Structural organization of the protein-tyrosine autokinase Wzc within Escherichia coli cells

Patricia Doublet, Christophe Grangeasse, Brice Obadia, Elisabeth Vaganay
and Alain J. Cozzone ‡

Institut de Biologie et Chimie des Protéines, Centre National de la Recherche Scientifique, Université de Lyon, France

Running title: E.coli protein-tyrosine autokinase

‡Correspondence to:

Alain J. Cozzone
Institut de Biologie et Chimie des Protéines
7 Passage du Vercors
69367 Lyon cedex 07
France
tel: 00 33 (0)4 72 72 26 72
fax: 00 33 (0)4 72 72 26 01
e.mail: aj.cozzone@ibcp.fr
Summary

Protein Wzc from *Escherichia coli* is member of a newly defined family of protein-tyrosine autokinases which are essential for surface polysaccharide production in both Gram-negative and Gram-positive bacteria. Although the catalytic mechanism of the autophosphorylation of Wzc was recently described, the *in vivo* structural organization of this protein remained unclear. Here, we have determined the membrane topology of Wzc by performing translational fusions of *lacZ* and *phoA* reporter genes to the *wzc* gene. It has been shown that Wzc consists of two main structural domains: a N-terminal domain, bordered by two transmembrane helices, which is located in the periplasm of cells, and a C-terminal domain, harboring all phosphorylation sites of the protein, which is located in the cytoplasm. In addition, it has been demonstrated, for the first time, that Wzc can oligomerize *in vivo* to form essentially trimers and hexamers. Cross-linking experiments performed on strains expressing various domains of Wzc, have shown that the cytoplasmic C-terminal domain is sufficient to generate oligomerization of Wzc. Mutant proteins, modified in either the ATP-binding site or the different phosphorylation sites, *i.e.* rendered unable to undergo autophosphorylation, have appeared to oligomerize into high-molecular-mass species identical to those formed by the wild-type protein. It was concluded that phosphorylation of Wzc is not essential to its oligomerization. These data, connected with the phosphorylation mechanism of Wzc, may be of biological significance in the regulatory role played by this kinase in polysaccharide synthesis.
Introduction

Capsule polysaccharides and exopolysaccharides are considered essential components of the surface of bacteria since they play a critical role in the interaction of cells with their environment and they are directly involved in the virulence of most pathogens (1). It has been shown in a variety of both Gram-negative and Gram-positive bacterial species, that the genes encoding the enzymes responsible for the production and transport of these polysaccharides are usually clustered in large operons that can comprise over twenty different loci. In addition, it has been found that each of these operons includes also a pair of genes which encode two opposing activities, a protein-tyrosine kinase and a phosphotyrosine-protein phosphatase, namely in *Acinetobacter johnsonii* (2), *Escherichia coli* (3), *Klebsiella pneumoniae* (4), *Sinorhizobium meliloti* (5) and *Streptococcus pneumoniae* (6, 7).

Phosphorylation of bacterial proteins on tyrosine has been demonstrated, for the first time, by overproducing and purifying a particular phosphoprotein, termed Ptk, from *A. johnsonii*, and by showing its capacity to autophosphorylate *in vitro* at the expense of ATP (8, 9). Then, other protein-tyrosine kinases, homologous to Ptk, have been characterized in several other species, namely protein Wzc from *E.coli* K-12 (10) and *E.coli* K-30 (11), protein Etk from *E.coli* K-12 (12) and *E.coli* K-30 (13), protein ExoP from *S.meliloti* (14) and protein YCO6 from *K.pneumoniae* (15). Furthermore, the reversibility of protein tyrosine phosphorylation has been evidenced by showing the presence, in the same bacterial species, of phosphotyrosine-protein phosphatases of low molecular mass able to specifically dephosphorylate the cognate protein-tyrosine kinases (10-12, 16), thus suggesting a possible regulatory role for tyrosine phosphorylation.

The occurrence of a biological link between tyrosine phosphorylation and exopolysaccharide / capsular polysaccharide production and, consequently, between tyrosine phosphorylation and bacterial pathogenicity, is supported by a number of recent observations. For instance, phosphorylation of the *E.coli* tyrosine autokinase Wzc is essential for the assembly of group I capsular polysaccharides (11). Conversely, when this protein is phosphorylated, the production of the particular exopolysaccharide, colanic acid, is blocked, but starts again when Wzc is dephosphorylated by its cognate phosphoprotein phosphatase, Wzb (12). In *S.pneumoniae*, phosphorylation of the protein-tyrosine kinase CpsD negatively regulates the biosynthesis of capsular polysaccharides (17), and this control is modulated by the phosphatase activity of protein CpsB (18, 19). Also, in *S.meliloti*, the molecular mass
distribution of the acidic exopolysaccharide, succinoglycan, is directly influenced by the specific phosphorylation of protein ExoP (14).

In none of these cases, however, has the process of tyrosine phosphorylation and its relationship to polysaccharide production been described at the molecular level. The only exception concerns the mechanism of autophosphorylation of protein Wzc from *E.coli* K-12 (20). This tyrosine kinase of 720 amino acids, bound to the inner membrane of cells, consists of two functional domains: a C-terminal domain which contains the tyrosine kinase activity and can undergo autophosphorylation, and a N-terminal domain which is not phosphorylated and does not influence the phosphorylation of the C-domain. Tyrosine phosphorylation occurs at the expense of ATP molecules that bind to Walker A and B motifs of the C-domain. This domain contains six different sites of phosphorylation among which five sites are located at the extreme C-end of the molecule in the form of a tyrosine cluster, and one site is located upstream, at Y569. It has been shown that Y569 can first autophosphorylate through an intramolecular reaction which induces an enhanced kinase activity of Wzc resulting, in turn, in the phosphorylation of the five terminal tyrosines by an intermolecular process. This cooperative two-step mechanism may be part of the cascade of reactions involved in signal transduction in bacteria (20).

In search of the mode of functioning of protein Wzc, it seemed of interest to analyze its structural organization within the cell and to determine whether the two functional domains previously characterized *in vitro* could be connected with particular structural domains *in vivo*. For this, we have examined the membrane topology of Wzc by performing a series of experiments based on translational fusions of *lacZ* and *phoA* reporter genes to the *wzc* gene. The corresponding results have been compared to the predictive data deduced from the theoretical hydropathic profile of the protein. In addition, we have tested the possibility that Wzc could oligomerize *in vivo* and we have assessed the effect of phosphorylation on oligomerization.

**Experimental procedures**

**Bacterial strains and plasmids**

The strains and plasmids used in this study are listed in Table 1. *E.coli* JM109 strain was used as template for PCR amplification of different DNA fragments of the *wzc* gene.
*E. coli* DH5α was used to test wzc::lacZ fusions. The *phoA* deletion strain CC118, in which the wzc::phoA fusions were expressed, was kindly provided by J.T. Beatty (25). *E. coli* JM83(pUC18-rcsA<sup>+</sup>), JM83 wzc::Km<sup>R</sup> (pUC18-rcsA<sup>+</sup>) and JM83 wzc::Km<sup>R</sup> strains, used in the oligomerization study, were previously described (12). Strain BL21(pREP4-groESL), in which overproduction of the C-domain of Wzc was obtained, was previously reported (24). Plasmids pSP72-lacZ and pUC19-phoA were obtained from J.T. Beatty and W.H. Bingle, respectively (25, 26).

**Growth conditions**

*E. coli* strains were grown in LB or 2YT medium at 37°C. Minimal medium M9 supplemented with 0.5mM IPTG was used for β-galactosidase assays and radioactive labeling of protein Wza. Antibiotics were added at the following concentrations: 50 µg/ml ampicillin, 25 µg/ml kanamycin, 15 µg/ml tetracyclin. Chromogenic substrates 5-bromo-4-chloro-3-indolyl phosphate (X-P) and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) were used at the concentration of 40 µg/ml each in Luria plate.

