), cloned in BamHI/HindIII sites, AmpR   | This study          |
| pQE30-42K          | Same as pQE30-41K, but mutated on K540M                                      | This study          |
| pQE30-42Y467F      | Same as pQE30-42, but mutated on Y467F                                       | This study          |
| pQE30-42Y491F      | Same as pQE30-42, but mutated on Y491F                                       | This study          |
| pQE30-42Y569F      | Same as pQE30-42, but mutated on Y569F                                       | This study          |
| pQE30-42Y636F      | Same as pQE30-42, but mutated on Y636F                                       | This study          |
| pQE30-42Y668F      | Same as pQE30-42, but mutated on Y668F                                       | This study          |
| pQE30-42Y705F      | Same as pQE30-42, but mutated on Y705F                                       | This study          |
| pQE30-42Y708F      | Same as pQE30-42, but mutated on Y708F                                       | This study          |
| pQE30-42Y710F      | Same as pQE30-42, but mutated on Y710F                                       | This study          |
| pQE30-42Y711F      | Same as pQE30-42, but mutated on Y711F                                       | This study          |
| pQE30-42Y713F      | Same as pQE30-42, but mutated on Y713F                                       | This study          |
| pQE30-42Y715F      | Same as pQE30-42, but mutated on Y715F                                       | This study          |
| pQE30-42Y705F-F5   | Same as pQE30-42Y705F, but mutated on Y569F                                   | This study          |
| pQE30-42Y706F-L6   | Same as pQE30-42Y706F, but mutated on Y705F, Y708F, Y710F, Y711F, Y713F, and Y715F | This study          |
| pQE30-42Y705F-L5   | Same as pQE30-42Y705F, but mutated on Y705F, Y708F, Y710F, Y711F, and Y715F | This study          |
| pQE30-42Y705F-F1L4 | Same as pQE30-42Y705F, but mutated on Y705F, Y708F, Y710F, Y711F, Y713F, and Y715F | This study          |
| pQE30-42Y705F-F2L3 | Same as pQE30-42Y705F, but mutated on Y705F, Y708F, Y710F, Y711F, Y713F, and Y715F | This study          |
| pQE30-42Y705F-F3L2 | Same as pQE30-42Y705F, but mutated on Y705F, Y708F, Y710F, Y711F, Y713F, and Y715F | This study          |
| pQE30-42Y705F-F4L1 | Same as pQE30-42Y705F, but mutated on Y705F, Y708F, Y710F, Y711F, and Y715F | This study          |
| pQE30-42Y705F-F5   | Same as pQE30-42Y705F, but mutated on Y705F, Y708F, Y710F, Y711F, and Y715F | This study          |
| pQE30-NTwzc1275    | Encoding Wzc N-terminal part from Thr15 to Gly425, His₆ Wzc-(Thr₁⁵) Gly₄²⁵, cloned in BamHI/Aec651 sites, AmpR | This study          |
| pQE30-NTwzc1356    | Encoding Wzc N-terminal part from Thr15 to Gly425, His₆ Wzc-(Thr₁⁵) Arg₄²⁵, cloned in BamHI/Aec651 sites, AmpR | This study          |
| pGEX-KT-41         | Encoding Wzc cytoplasmic fragment from Ser447 to Lys720, GSTWzc-(Ser447-Lys720), cloned in BamHI/EcoRI sites, AmpR | This study          |
| pGEX-KT-41K        | Same as pGEX-KT-41, but mutated on K540M                                      | This study          |
| pGEX-KT-42         | Encoding Wzc cytoplasmic fragment from Ser447 to Arg701, GSTWzc-(Ser447-Lys701), cloned in BamHI/EcoRI sites, AmpR | This study          |
| pGEX-KT-42K        | Same as pGEX-KT-42, but mutated on K540M                                      | This study          |

FIG. 5. Characterization of the phosphorylation sites in the Wzc C-terminal tyrosine cluster. Detection of phosphorylation sites located in the tyrosine cluster of the Wzc C-terminal domain was carried out by site-directed mutagenesis experiments so as to generate, first, Y569F substitution to obtain the Wzc fragment His₆-Wzc-(Ser⁴⁴⁷-Lys⁷₂⁰)-Y569F (lane 2). Then, the DNA fragment encoding this peptide was used as a template to substitute all six tyrosines to phenylalanine, i.e., Y705F, Y708F, Y701F, Y711F, Y713F, and Y715F; yielding His₆-Wzc-(Ser⁴⁴⁷-Lys⁷₂⁰)-Y569F-L6 (lane 3). Finally, tyrosine residues were restored, one by one, to generate a series of peptides with either Tyr⁷₀⁸, Tyr⁷₁₀, Tyr⁷₁₁, or Tyr⁷₁₅, termed, respectively His₆-Wzc-(Ser⁴⁴⁷-Lys⁷₂⁰)-Y569F-L5 (lane 4), His₆-Wzc-(Ser⁴⁷⁴-Lys⁷₂⁰)-Y569F-F1L4 (lane 5), His₆-Wzc-(Ser⁴⁴⁷-Lys⁷₂⁰)-Y569F-P₂L₃ (lane 6), His₆-Wzc-(Ser⁴⁴⁷-Lys⁷₂⁰)-Y569F-P₃L₂ (lane 7), His₆-Wzc-(Ser⁴⁴⁷-Lys⁷₂⁰)-Y569F-F₄L₁ (lane 8), and His₆-Wzc-(Ser⁴⁴⁷-Lys⁷₂⁰)-Y569F-F₅ (lane 9). The different mutated fragments were overproduced, purified, and assayed individually for in vitro phosphorylation with [γ³²P]ATP. His₆-Wzc-(Ser⁴⁴⁷-Lys⁷₂⁰) was run in parallel as a control (lane 1).

respectively, 380,740 and 560 cpm. Similarly, when comparing directly the extent of phosphorylation of the complete C-terminal fragment His₆-Wzc-(Ser⁴⁴⁷-Lys⁷₂⁰) (Fig. 2, lane 1) with that of fragment His₆-Wzc-(Ser⁴⁴⁷-Lys⁷₂⁰)-Y569F containing the tyrosine cluster but missing Tyr⁵₆⁹ (Fig. 5, lane 2), a

45-fold difference was emerging, as confirmed by the average scintillation counting of the corresponding bands: 380,740 cpm in the former fragment and 8580 cpm in the latter. Together, these data suggested the existence of a cooperative effect between the phosphorylation sites of Wzc, the tyrosine at position 569 and the 5 tyrosines of the cluster, leading to a substantial increase of the overall degree of phosphorylation of the C-terminal fragment. More precisely, at this point, it seemed that the presence of Tyr⁵₆⁹ would enhance the phosphorylation of the tyrosine cluster.

To check this hypothesis, we investigated in more detail the process of phosphorylation. First, we produced a class of C-fragments unable to sustain phosphorylation. For this, the lysine residue located at position 540 in the Walker A motif essential for ATP binding (9, 10) was substituted to methionine so as to yield Wzc-(Ser⁴⁴⁷-Lys⁷₂⁰)-K540M or Wzc-(Ser⁴⁴⁷-Ala⁷₀⁴)K540M, depending on the plasmid used for site-directed mutagenesis (Table II). In addition, to mark out these peptides, the corresponding DNA fragments were cloned in the pGEX-KT vector (Table II) to obtain GST-fused peptides, GST-Wzc-(Ser⁴⁴⁷-Lys⁷₂⁰)-K540M and GST-Wzc-(Ser⁴⁴⁷-Ala⁷₀⁴)-K540M. A similar population of peptides, also fused to GST but containing a non-mutated K540, was also prepared for control assays: GST-Wzc-(Ser⁴⁴⁷-Lys⁷₂⁰) and GST-Wzc-(Ser⁴⁴⁷-Ala⁷₀⁴).