**DNA manipulation**

Plasmids were purified by using a Qiaprep Purification Kit (Qiagen). All restriction enzymes, calf intestine phosphatase, T4 DNA ligase, Klenow fragment and Taq DNA polymerase, were used as recommended by the manufacturer (Promega). Transformation of *E. coli* cells was performed as previously reported (27).

**Construction of wzc::phoA and wzc::lacZ translational fusions**

To construct translational fusions of alkaline phosphatase (PhoA) at various points within Wzc, PCR products were prepared by using a forward primer for 5' wzc gene (Pho1), paired with 9 different downstream reverse primers (Pho2 to Pho10) (Table 2). Restriction sites for *BamHI* and *KpnI* enzymes were included in Pho1 and Pho2-10 primers, respectively. Genomic DNA from *E. coli* JM109 strain was used as template. The amplified fragments were restricted by *BamHI* and *KpnI*, and inserted into the pUC19-phoA vector opened with the same enzymes. The resulting plasmids were used to transform the *E. coli* strain CC118.

To obtain in-frame fusions of lacZ gene to wzc, DNA fragments encoding various Wzc polypeptides were synthesized by PCR amplification, using genomic DNA from JM109 as template and the following primers: a forward primer complementary to the 5' end of wzc
(Lac1) and 8 different reverse primers complementary to different sequences at the 3’ end of the wzc gene (Lac2 to Lac9) (Table 2). Restriction sites, namely a KpnI site in Lac1 primer and a BamHI site in Lac2-9 oligonucleotides, were included. The PCR products were digested by BamHI and KpnI, and cloned into the pSP72-lacZ vector restricted with the same enzymes. The wzc::lacZ fusions were then subcloned into plasmid pUC18-ΔlacZ. This vector was first obtained by deleting the lacZ’ gene of pUC18 with NdeI and HindIII restriction enzymes, and treating with Klenow fragment to fill in the 3’-recessed ends. The wzc::lacZ fusions were then excised from pSP72 with KpnI and HindIII, and subcloned into the polylinker of the resulting pUC18-ΔlacZ vector, opened with the same enzymes. The plasmids thus obtained were used to transform the DH5α strain of E.coli.

**Construction of plasmids expressing wild or mutated Wzc protein**

Various DNA fragments encoding the entire Wzc protein or its N- and C-terminal domains were obtained by PCR amplification, using JM109 E.coli DNA as template and 6 different oligonucleotides (Table 2, oligos 1 to 6). A restriction site for EcoRI, a start codon ATG, and a ribosome-binding site were included in forward primers. A site for KpnI enzyme was added in reverse primers. The PCR products were digested by EcoRI and KpnI enzymes, and cloned into pUC18-rcsA+ opened with the same enzymes. The resulting plasmid pUC18-wzc1-2163-rcsA+ encoded the entire Wzc protein. Plasmid pUC18-wzc1-1356-rcsA+ expressed the N-terminal domain of Wzc (from codon 1 to codon 452) including the two transmembrane helices, TM1 and TM2. Plasmid pUC18-wzc1260-2163-rcsA+ encoded the C-terminal domain including the transmembrane helix TM2. The pUC18-wzc1345-2163-rcsA+ construct expressed the cytoplasmic C-terminal domain of Wzc, without any transmembrane helix, from codon 449 to the stop codon. Plasmid pUC18-wzc1260-2112-rcsA+ encoded the C-terminal domain, from codon 421 to codon 704, deleted from the C-terminal tyrosine cluster (Fig. 4).

The N-terminal domain of Wzc with the two transmembrane helices, tagged with 6 histidine residues at its N-terminus, was expressed from pQE30-wzc1-1356. This construct was obtained by synthesizing the corresponding 1356-bp DNA fragment by PCR amplification, using oligos 7 and 8 as primer pair (Table 2). The amplified DNA fragment was restricted by BamHI and KpnI enzymes, and inserted into the pQE30 vector opened with the same enzymes.

Mutant Wzc proteins modified on the sites of intra- or inter- phosphorylation were expressed, respectively, from pUC18-wzcY569F-rcsA+ and pUC18-wzcL6-rcsA+. These
constructs were obtained by PCR amplification, using plasmids pQE30-41-Y569F and pQE30-41-L6 as templates (20). Restriction sites for MluI and KpnI were included respectively in forward (Table 2, oligo 9) and reverse (Table 2, oligo 10) primers. Amplified DNA fragments were digested by MluI and KpnI enzymes, and cloned into pUC18-wzc-rcsA+, previously deleted from the wild MluI-KpnI wzc fragment.

Plasmid pUC18-wzc\textsubscript{K540M}-rcsA encoding a mutant Wzc protein, modified on the lysine residue in the Walker A motif involved in ATP binding, was obtained by site-directed mutagenesis by using the Transformer Site-directed Mutagenesis Kit from Clontech, based on the method developed in (28). The strategy of this method lies in the fact that, in addition to the mutagenic oligonucleotide, a selection oligonucleotide containing a mutation in a unique restriction site within the target plasmid is used. Such procedure was applied directly to pUC18-wzc\textsubscript{1-2163}-rcsA+ by using an oligonucleotide that introduced the expected substitution, K540M, and a selection primer that eliminated the unique EcoO109I restriction site present on the pUC18-wzc\textsubscript{1-2163}-rcsA+ vector (Table 2). The occurrence of the mutation was checked by DNA sequencing (29).

**Construction of plasmids expressing the outer membrane protein Wza**

A DNA fragment encoding protein Wza was obtained by PCR amplification, using the forward primer Wza1 that contained a KpnI site and the reverse primer Wza2 in which a BamHI site was included. The PCR product was restricted with KpnI and BamHI enzymes, and inserted in the corresponding sites of either the pUC18-rcsA+ plasmid or the pUC18-wzc\textsubscript{1-2163}-rcsA+ construct. The resulting plasmids, pUC18-wza-rcsA+ and pUC18-wzc-wza-rcsA+, encoded either protein Wza alone or proteins Wza and Wzc, respectively.

**Alkaline phosphatase and β-galactosidase assays**

Alkaline phosphatase activity of translational fusions Wzc-PhoA was first assessed by plating cells of *E.coli* strain CC118 transformed by each fusion onto LB agar containing the chromogenic X-P substrate. Alkaline phosphatase assay was then performed as previously described (23).

β-galactosidase activity was assayed on LB agar containing X-gal substrate. It was further quantified by using the procedure described in (30), except that cells were permeabilized with 0.1% SDS and chloroform instead of toluene.
The activity of enzymes was calculated relatively to the number of bacterial cells according to (23, 30).

**Preparation of a Wzc-specific monoclonal antibody**

A monoclonal antibody specific to Wzc was prepared by immunizing mice with the C-terminal domain (amino acids 447 to 720) of Wzc mutated in the ATP-binding site (lysine residue 540). This mutant was unable to bind ATP and, therefore, to autophosphorylate. This procedure avoided the production of non-specific antibodies directed against phosphotyrosine. Large scale production of the C-terminal fragment of Wzc was obtained by overexpression of the corresponding 824-bp DNA fragment in the pQE30 vector, as previously described (12). K540M substitution was carried out by site-directed mutagenesis using the Transformer Site-directed Mutagenesis Kit from Clontech, as described above. Briefly, plasmid pQE30-wzc1345-2163 was used as template for mutagenesis with an oligonucleotide that introduced the expected substitution, and a selective primer that eliminated a unique XbaI restriction site on pQE30-wzc1345-2163. The mutated plasmid was transferred into the E.coli BL21(pREP4-groESL) strain. Cells were then grown at 37°C until A<sub>600</sub> reached 0.5, IPTG was added at a final concentration of 0.5 mM, and growth was continued for 4h at the same temperature. The fusion protein 6His-Wzc<sub>447-720/K540M</sub> was extracted and purified to homogeneity by using a Ni<sup>2+</sup>-immobilized matrix. Mice were immunized with the corresponding purified fusion protein. Several monoclonal antibodies were thus prepared as previously described (31).