The various GST peptides could thus be distinguished from His₆-tagged peptides on the basis of a larger molecular mass. The various GST peptides could thus be distinguished from His₆-tagged peptides on the basis of a larger molecular mass. Moreover, in view of testing the possible effect of the nature of the tag on the phosphorylation reaction, a symmetrical class of...
peptides bearing a mutated Lys and fused with 6 histidine residues was also constructed by using pQE30-41 and pQE30-42 as templates (Table II): His6-Wzc-(Ser447-Lys720) was also constructed by using pQE30-41 and was incubated with either active His6-Wzc-(Ser447-Lys720) or His6-Wzc-(Ser447-Ala704)-Y569F (lane 7). When the same two peptides were mutated at Lys540, no phosphorylation occurred, which confirmed the crucial role played by this lysine residue in the kinase activity of Wzc (lanes 3 and 4).

When inactive GST-Wzc-(Ser447-Ala704)-K540M was incubated with either active His6-Wzc-(Ser447-Lys720) or active His6-Wzc-(Ser447-Ala704), no phosphorylation was detected at the level of the GST-fused peptide (lanes 5 and 6), whereas the active 6 His-tagged peptides were phosphorylated. The same result was obtained when, conversely, inactive His6-Wzc-(Ser447-Ala704)-K540M was incubated with either active GST-Wzc-(Ser447-Lys720) or active GST-Wzc-(Ser447-Ala704) (data not shown). This means that, in both situations, whatever the nature of the tag fused, no transfer of the radioactive moiety present on a tagged C-fragment, with or without tyrosine cluster, to the phosphorylation site Tyr569 of another wzc-(Ser447-Ala704) peptide, inefficient in phosphorylating activity (Fig. 7).

In contrast, when inactive GST-Wzc-(Ser447-Lys720)-K540M was incubated with either active His6-Wzc-(Ser447-Lys720) or active His6-Wzc-(Ser447-Ala704), the GST-fused peptide was effectively labeled (lanes 7 and 8), like the 6 His-tagged peptides. An identical result was obtained when inactive His6-Wzc-(Ser447-Lys720)-K540M was incubated with either active GST-Wzc-(Ser447-Lys720) or active GST-Wzc-(Ser447-Ala704) (data not shown). It can be concluded that, in these conditions, the phosphorylated residues belong to the tyrosine cluster and accept radioactivity through an intermolecular transfer of phosphoryl groups arising from ATP hydrolysis and catalyzed by another copy of the C-fragment of Wzc. In other words, tyrosine 569 would be specifically phosphorylated in an intramolecular phosphorylating process, whereas the tyrosine cluster would be phosphorylated in an intermolecular reaction between two distinct molecules of Wzc.

Finally, to test further the possible stimulatory effect of Tyr569 on the phosphorylation of the tyrosine cluster, the GST-Wzc-(Ser447-Lys720)-K540M peptide, inefficient in phosphorylation, was incubated with either His6-Wzc-(Ser447-Lys720)-Y569F or His6-Wzc-(Ser447-Ala704)-Y569F, which both lack a phosphorylatable native Tyr569 residue. The phosphorylation of GST-Wzc-(Ser447-Lys720)-K540M was drastically diminished (lanes 9 and 10) compared with the assays with peptides containing an active Tyr569, suggesting that Wzc needs to autophosphorylate before acquiring its maximal interphosphorylating activity (Fig. 7).

**DISCUSSION**

Protein Wzc is an inner-membrane protein from *E. coli* K12 able to undergo autophosphorylation on tyrosine residues and required for the production of a particular exopolysaccharide, colanic acid (14). In this study, we determined, for the first time, the precise number and location of the different phosphorylation sites within the protein molecule. Then we newly showed that phosphorylation of Wzc proceeds through a cooperative two-step mechanism that involves both intramolecular and intermolecular phosphorylation.