**In vivo cross-linking and protein analysis**

Overnight cultures of strain JM83 (pUC18-rcsA<sup>+</sup>) expressing endogenous protein Wzc, strains JM83 wzc::Km<sup>R</sup> (pUC18-Δwzc-rcsA<sup>+</sup>) and JM83 wzc::Km<sup>R</sup> (pQE30-wzc1-1356) encoding various domains of Wzc, were subcultured and grown at 37°C in LB medium supplemented with 1mM IPTG until A<sub>600</sub> reached 1.0. Cross-linking experiments were then carried out with two different cross-linkers, formaldehyde and dithio-bis(succinimidylpropionate) (DSP).

Cells were harvested by centrifugation, washed once in ice-cold 10mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, pH 6.8, and resuspended in the same buffer to obtain an A<sub>600</sub> of 1.0. Formaldehyde (37%, w/w) was added to a final concentration varying from 0.01 to 2%, and samples were incubated for 1h at 23°C. Cross-linked samples (4 ml) were washed once in 6 ml of ice-cold 10mM K<sub>2</sub>HPO<sub>4</sub>/KH<sub>2</sub>PO<sub>4</sub>, pH 6.8, and resuspended in 160 µl of loading buffer.
Aliquots of 20 µl were heated either at 60°C for 10 min or at 100°C for 20 min to reverse cross-linking, then loaded on polyacrylamide gel.

For DSP experiments, cells were washed and resuspended in an appropriate volume of PBS buffer (150 mM NaCl, 20mM Na₂HPO₄, pH 7.4) to reach A₆₀₀=2. DSP was used at a final concentration ranging from 10 to 100 µM. After 30 min of incubation at room temperature, the cross-linking reagent was quenched by addition of 100 mM Tris-HCl, pH 7.4. Cross-linked samples (4 ml) were resuspended in 160 µl of loading buffer (32) without or with β-mercaptoethanol to reverse DSP cross-linking. Aliquots were then heated at 100°C for 5 min before loading on gel.

Samples were fractionated by SDS-PAGE and analyzed by Western immunoblotting by using one of the monoclonal anti-Wzc antibodies described above. The secondary antibody, horse radish peroxidase-coupled anti-mouse IgG, was revealed by enhanced chemiluminescence using the chemiluminescence reagent from Amersham. In the case of the N-terminal domain of Wzc, tagged with 6 histidine residues, blots were revealed with the "SuperSignal West HisProbe Kit" from Pierce. Commercial protein markers and purified high-molecular-mass proteins such as laminine 1 (400 kDa and 220 kDa in the presence of reducing agents) were used to calibrate SDS-PAGE.

Palmitate labeling of Wza protein

The E.coli strains JM83 (pUC18-wzc-wza-rcsA⁺) and JM83 wzc::Km R(pUC18-wza-rcsA⁺) were grown in M9 medium. At A₆₀₀= 0.3, 1 mM IPTG and [³H]-palmitic acid (5 µCi/ml) were added, and growth was continued until A₆₀₀ reached 1. Cells were then washed three times in ice-cold 10mM K₂HPO₄/KH₂PO₄, pH 6.8, resuspended in the same buffer to yield A₆₀₀=1, and submitted to cross-linking in the presence of 0.5 % formaldehyde for 1h at 23°C. Cross-linked samples were washed once with 10 mM K₂HPO₄/KH₂PO₄, pH 6.8, and resuspended in loading buffer (32). Samples containing about 10⁶ cpm were loaded on polyacrylamide gel, blotted onto PVDF membrane and revealed by autoradiography with an intensifying screen (Kodak Biomax Transcreen system).
Results

Membrane topology of Wzc

Previous studies have shown that Wzc from *E.coli* is located in the inner membrane of bacteria (11). To investigate the membrane topology of Wzc, we analyzed the hydrophatic profile of its amino acid sequence. The hydrophobic plot, according to (33), suggested the presence of two putative transmembrane α-helices, TM1 and TM2, located between amino acids W_{32}-A_{32} and L_{426}-L_{445}, respectively (Fig. 1A). To test this prediction, we constructed a series of translational fusions of *wzc* gene fragments to the *E.coli lacZ* gene encoding β-galactosidase (LacZ) and to the *E.coli phoA* gene expressing a truncated alkaline phosphatase lacking its leader peptide (PhoA). The β-galactosidase protein is active only when expressed in the cytoplasm. On the other hand, alkaline phosphatase, which requires the formation of disulfide bonds for activity, exhibits enzymatic activity exclusively when present in the periplasm. These two types of fusion were therefore complementary to each other since they allowed mapping of both the cytoplasmic and periplasmic segments of Wzc.

To construct translational fusions of *phoA* and *lacZ* at various points within *wzc*, PCR products were synthesized by using a forward primer which could bind to the 5' end of the *wzc* gene (Pho1 or Lac1) paired with 9 different downstream reverse primers (Pho2-Pho10 or Lac2- Lac9) complementary to various codons inside the *wzc* gene (Table 2). The amplified fragments were cloned into pUC19-*phoA* and pUC18-ΔlacZ vectors. The resulting plasmids were used to transform the *phoA* – *E.coli* strain CC118 or the *lacZ* - *E.coli* strain DH5α.

Alkaline phosphatase activity of the *E.coli phoA* deletion CC118 cells, transformed with these *wzc::phoA* fusions, was measured (Fig. 1B). Two levels of activity were observed : strains expressing *wzc::phoA* constructs with fusion sites located between the two potential transmembrane helices, TM1 and TM2, of Wzc (A58, Q241, T363, Q416) exhibited PhoA activities of about 1000 units, whereas strains carrying fusion sites located either at the N-terminal end, before TM1 (A30), or after the second hypothetical transmembrane helix TM2 (P456, Q553, V670, A719), displayed low PhoA activities, in the same range as the background PhoA activity of the CC118 (pUC19-ΔphoA) strain (Fig. 1B). To assess the stability of the Wzc::PhoA fusions, Western-blotting of cell fractions expressing various PhoA fusions was performed using anti-PhoA antibody. All fusions were detectable and expressed to about the same level, which suggested that the differences observed between the PhoA activities measured for various fusions were not due to differences between fusion...
stabilities (data not shown). These data confirmed the positions of the two putative transmembrane helices and supported the concept that both the N-terminal end and the C-terminal domain of Wzc are located in the cytoplasm. They also provided evidence of the periplasmic localization of an internal Wzc domain bordered by the two transmembrane helices, TM1 and TM2.

To check further the validity of these observations, \textit{wzc::lacZ} fusions introduced into the \textit{lac} DH5α strain were assayed for β-galactosidase activity. It was observed that the \textit{wzc::lacZ} fusions with Wzc peptides exhibiting a high level of phosphatase activity, displayed instead low level β-galactosidase activity (A58, Q241, Q416), comparable to the LacZ activity of DH5α (pUC18-ΔlacZ). This finding supported the periplasmic localization of the fragment located between TM1 and TM2. Conversely, strains which encoded Wzc-LacZ hybrid proteins carrying fusion sites located at the N-terminal end (A30) and in the C-terminal domain (P456, Q553, A719), exhibited LacZ activities of about 3000 units, which was consistent with a cytoplasmic localization of the corresponding Wzc sequences. It is noteworthy that the LacZ fusion at amino acid V670 had a rather low β-galactosidase activity in comparison to the C-terminal domain fusions (450 vs. 3000 units). This fusion concerned a hydrophobic fragment of Wzc which could be either associated with the membrane or folded inside the protein structure. The corresponding PhoA fusion demonstrated that this region is, in fact, located in the cytoplasm of the cell.

Based on these data, a schematic view of the topology of Wzc is presented in Fig. 1B, showing the different parts of the molecule present respectively in the cytoplasm, the inner membrane and the periplasm.

\textbf{Detection of Wzc oligomer formation by \textit{in vivo} cross-linking}

Since the overall process of Wzc phosphorylation involves at least two different molecules which participate in an interphosphorylation reaction (20), we investigated the possibility that Wzc would exist in the cell as an oligomer. For this, \textit{in vivo} cross-linking experiments were performed by using formaldehyde on intact cells of \textit{E.coli} JM83(pUC18-rcsA\textsuperscript{+}). This strain is known to overproduce the transcriptional activator RcsA, and thus to activate the transcription of colanic acid genes (\textit{cps} cluster), including the \textit{wzc} gene encoding the autokinase Wzc (12). Cells of \textit{E.coli} JM83(pUC18-rcsA\textsuperscript{+}) were grown in BL medium supplemented with IPTG to induce RcsA synthesis and, consequently, high level transcription of the \textit{wzc} gene. Cross-linking was performed with a concentration of formaldehyde varying
from 0.01 to 2% (w/w). Total protein extracts were separated by SDS-PAGE and analyzed by immunoblotting using a monoclonal anti-Wzc antibody against the C-terminal domain of Wzc. As shown in Fig. 2A, treatment with formaldehyde generated a minor protein species of about 160 kDa, a predominant form with a high molecular mass around 240 kDa, and a heavy complex over 400 kDa. Production of these molecular species began to occur at a formaldehyde concentration as low as 0.1%, but it was optimal at 0.5% formaldehyde. It could be noted that the molecular species of 240 kDa appeared in the form of a doublet, which was likely be due to two different conformations of the complex, as previously described (34). The presence of heavy forms at > 400 kDa and 240 kDa could be reversed by heating samples at 100°C for 20 min before loading on polyacrylamide gel. This finding was consistent with the known lability of the cross-links produced by formaldehyde. As a control, the cross-linking experiment performed on the *E. coli* strain JM83*wzc::Km R (pUC18-rcsA*) which cannot synthesize Wzc, did not reveal any band by immunoblotting, thus confirming the specificity of the labeling by the anti-Wzc antibody.

To confirm the data obtained with formaldehyde, cross-linking was performed on the same *E. coli* JM83(pUC18-rcsA*) strain by using another reagent, dithiobis(succinimidylpropionate) (DSP). Formaldehyde is a small reactive molecule capable of polymerization to a variety of lengths (35) and, therefore, is able to cross-link polypeptides which are separated from one another by a variable distance. By contrast, DSP is a fixed-arm-length cross-linking reagent, 12Å in length (34). Experiments were performed with a DSP concentration varying from 10 to 100 µM. Total extracts were fractionated by SDS-PAGE and revealed with anti-Wzc antibody. The profiles of cross-linked proteins obtained after DSP treatment were identical to those obtained with formaldehyde experiments, showing namely the same molecular species of about 160 kDa, 240 kDa and over 400 kDa (Fig. 2B). These heavy species were sensitive to treatment by β-mercaptoethanol and disappeared in the presence of this reducing reagent, as expected from the chemical nature of DSP which contains a disulfide bridge. Interestingly, the samples which were not treated by DSP contained a major band corresponding to the Wzc monomer and also a minor species of 160 kDa. This 160 kDa species disappeared under treatment with β-mercaptoethanol, which suggested that it is a relatively stable complex resistant to SDS treatment at 100°C and stabilized by disulfide linkage.

Together, these results indicated that Wzc could oligomerize, essentially in the form of a trimer and, possibly, of a hexamer. But, they could not exclude the possibility that Wzc
would interact as well with another type of protein, lying in its vicinity and having a similar 
molecular mass of about 80 kDa (alone or in a multimeric state), to form a heterologous 
protein complex. It was therefore useful to analyze further the composition of the heavy 
molecular species found in these experiments.

**Absence of interaction between Wzc and Wza**

The protein molecule that would represent the most probable candidate for interacting 
with Wzc to yield a heterologous complex, was protein Wza. Indeed, Wza has a molecular 
mass of 41.9 kDa and could be present as a multimer in the outer membrane of *E.coli*. In 
addition, it has been recently described to form a pore through which the capsule antigen 
would be translocated (36). On the other hand, Wzc seems to be essential for capsule 
translocation from the inner membrane to the cell surface of *E.coli* (37). Moreover, electron 
microscopy analysis of the insertion of nascent group I capsule on the *E.coli* surface has 
shown that the sites of insertion are located above regions where the inner and outer 
membranes are in apposition (38). These various observations support the occurrence of a 
translocation complex which would span the periplasm and involve both Wza and Wzc in the 
process of polysaccharide export (39).

To check the possible interaction between Wzc and Wza, the *E.coli* strain JM83 
(pUC18-wzc-wza-rcsA*) which expressed both proteins upon induction by IPTG, was grown 
in M9 medium in the presence of [3H]-palmitic acid. Such radioactive labeling was chosen 
because Wza is a lipoprotein which can be effectively labeled in the presence of this lipid 
(36). Cells were then submitted to cross-linking with 0.5% formaldehyde. Total protein 
extracts were separated by SDS-PAGE, blotted onto membrane and revealed, first, by 
incubation with an anti-Wzc antibody to identify the molecular species containing Wzc and, 
second, by autoradiography to detect the presence of Wza and its putative oligomers. As 
shown in Fig. 3, immunoblotting with anti-Wzc revealed the monomeric form of Wzc (lanes 1 
and 5) and, after cross-linking, the heavy species described above, *i.e.* with a molecular mass 
of 160 kDa, 240 kDa, and > 400 kDa, respectively (Fig. 3, lanes 2 and 6). Autoradiography of 
the same membrane revealed a protein of about 40 kDa consistent with the molecular mass of 
Wza (Fig. 3, lanes 7 and 8). In the presence of formaldehyde, additional minor species, with a 
molecular mass of 80 kDa and 120 kDa, were detected (Fig. 3, lane 8). These species, which 
could correspond respectively to a dimer and a trimer of Wza, or to the oligomerization of 
another lipoprotein detected by palmitate labeling, did not comigrate with the protein 
complexes containing Wzc (Fig.3, lanes 2 and 6). Moreover, similar labeling and cross-
linking experiments were performed on the *E. coli* strain JM83 *wzc::Km*\(^R\)* (pUC18-wza-rcsA\(^+\)*) which expressed Wza but could not synthesize functional Wzc. In this case, immunoblotting with anti-Wzc antibody did not reveal any band, which confirmed that Wzc was not expressed, whereas autoradiography revealed the presence of Wza, and the 80-kDa and 120-kDa species after cross-linking (data not shown), thus demonstrating that these species did not contain Wzc. These results therefore indicated that, in our experimental conditions, Wza and Wzc were not associated in a protein complex.

**The C-terminal domain of Wzc is sufficient for oligomerization**

A more detailed analysis of the oligomerization of Wzc was undertaken to identify the protein domains specifically required for this reaction. For that purpose, cross-linking experiments were performed on *E. coli* strains expressing various domains of Wzc, namely the cytoplasmic C-terminal domain and the periplasmic N-terminal domain. These experiments allowed also to check whether Wzc was capable to oligomerize *per se* or was interacting with other proteins to form heavy complexes. Indeed, in the case of strict oligomerization, cross-linking experiments were expected to yield molecular species whose molecular mass was a multiple of the mass of the Wzc domain, whereas in the case of interaction with a different protein, the corresponding heterologous complexes should have a mass equal to the sum of the Wzc domain and the other protein.