Based on the hydropathic profile of its amino acid sequence, it was predicted that Wzc contained two separable domains: an N-terminal domain, with two transmembrane α-helices, and a C-terminal cytoplasmic domain, harboring Walker A and B ATP-binding motifs. This type of molecule topology was previously demonstrated in the case of protein ExoP, a Wzc homologue in *S. meliloti*, and the C-terminal domain alone was shown to autophosphorylate in *vitro* (12). Our data confirmed this finding, because we observed that the C-terminal fragment of Wzc, Wzc-(Ser447-Lys720), exhibits an intrinsic protein-tyrosine kinase activity. Discrepant results were, however, described for another class of *E. coli* strain, K30, and for the Gram-positive bacterium *S. pneumoniae*. Indeed, it was reported (10) that a C-terminal fragment of Wzc from *E. coli* K30 is unable to autophosphorylate, per se but needs the presence of the N-terminal part of the protein to be phosphorylated. Similarly, it was found that in *S. pneumoniae* the phosphorylation at the tyrosine of protein CpsD requires the presence of another.
protein, CpsC (18, 22). Interestingly, proteins CpsC and CpsD share common structural features with the N-terminal and C-terminal regions of Wzc, respectively (14). In fact, this dual sequence similarity is encountered in a variety of pairs of separate proteins in Gram-positive cells, including CpsC/CpsD from *S. agalactiae* (24), CapA/CapB from *S. aureus* (26), EpsC/EpsD from *Streptococcus thermophilus* (38), and EpsD/EpsA from *Lactococcus lactis* (39). By contrast, the presence of the N-terminal fragment of protein Wzc from *E. coli* K12 does not influence the phosphorylation of the C-terminal fragment. Conversely, this N-terminal fragment is not phosphorylated by the C-fragment. Thus, the N-terminal region of Wzc does not participate *per se* in the phosphorylation reaction, neither as a modulator, nor as a substrate. Therefore, the mechanism responsible for protein tyrosine phosphorylation in the Gram-negative bacterium *E. coli* K12 is different from that in Gram-positive bacteria in terms of nature and number of protein components required for this reaction to occur.

From a series of experiments based on site-directed mutagenesis and specific phosphorylation assays *in vitro*, we identified the different sites of phosphorylation on Wzc. Previous work suggested that Wzc, as well as certain homologues, was phosphorylated on multiple tyrosine residues (14, 10), namely in the tyrosine cluster found in the extreme C-region of several proteins from various bacteria (Fig. 3). The involvement of this cluster in the phosphorylation process was suggested, namely for protein Wzc from *E. coli* K30 (10), protein ExoP from *S. meliloti* (12), and protein CpsD from *S. pneumoniae* (18, 22). However, in these studies, the precise number of phosphorylation sites and their location along the protein molecule were not determined. Some estimates were made, in particular on the basis of homology with consensus autophosphorylation motifs present in eukaryotic kinases, but no comprehensive analysis was performed (8, 12). We have now demonstrated that Wzc harbors six distinct phosphorylation sites. Of interest is the finding that these sites are not all located in the C-terminal tyrosine cluster of the molecule. Indeed, one of them, tyrosine 569, is present rather far upstream of this cluster, in the vicinity of the Walker A ATP-binding sequence. No previous report indicated the existence of this phosphorylation site, even though it seems to be present in all Gram-negative bacterial proteins homologous to Wzc that were analyzed so far by comparative sequence alignment (Fig. 3). It would be interesting to assess whether this tyrosine residue represents also a phosphorylation site in these various proteins. A possibly similar site might be a phosphorylated tyrosine residue located elsewhere, at position 505, in protein ExoP (12), but further analysis is required to determine whether it really behaves like Tyr<sup>569</sup> in Wzc. On the other hand, it is worth noticing that, in the C-terminal cluster of Wzc, only 5 tyrosine residues (Tyr<sup>708</sup>, Tyr<sup>710</sup>, Tyr<sup>711</sup>, Tyr<sup>713</sup>, and Tyr<sup>715</sup>) out of the 6 residues present in that part of the molecule can be phosphorylated by the intrinsic protein kinase activity. The most upstream residue, Tyr<sup>705</sup>, is insensitive to modification catalyzed by this activity. In other words, a tyrosine belonging to the C-terminal cluster does not necessarily mean that it will constitute a phosphorylation site. In addition, the number of tyrosine residues in this part of the molecule widely varies from one protein to the other. For instance, the tyrosine cluster of protein YwqD from *Bacillus subtilis* contains only 3 residues, whereas that of protein EpsB from *R. solanacearum* contains up to 8 residues (Fig. 3), all of them being present among the last 20 amino acids of each molecule. In these conditions, it seems difficult to predict merely from the comparison of the amino acid sequences of different phosphorylatable proteins, even if they are homologues, which tyrosine residues will be phosphorylated. In consequence of that, the identification of the phosphorylation sites will necessitate, in all cases, a direct experimental analysis so as to provide a reliable basis for studying at the molecular level the involvement of tyrosine phosphorylation in the regulation of bacterial metabolism. In addition, it would be important to determine whether these different sites are actually phosphorylated *in vivo*. Further work is required to check this point, but our data likely reflect the physiological situation, because other results have previously shown that the C-end tyrosine cluster is phosphorylated *in vivo* both in *E. coli* (10) and *S. pneumoniae* (18).