In all experiments described above, Wzc was synthesized, in various conditions, from the corresponding *wzc* gene present in the chromosome of bacteria. In the following experiments, Wzc or Wzc fragments were produced selectively from the complete or truncated *wzc* gene carried by a plasmid vector. To do so, we used the *E. coli* strain JM83 *wzc::Km*\(^R\)* in which the genomic copy of *wzc* had been inactivated by kanamycin cassette insertion, and we transformed this strain by plasmid pUC18-*wzc-rcsA*\(^+\)* bearing various mutations in *wzc* (Fig. 4). In a preliminary assay, this expression system was tested through the synthesis of entire wild Wzc. Strain JM83 *wzc::Km*\(^R\)* was transformed by a pUC18-*wzc*_1-2163\(^-\)*-rcsA\(^+\)* construct and shown to actually produce full-length Wzc (Fig. 5A, lane 3). Moreover, cross-linking with formaldehyde on the strain transformed by pUC18-*wzc*_1-2163\(^-\)*-rcsA\(^+\)* generated the same molecular species as those previously observed for the genome-encoded Wzc protein (Fig. 5A, lanes 2 and 4), which confirmed the validity of the plasmid expression system used.

Experiments were then performed on strain JM83 *wzc::Km*\(^R\)* transformed by the pUC18-*wzc*_1260-2163\(^-\)*-rcsA\(^+\)* vector encoding the Wzc\(_{421-720}\) C-terminal domain anchored to the
membrane by the hydrophobic helix TM2 (Fig. 4). This 33-kDa domain was able to yield two molecular species of 66 kDa and ~ 97 kDa, respectively, i.e. twice and three times as large as its apparent molecular mass (Fig. 5B , lanes 1 and 2), suggesting the occurrence of oligomerization.

To check the role of the anchorage to the membrane in Wzc oligomerization, cells of E.coli JM83 wzc::KmR(pUC18-wzc 1345-2163rcsA) which expressed the cytoplasmic C-terminal domain of Wzc but not the transmembrane helix TM2 (Fig. 4), were assayed for oligomerization. Immunoblotting with anti-Wzc antibody showed that this construct produced oligomers, namely a dimer and a trimer, in the presence of formaldehyde (Fig. 5B, lanes 3 and 4). These data indicated that membrane association is not required for Wzc oligomerization and, therefore, that protein association is rather due to interaction between amino acids present in the cytoplasmic domain of Wzc.

In order to characterize further the sequence in Wzc required for oligomerization, the effect of the C-terminal tyrosine cluster on oligomerization was analyzed. Cells of E.coli JM83 wzc::KmR(pUC18-wzc 1260-2112rcsA) which encoded the C-terminal domain of Wzc deleted from its tyrosine cluster (Fig. 4), were treated with the cross-linking reagent, formaldehyde. This treatment generated molecular species whose mass corresponded to dimeric and trimeric forms of the domain, thus indicating that the tyrosine cluster is not essential to oligomerization (Fig. 5B, lanes 5 and 6). Therefore, neither the transmembrane helix TM2 nor the C-terminal tyrosine cluster of Wzc seemed to be essential to its oligomerization. Only the segment of protein encompassing amino acid residues 449 to 704 appeared to be sufficient to induce oligomerization.

On the other hand, the ability of the N-terminal domain of Wzc to oligomerize was assayed by treating with formaldehyde the E.coli strain JM83 transformed by pUC18-wzc1-1356rcsA+ which expressed the N-terminal domain of Wzc anchored to the membrane by the two helices, TM1 and TM2 (Fig. 4). In this special case, the protein domain was expressed in wild-type JM83 cells instead of deleted wzc::KmR cells, so as to allow detection of the interaction between the N-domain and the complete Wzc protein. The reason lay in the fact that no antibody raised specifically against the N-domain was available. Therefore, immunodetection could be achieved only by using the antibody against the C-domain of the protein which was also active against the complete protein including the N-domain. As shown in Fig. 5A (lanes 11 and 12), no interaction between the N-domain and entire Wzc was found, and the cross-linking profile was identical to that of complete Wzc alone. This suggested that the N-domain alone could not interact with Wzc and, therefore, could not oligomerize as such.
However, in our experimental conditions, it could not be excluded that the entire Wzc protein would auto-associate with high affinity and thus would mask a possible role of the N-terminal domain, separately, in the process of oligomerization. Since antibodies against the N-terminal domain were not available, the latter possibility was checked by constructing plasmid pQE30-wzc1-1356, which expressed the N-terminal domain of Wzc bearing a 6His-tag, and assaying cells of *E.coli* JM83 *wzc::Km* R (pQE30-*wzc*1-1356) for oligomerization. After cross-linking with formaldehyde, protein extracts were separated by SDS-PAGE, blotted to nitrocellulose membrane and revealed by using a nickel derivative of horseradish peroxydase for direct detection of recombinant polyhistidine-tagged fusion proteins. As shown in Fig. 5B (lanes 7 and 8), the N-terminal His_6-tagged domain was effectively produced, but could not form high molecular-mass species in the presence of cross-linker.

It thus could be concluded that the formation of Wzc oligomers was due to interaction between different molecules of protein via their C-terminal domain rather than their N-terminal domain.

**Phosphorylation does not influence oligomerization of Wzc**

An attempt was made to correlate the structural organization of Wzc with its function by studying the possible relationship between its ability to oligomerize and its capacity to autophosphorylate on tyrosine residues. In a recent work, we had established that Wzc could be phosphorylated on two types of sites: first, on five tyrosine residues in a "tyrosine cluster" located at the C-terminal end of Wzc which are phosphorylated by an interphosphorylation reaction between two molecules of protein; second, on a particular tyrosine residue, Y569, which is modified in an intramolecular reaction.

The possible effect of interphosphorylation on Wzc oligomerization was studied by using a mutant protein, expressed from pUC18-*wzc*<sub>L6</sub>-rcsA<sup>+</sup>, bearing five phenylalanine residues in the place of the five tyrosine residues (Y708, Y710, Y711, Y713 and Y715) which constitute the phosphorylation sites clustered at the C-terminus of the protein. Cross-linking experiments showed that this mutant protein could form the same high-molecular-mass species as the wild type (Fig 5A, lanes 5 and 6). Similarly, to check the effect of intraphosphorylation on oligomerization, the Wzc-Y569F protein containing a phenylalanine residue arising from site-directed mutagenesis of the tyrosine residue at position 569, was expressed from the pUC18-*wzc*<sub>Y569F</sub>-rcsA<sup>+</sup> plasmid and treated by formaldehyde. As shown in Fig. 5A (lanes 7 and 8), this mutant protein also could oligomerize into dimer, trimer and hexamer.
To assess further the possible relationship between the phosphorylation of Wzc and its oligomerization, another construct, pUC18-\textit{wzc}_{K540M}-\textit{rcsA}^{+}, was prepared. This plasmid encoded a Wzc protein modified in the ATP-binding site, at the level of the lysine residue at position 540 which is essential for overall phosphorylation (11, 40). \textit{E.coli} JM83 \textit{wzc::Km}^{R} cells carrying this construct were subjected to 0.5 % formaldehyde treatment, then the protein content was analyzed by electrophoresis and immunoblotting with anti-Wzc. This mutant protein produced the same high-molecular-mass species as the wild-type protein (Fig. 5A, lanes 9 and 10). These various data therefore concurred in showing that phosphorylation is not required for Wzc oligomer formation.

Conversely, we checked whether Wzc oligomerization could influence the phosphorylation state of the protein, \textit{i.e.} whether the Wzc protein was found phosphorylated in oligomers. \textit{E.coli} JM83(pUC18-\textit{rcsA}^{+}) cells which expressed the endogenous wild Wzc protein, were treated with cross-linker, then a protein extract was analyzed by immunoblotting using an antibody against phosphorylated tyrosine (PY20). As shown in Fig. 6, Wzc dimers, trimers and hexamers as well as monomers, were detected, which indicated that Wzc was phosphorylated in these different molecular species.

**Discussion**

Protein Wzc is member of a newly defined family of protein-tyrosine autokinases involved in surface polysaccharide synthesis in both Gram-negative and Gram-positive bacteria. This kinase, located in the inner-membrane of \textit{E.coli} K-12, is able to undergo autophosphorylation on six different tyrosine residues, and is required for the production of the widespread exopolysaccharide, colanic acid (12). The catalytic mechanism of autophosphorylation of Wzc was recently described from \textit{in vitro} experiments (20), but so far little was known about its structural organization \textit{in vivo}. The main purpose of this study was to investigate such organization and try to relate the corresponding data to the functional properties of the protein. Our results allow to propose a model for the topology of Wzc within the cell, to demonstrate for the first time that this bacterial protein kinase can oligomerize, and to provide evidence that oligomerization is not dependent on its phosphorylation.

Concerning the membrane topology of Wzc, our results support the theoretical predictive data suggesting that Wzc can be divided into two main structural domains: a N-
terminal domain, bordered by two transmembrane helices located between \( W_{32} - A_{52} \) and \( L_{426} - L_{445} \), which is located in the periplasmic region of the cell, and a C-terminal domain, extending from \( R_{446} \) to \( K_{720} \), which is located in the cytoplasm. These structural domains are connected with the functional domains previously defined in terms of phosphorylation (20). Indeed, the C-terminal fragment of Wzc (\( S_{447} - K_{720} \)) can autophosphorylate, whereas the N-terminal fragment of Wzc (\( M_{1} - G_{452} \)) cannot and, moreover, does not influence the \textit{in vitro} phosphorylation of the C-terminal fragment. Interestingly, the C-terminal domain harbors the Walker A and B motifs necessary for phosphorylation as well as the six different phosphorylation sites of Wzc. The present data support that the C-terminal fragment of Wzc is structurally independent, which coincides with the fact that it possesses an intrinsic protein-tyrosine kinase activity. A membrane topology similar to that of Wzc has been previously described in the case of protein ExoP, a Wzc homologue from \textit{Sinorhizobium meliloti} (41), whose C-terminal fragment also can autophosphorylate \textit{in vitro} (14). It therefore appears that these two members of the tyrosine-kinase family share similar structural and functional features. Different results have however been reported for the Wzc protein of \textit{E.coli} K-30. In this case, the C-terminal fragment is unable to autophosphorylate and needs the presence of the N-terminal part of the protein to become phosphorylated (11). Similarly, it has been found that in Gram-positive bacteria, namely \textit{Streptococcus pneumoniae}, the tyrosine kinase activity requires the presence of two separate proteins, CpsC and CpsD, which exhibit homology respectively with the N- and C-terminal domains of Wzc. Although proteins CpsC and CpsD can be compared separately to the structurally independent N-domain and C-domain of Wzc, the autophosphorylation of CpsD needs the presence of CpsC (17). Thus, in spite of relatively high structural similarity between bacterial tyrosine kinases, the catalytic mechanism of autophosphorylation seems to differ from one species to the other.

\textit{In vivo} cross-linking experiments provide the first demonstration that a bacterial kinase can oligomerize. Recently, it has been observed that protein Wzc autophosphorylates \textit{in vitro} through a cooperative two-step mechanism (20). First, the tyrosine residue \( Y_{569} \) is phosphorylated in an intramolecular process, which results in an increased protein-kinase activity of Wzc that can, in turn, phosphorylate the five C-terminal tyrosines in an intermolecular process. A mechanism of this type implies that at least two molecules of Wzc can interact to undergo interphosphorylation. Our data demonstrate that such interaction between Wzc molecules does exist in the inner membrane of the cell.

The observation that the C-terminal fragment of Wzc is sufficient for triggering oligomerization is consistent with the functional capacity of this fragment to
autophosphorylate, in the absence of the N-terminal fragment. It can be noted that protein Wzz from *Shigella flexneri*, which exhibits slight homology with the N-terminal domain of Wzc even though it does not possess tyrosine-kinase activity, has been reported to form oligomers of at least six units (34). From this finding, it could be envisaged that the N-terminal domain of Wzc would also be able to form high-molecular-mass oligomers and/or contribute to the oligomerization of the C-terminal domain. However, no such possibility was observed in our experiments.

Neither the anchorage of the protein to the inner membrane nor the presence of the C-terminal tyrosine cluster appears to be essential for the oligomerization of Wzc. Therefore, it seems that the amino acid sequences involved in oligomerization are located in the central part of the cytoplasmic C-fragment. In this regard, we have observed that Wzc oligomers, mainly the dimer species, are sensitive to reducing agents such as β-mercaptoethanol, which suggests that oligomers might be stabilized by disulfide bonds.

The mutant Wzc protein, altered in the Walker A motif, which can no longer undergo phosphorylation, still forms the same high-molecular-mass species as the wild-type protein does. Similarly, various Wzc proteins mutated either on Y569 or on the phosphorylation sites of the C-terminal tyrosine cluster, keep the capacity to form trimers and hexamers. Therefore, neither Wzc intraphosphorylation nor interphosphorylation is necessary for Wzc oligomerization. Inversely, however, it cannot be concluded whether oligomerization is essential to phosphorylation. Indeed, the observation that monomers as well as oligomers are found phosphorylated could be due to the dissociation of previously phosphorylated oligomers into monomers. If so, this would mean that monomers are unable to phosphorylate *per se*. But, alternatively, it cannot be excluded that phosphorylation would in fact occur first on monomers which, then, would associate into oligomers. Further work is therefore needed to decide between these two possibilities. Still, in any case, considering the capacity of Wzc to interphosphorylate, it seems likely that at least two molecules of Wzc must interact, even transiently, to allow interphosphorylation.

The question left is to understand the physiological function of oligomerization of Wzc. In particular, it would be interesting to determine whether oligomerization is an essential step in a signal transduction process possibly mediated by Wzc, and/or is necessary for the polysaccharide synthesis catalyzed by Wzc. When referring to eukaryotic systems, a large number of protein-tyrosine kinases are known to oligomerize in response to extracellular signals, which results in interphosphorylation of two kinase molecules and triggers signal
transduction in the cell. By analogy, it could be suggested that Wzc oligomerization occurs in response to certain environmental signals, to be identified, then allows Wzc interphosphorylation, which would result in the regulation of colanic acid biosynthesis. It could be envisaged, as well, that colanic acid biosynthesis requires by itself oligomerization of Wzc. Indeed, it has been reported that the Wzc protein from *E.coli* K-30 is essential to translocation and cell-surface assembly of the capsular polysaccharides (11, 37). Moreover, the outer-membrane protein Wza is able to oligomerize so as to form a pore through which the capsule antigen can be translocated (36). Considering that *wza* and *wzc* deleted mutants exhibit the same capsule translocation defective phenotype (39), it can be proposed that Wzc would be the inner membrane counterpart of Wza and would form a complex allowing capsular translocation of the polysaccharides through the inner-membrane, from cytoplasm to periplasm. Further experiments are however needed to check the validity of this hypothesis.
Acknowledgements

Thanks are due to J.T. Beatty and W.H. Bingle for their gift of strain CC118 and plasmids pSP72-lacZ and pUC19-phoA, to P. Rousselle for providing purified laminine 1, and to Y. Descollonges for preparing monoclonal antibodies. The assistance of N. Moreno is gratefully acknowledged. This work was supported by grants from the Société Ezus-Lyon 1 (contract 482.022), the Ministère de la Recherche (contract FNS 2000 Microbiologie) and the Institut Universitaire de France.
References

Figure legends

FIG. 1. Analysis of the membrane topology of Wzc. A. Hydropathic profile of protein Wzc from *E.coli* K-12 according to Kyte and Doolittle (33). B. Schematic presentation of the topology of protein Wzc. The different amino acids on which translational fusions *wzc::phoA* and *wzc::lacZ* were performed are indicated by single letter code and number. The alkaline phosphatase and β-galactosidase activities, as expressed in total units, are presented respectively in an oval and a rectangle close to each amino acid.