Still, by using site-directed mutagenesis followed by phosphorylation assays with polypeptide constructs tagged with either glutathione-S-transferase or a sequence of 6 histidines, a number of novel data were obtained concerning the process of phosphorylation of Wzc and, most likely, that of the other autokinases from Gram-negative bacteria. First, when tyrosine 569 is changed to phenylalanine, the overall phosphorylation of Wzc is drastically diminished, namely the phosphorylation of the C-terminal tyrosine cluster. Second, Tyr<sup>569</sup> can be phosphorylated exclusively through an intramolecular reaction catalyzed by the endogenous protein-tyrosine kinase activity of Wzc. On the other hand, the 5 tyrosine residues of the cluster can be modified by an intermolecular phosphorylation of Wzc. Moreover, the intraphosphorylation of Tyr<sup>569</sup> induces an important increase of the protein-tyrosine kinase activity of Wzc, which results in an enhanced phosphorylation of the tyrosine cluster. The latter finding represents the first demonstration that a bacterial protein-tyrosine kinase is activated by autophosphorylation and that such activation is promoted by the phosphorylation of a single tyrosine residue. To characterize further the molecular mechanism of Wzc phosphorylation, it would be interesting to determine whether all 5 tyrosine residues in the C-terminal cluster are phosphorylated simultaneously or sequentially. Also, it would be worth finding the reason for the ample increase, by 700-fold, observed for *in vitro* Wzc phosphorylation as induced by Tyr<sup>569</sup> activation. This increase could imply that phosphorylation of the Tyr<sup>569</sup> site would be basically very low but sufficient to elicit extensive interphosphorylation. Obviously, additional experiments are needed to answer this question. These include kinetic analysis of the Wzc phosphorylation reaction and assessment of the effect of a particular structural organization that Wzc could acquire *in vivo*, such as oligomerization.

Our data indicate that phosphorylation of Wzc proceeds through a cooperative two-step mechanism: First, Wzc is phosphorylated at Tyr<sup>569</sup> in an intraphosphorylation reaction that generates a significant increase of protein kinase activity, then, the activated kinase phosphorylates the 5 tyrosines at the C-terminal end of another molecule of Wzc in an interphosphorylation reaction (Fig. 7). The question left is to characterize the effectors that would trigger the first reaction, *i.e.*, the intraphosphorylation at Tyr<sup>569</sup>. When referring to eukaryotic systems, a large number of protein-tyrosine kinases are known to catalyze autophosphorylation in an intramolecular process generally modulated by regulatory ligands, which allows rapid switching of numerous cellular functions. A similar situation can be envisaged in prokaryotes, namely for Wzc that would behave like a receptor-tyrosine kinase and would regulate a particular metabolic pathway, the production and export of exopolysaccharides. In this scheme, the cascade of successive events would be as follows: the N-terminal part of Wzc anchored in the inner-membrane interacts with an external effector to be characterized; this interaction generates a signal that triggers the phosphorylation of Wzc at Tyr<sup>569</sup> (intraphosphorylation), the resulting activation of the protein kinase ac-
tivity promotes extensive phosphorylation of the tyrosine cluster (interphosphorylation) in the C-terminal region; then the phosphorylation of Wzc affects, directly or indirectly, the production of the exopolysaccharide, colanic acid. Further experiments are now needed to check the plausibility of this hypothesis and to decipher the molecular mechanism that interconnects protein tyrosine phosphorylation and bacterial pathogenicity, viz. the production of capsular and/or extracellular polysaccharides.

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Tyrosine Phosphorylation of Protein Kinase Wzc from *Escherichia coli* K12 Occurs through a Two-step Process

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