FIG. 2. *In vivo* cross-linking of Wzc in *E.coli* cells. A. Formaldehyde cross-linking experiments were performed on cells of either *E.coli* JM83(pUC18-rcsA*) or *E.coli* JM83 *wzc::Km R*(pUC18-rcsA*) with a concentration of formaldehyde varying from 0.01 to 2%. Total extracts were then heated at 60°C for 10 min or at 100°C for 20 min, separated by SDS-PAGE on a 6% gel, and analyzed by immunoblotting using a monoclonal anti-Wzc antibody. B. Dithio-bis(succinimidylpropionate) (DSP) cross-linking was carried out on cells of *E.coli* JM83(pUC18-rcsA*) with a reagent concentration varying from 10 to 100 µM. Cells were resuspended in loading buffer with (+) or without (-) β-mercaptoethanol. Total extracts were separated by SDS-PAGE on a 6% gel and revealed by anti-Wzc antibody.

FIG. 3. *In vivo* labeling and cross-linking of Wza. Cells of *E.coli* strain JM83 (pUC18-*wzc-wza-rcsA*) were grown in M9 medium in the presence of [*3 H*-palmitic acid, then submitted to 0.5% formaldehyde treatment. Total extracts were separated either on a 6% (A) or 10% (B) polyacrylamide gel and revealed by immunoblotting with a monoclonal anti-Wzc antibody (lanes 1,2,5,6) or by autoradiography (lanes 3,4,7,8).

FIG. 4. Schematic presentation of mutant Wzc proteins. Protein Wzc in its wild form is presented in the upper line of the figure: the two transmembrane helices, TM1 and TM2, are indicated by grey shaded boxes; the tyrosine cluster at the C-terminal end of Wzc is shown as a dark box. The various mutant proteins used in this study are presented. They are designated, on the left, by numbering either the amino acids of their N- and C-termini or the amino acids modified by mutagenesis. The nomenclature of the corresponding plasmids is given on the right.
FIG. 5. Formaldehyde cross-linking of mutant Wzc proteins. The *E.coli* JM83 wzc::KmR strain was transformed by the different constructs (pUC18-Δwzc-rcsA+) and (pQE30-wzc\_1-1356) described in Fig. 4, which expressed either the wild protein Wzc or various mutants of this protein. Total cells were treated with 0.5% formaldehyde, electrophorezed on SDS-polyacrylamide gels, and immunoblotted using a monoclonal anti-Wzc antibody or an anti His\_6-tag in the particular case of (B) lanes 7 and 8. **A.** 6% polyacrylamide gel electrophoresis. Lanes 1 and 2: JM83 (pUC18-rcsA+); lanes 3 and 4: JM83 wzc::KmR(pUC18-wzc\_1-2163-rcsA+); lanes 5 and 6: JM83 wzc::KmR(pUC18-wzc\_16-rcsA+); lanes 7 and 8: JM83 wzc::KmR (pUC18-wzc\_Y569F-rcsA+); lanes 9 and 10: JM83 wzc::KmR (pUC18-wzc\_K540M-rcsA+); lanes 11 and 12: JM83 (pUC18-wzc\_1-1356-rcsA+). **B.** 10% polyacrylamide gel electrophoresis. Lanes 1 and 2: JM83 wzc::KmR(pUC18-wzc\_1260-2163-rcsA+); lanes 3 and 4: JM83 wzc::KmR(pUC18-wzc\_1260-2112-rcsA+); lanes 5 and 6: JM83 wzc::KmR(pUC18-wzc\_1260-2112-rcsA+); lanes 7 and 8: JM83 wzc::KmR(pQE30-wzc\_1-1356).

FIG. 6. Phosphorylation of Wzc monomer and oligomers. *E.coli* JM83(pUC18-rcsA+) strain was treated with 0.5% formaldehyde. Total protein extracts were separated by SDS-PAGE on 6% gel, blotted and revealed by anti-phosphotyrosine antibody.
<table>
<thead>
<tr>
<th>Strain or plasmid</th>
<th>Genotype or description</th>
<th>Reference or source</th>
</tr>
</thead>
<tbody>
<tr>
<td>JM109</td>
<td>recA1 supE44 endA1 hsdR17 gyrA96 relA1 thiΔ(lac-proAB) F[traD36 proAB·lacZΔM15]</td>
<td>(21)</td>
</tr>
<tr>
<td>DH5α</td>
<td>supE44 ΔlacU169(Φ80lacZΔM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</td>
<td>(22)</td>
</tr>
<tr>
<td>CC118</td>
<td>araD139 (ara,leu)7697 ΔlacX74 phoAΔ20 galE galK thi rps rpoB argE am recA1</td>
<td>(23)</td>
</tr>
<tr>
<td>BL21(pRep4-groESL)</td>
<td>F·ompT dcm (r_B m_B) gal λ[pRep4GroESL]</td>
<td>(24)</td>
</tr>
<tr>
<td>JM83</td>
<td>F·ara Δ(lac-proAB) rpsL (Str^R) [Φ 80 Δlac(lacZ)M15] thi</td>
<td>(21)</td>
</tr>
<tr>
<td>JM83 wzc::Km^R</td>
<td>F·ara Δ(lac-proAB) rpsL (Str^R) [Φ 80 Δlac(lacZ)M15] thi wzc</td>
<td>(12)</td>
</tr>
<tr>
<td>pSP72-lacZ</td>
<td>LacZ-fusion pT7 expression vector</td>
<td>(25)</td>
</tr>
<tr>
<td>pUC19-phoA</td>
<td>PhoA-fusion vector</td>
<td>(26)</td>
</tr>
<tr>
<td>pUC18-ΔlacZ</td>
<td>LacZ-fusion plac expression vector</td>
<td>this study</td>
</tr>
<tr>
<td>pQE30</td>
<td>Expression vector generating 6His fusion proteins</td>
<td>purchased from Qiagen</td>
</tr>
<tr>
<td>pQE30-wzc1339-2163</td>
<td>pQE30 with a 824-bp BamHI/HindIII fragment encoding the cytoplasmic domain of Wzc</td>
<td>(12)</td>
</tr>
<tr>
<td>pQE30-wzc1-1356</td>
<td>pQE30 with a 1356-bp BamHI/KpnI fragment encoding the N-terminal domain Wzc_1-1356 fused to 6His tag</td>
<td>this study</td>
</tr>
<tr>
<td>pUC18-rcsA^+</td>
<td>pUC18 with a 818-bp BamHI/HindIII fragment encoding the RcsA transcriptional activator</td>
<td>(12)</td>
</tr>
<tr>
<td>pUC18-wzc1-2163rcsA^+</td>
<td>pUC18-rcsA^+ with a 2163-bp EcoRI/KpnI fragment encoding entire Wzc_1-2163 protein</td>
<td>this study</td>
</tr>
<tr>
<td>pUC18-wzc1-1336rcsA^+</td>
<td>pUC18-rcsA^+ with a 1336-bp EcoRI/KpnI fragment encoding the N-terminal domain Wzc_1-1336</td>
<td>this study</td>
</tr>
<tr>
<td>pUC18-wzc1260-2163rcsA^+</td>
<td>pUC18-rcsA^+ with a 903-bp EcoRI/KpnI fragment encoding the C-terminal domain Wzc_1260-2163</td>
<td>this study</td>
</tr>
<tr>
<td>pUC18-wzc1345-2163rcsA^+</td>
<td>pUC18-rcsA^+ with a 819-bp EcoRI/KpnI fragment encoding the C-terminal domain Wzc_1345-2163</td>
<td>this study</td>
</tr>
<tr>
<td>pUC18-wzc1260-2112rcsA^+</td>
<td>pUC18-rcsA^+ with a 52-bp EcoRI/KpnI fragment encoding the C-terminal domain Wzc_1260-2112</td>
<td>this study</td>
</tr>
<tr>
<td>pUC18-wzcY569rcsA^+</td>
<td>pUC18-rcsA^+ with a 2163-bp EcoRI/KpnI fragment encoding entire Wzc_1-720 protein, mutated on Y_569</td>
<td>this study</td>
</tr>
<tr>
<td>pUC18-wzcL6rcsA^+</td>
<td>pUC18-rcsA^+ with a 2163-bp EcoRI/KpnI fragment encoding entire Wzc_1-720 protein, mutated on all inter-phosphorylation sites from Y_508 to Y_715</td>
<td>this study</td>
</tr>
<tr>
<td>pUC18-wzcK540rcsA^+</td>
<td>pUC18-rcsA^+ with a 2163-bp EcoRI/KpnI fragment encoding entire Wzc_1-720 protein, mutated on K_540</td>
<td>this study</td>
</tr>
<tr>
<td>pUC18-wzc-wza-rcsA^+</td>
<td>pUC18-wzc1-2163rcsA^+ with a 1139-bp KpnI/BamHI fragment encoding Wza</td>
<td>this study</td>
</tr>
<tr>
<td>pUC18-wza-rcsA^+</td>
<td>pUC18-rcsA^+ with a 1139-bp KpnI/BamHI fragment encoding Wza</td>
<td>this study</td>
</tr>
</tbody>
</table>
TABLE 2. Oligonucleotides used in this study.

<table>
<thead>
<tr>
<th>Primer</th>
<th>5' to 3' Sequence a,b,c</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pho1</td>
<td>TATGGATCCATGACAGAAAAAGTAAACACATGCCGCTCCGG</td>
</tr>
<tr>
<td>Pho2</td>
<td>TATGGTACCGCTTTCAATGACGGTGCCG</td>
</tr>
<tr>
<td>Pho3</td>
<td>TATGGTACCGCACTATAAATCGGCGTGGCG</td>
</tr>
<tr>
<td>Pho4</td>
<td>TATGGTACCGCTTTCAATGACCATTCACC</td>
</tr>
<tr>
<td>Pho5</td>
<td>TATGGTACCGCTTTCAATGACGCCGTAAG</td>
</tr>
<tr>
<td>Pho6</td>
<td>TATGGTACCGCACTATAAATCGGCGTGGCG</td>
</tr>
<tr>
<td>Pho7</td>
<td>TATGGTACCGCTTTCAATGACGCCGTAAG</td>
</tr>
<tr>
<td>Pho8</td>
<td>TATGGTACCGCTTTCAATGACGCCGTAAG</td>
</tr>
<tr>
<td>Pho9</td>
<td>TATGGTACCGCTTTCAATGACGCCGTAAG</td>
</tr>
<tr>
<td>Pho10</td>
<td>TATGGTACCGCTTTCAATGACGCCGTAAG</td>
</tr>
<tr>
<td>Lac1</td>
<td>TATGGTACCGCTTTCAATGACGCCGTAAG</td>
</tr>
<tr>
<td>Lac2</td>
<td>TATGGTACCGCCTTTCAATGACGGTGCCG</td>
</tr>
<tr>
<td>Lac3</td>
<td>TATGGTACCGCCTTTCAATGACGGTGCCG</td>
</tr>
<tr>
<td>Lac4</td>
<td>TATGGTACCGCCTTTCAATGACGGTGCCG</td>
</tr>
<tr>
<td>Lac5</td>
<td>TATGGTACCGCCTTTCAATGACGGTGCCG</td>
</tr>
<tr>
<td>Lac6</td>
<td>TATGGTACCGCCTTTCAATGACGGTGCCG</td>
</tr>
<tr>
<td>Lac7</td>
<td>TATGGTACCGCCTTTCAATGACGGTGCCG</td>
</tr>
<tr>
<td>Lac8</td>
<td>TATGGTACCGCCTTTCAATGACGGTGCCG</td>
</tr>
<tr>
<td>Lac9</td>
<td>TATGGTACCGCCTTTCAATGACGGTGCCG</td>
</tr>
<tr>
<td>Oligo1</td>
<td>CCGGAATTCAGGAGGTATAAGATGACAGAAAAAG</td>
</tr>
<tr>
<td>Oligo2</td>
<td>CGGGGTACCTTTATTTTCGATCCGACTTTATTTG</td>
</tr>
<tr>
<td>Oligo3</td>
<td>CGGGGTACCTTTATTTTCGATCCGACTTTATTTG</td>
</tr>
<tr>
<td>Oligo4</td>
<td>CGGGGTACCTTTATTTTCGATCCGACTTTATTTG</td>
</tr>
<tr>
<td>Oligo5</td>
<td>CGGGGTACCTTTATTTTCGATCCGACTTTATTTG</td>
</tr>
<tr>
<td>Oligo6</td>
<td>CGGGGTACCTTTATTTTCGATCCGACTTTATTTG</td>
</tr>
<tr>
<td>Oligo7</td>
<td>CGGGGTACCTTTATTTTCGATCCGACTTTATTTG</td>
</tr>
<tr>
<td>Oligo8</td>
<td>CGGGGTACCTTTATTTTCGATCCGACTTTATTTG</td>
</tr>
<tr>
<td>Oligo9</td>
<td>CGGGGTACCTTTATTTTCGATCCGACTTTATTTG</td>
</tr>
<tr>
<td>Oligo10</td>
<td>CGGGGTACCTTTATTTTCGATCCGACTTTATTTG</td>
</tr>
<tr>
<td>Oligo K540M</td>
<td>GCCCGTCAATTGGTATGACCTTTGTCTGCGCC</td>
</tr>
<tr>
<td>EcoO109I/StuI primer</td>
<td>GAGACGAAAAGGCCTCGTGATAC</td>
</tr>
<tr>
<td>Wza1</td>
<td>CGGGGTACCTTTATTTTCGATCCGACTTTATTTG</td>
</tr>
<tr>
<td>Wza2</td>
<td>CGGGGTACCTTTATTTTCGATCCGACTTTATTTG</td>
</tr>
</tbody>
</table>

**Notes:**

a Start and stop codons are bolded
b Restriction sites are italicized
c RBS sequences are underlined.
FIG. 1
FIG. 2
FIG. 3
FIG. 4
FIG. 5
FIG. 6
Structural organization of protein-tyrosine autokinase Wzc within Escherichia coli cells
Patricia Doublet, Christophe Grangeasse, Brice Obadia, Elisabeth Vaganay and Alain J. Cozzone

J. Biol. Chem. published online July 22, 2002

Access the most updated version of this article at doi: 10.1074/jbc.M204465200

Alerts:
- When this article is cited
- When a correction for this article is posted

Click here to choose from all of JBC's e-mail alerts