Cells of *Escherichia coli* Contain a Protein-Tyrosine Kinase, Wzc, and a Phosphotyrosine-Protein Phosphatase, Wzb

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Two proteins of *Escherichia coli*, termed Wzc and Wzb, were analyzed for their capacity to participate in the reversible phosphorylation of proteins on tyrosine. First, Wzc was overproduced from its specific gene and purified to homogeneity by affinity chromatography. Upon incubation in the presence of radioactive ATP, it was found to effectively autophosphorylate. Two-dimensional analysis of its phosphoamino acid content revealed that it was modified exclusively at tyrosine. Second, Wzb was also overproduced from the corresponding gene and purified to homogeneity by affinity chromatography. It was shown to contain a phosphatase activity capable of cleaving the synthetic substrate *p*-nitrophenyl phosphate into *p*-nitrophenol and free phosphate. In addition, it was assayed on individual phosphorylated amino acids and appeared to dephosphorylate specifically phosphotyrosine, with no effect on phosphoserine or phosphothreonine. Such specificity for phosphotyrosine was confirmed by the observation that Wzb was able to dephosphorylate previously autophosphorylated Wzc. Together, these data demonstrate, for the first time, that *E. coli* cells contain both a protein-tyrosine kinase and a phosphotyrosine-protein phosphatase. They also provide evidence that this phosphatase can utilize the kinase as an endogenous substrate, which suggests the occurrence of a regulatory mechanism connected with reversible protein phosphorylation on tyrosine. From comparative analysis of amino acid sequences, Wzc was found to be similar to a number of proteins present in other bacterial species which are all involved in the synthesis or export of exopolysaccharides. Since these polymers are considered important virulence factors, we suggest that reversible protein phosphorylation on tyrosine may be part of the cascade of reactions that determine the pathogenicity of bacteria.

In eukaryotes, a plethora of protein-tyrosine kinases and phosphotyrosine-protein phosphatases that catalyze the reversible phosphorylation of proteins on tyrosine residues have been detected and shown to play a key role in the regulation of various important biological functions, including signal transduction, growth control, and malignant transformation (15, 22). In prokaryotes, the presence of protein-tyrosine kinase activities was suggested, much later than in eukaryotes, by the finding of phosphotyrosine, first in the proteins of *Escherichia coli* (9) and then in the proteins of a series of other bacterial species (10, 11, 24). On the other hand, the occurrence of phosphotyrosine-protein-phosphatase activities was recently reported for a few examples, such as the IphP protein of *Nostoc commune* UTEX 584 (20), the YopH protein of *Yersinia pseudotuberculosis* (4, 19), and the PtpA protein of *Streptomyces coelicolor* (26). However, in bacteria, the biological significance of reversible protein phosphorylation on tyrosine is still unclear, essentially because for a long time, no individual protein-tyrosine kinase was characterized and no endogenous protein substrate for a phosphotyrosine-protein phosphatase was identified. The only exception so far reported concerns two proteins of *Acinetobacter johnsonii* that harbor opposing activities: the Ptk protein, which has been recently demonstrated to autophosphorylate on several tyrosine residues (14), and the Ptp protein, which has been identified as a phosphotyrosine-protein phosphatase (18). Moreover, in vitro experiments have shown that Ptp is able to specifically dephosphorylate Ptk, which constitutes the first evidence for a reversible protein phosphorylation reaction on tyrosine in bacteria. From these observations, it seemed interesting to determine whether such a reversible tyrosine phosphorylation system was unique and restricted to the bacterial genus *Acinetobacter* or was applicable to other types of bacteria as well.

For that purpose, we analyzed comparatively two proteins of *E. coli*, Wzc and Wzb (33), which exhibit striking sequence similarity with proteins Ptk and Ptp of *A. johnsonii*, respectively, and we checked whether such sequence relationships were linked to functional homologies. Wzc and Wzb are known to participate in the export of the extracellular polysaccharide colanic acid from the cell to medium (33). Wzc is an inner membrane protein that possesses an ATP-binding domain and three predicted transmembrane segments, while Wzb has an amino acid sequence homologous to that of acid phosphatases. The corresponding genes, *wzc* and *wzb*, are adjacent at 46 min on the *E. coli* chromosome and located at the second and third positions, respectively, in order of transcription, within the colanic acid cluster that comprises a total of 19 different genes (33).

In this work, Wzc was overproduced, purified to homogeneity, and shown to autophosphorylate on tyrosine. Wzb, also overproduced and purified, was found to exhibit a protein phosphatase activity with a strict specificity for phosphotyrosine. The functional properties of these two proteins were analyzed, and the phosphorylated form of Wzc was shown to be sensitive to dephosphorylation by Wzb, thus indicating that the Wzc-Wzb pair of *E. coli* is homolog of the Ptk-Ptp pair of *A. johnsonii*.

**MATERIALS AND METHODS**

Bacterial strains and plasmids. *E. coli* JM109 was used as template for PCR amplification of the *wzc* and *wzb* genes. *E. coli* XL1-Blue was used to propagate
plasmids in cloning experiments. E. coli BL21(pREP4-groESL), used for expression experiments, was previously described (1); it was a gift from I. Martin-Verastrae (Pasteur Institute, Paris, France). Plasmid vectors pQE30 and pGEX were kindly provided by T. Verstraete (Pasteur Institute, Paris, France).

**Culture media and growth conditions.** E. coli strains were grown in LB or 2YT medium at 37°C. In the case of strains carrying drug resistance genes, the antibiotics kanamycin, ampicillin, and tetracycline were added to the medium at concentrations of 50 μg/ml, 15 μg/ml, and 10 μg/ml, respectively. Growth was monitored by measuring the absorbance at 660 nm ($A_{660}$).

**DNA manipulation.** Small- and large-scale plasmid isolations were carried out by the alkaline lysis method, and plasmids were purified by using cesium chloride/ethidium bromide gradients. DNA was digested with restriction enzymes purchased from E. coli K-12 (31). All restriction enzymes, calf intestine phosphatase, T4 DNA ligase, and T4 DNA polymerase were used as recommended by the manufacturer (Promega). Transformation of E. coli cells was performed as previously described (31).

**Construction of the wzc and wzb expression plasmids.** Total DNA from E. coli JM109 served as the template in PCR amplification for preparing the wzc and wzb genes with appropriate restriction sites at both ends. For wzc gene cloning, the sequences of the two primers were 5'-GCCGGTA TACCAAGAAAGGATGTTAAAACATCCGCCTGCGG-3' at the N terminus (the BamHI site is italicized; the second codon of wzc is underlined) and 5'-CGGCATTATTCGACCGAACATTCCTTACGATTCG-3' at the C terminus (the EcoRI site is italicized; the stop codon of wzb is underlined). The amplified fragment was digested with restriction enzymes BamHI and EcoRI and ligated into pGEX-KT vector, opened with the same enzymes, to yield plasmid pGEX-wzc.

For wzb gene amplification, the sequences of the primers used were 5'-TAT GCACTGTAAAACATCTTAGTGGTGTGCCTGGC-3' at the N terminus (the BamHI site is italicized; the second codon of wzb is underlined) and 5'-CGGGGATCCATTAATACGCTGTGCTTAGTACG-3' at the C terminus (the KpnI site is italicized; the stop codon of wzb is underlined). The synthesized DNA was digested with BamHI and KpnI and ligated into pQE30 vector, opened with the same enzymes. The resulting plasmid was termed pQE30-wzb.

In each case, the nucleotide sequence of the synthesized gene was checked by dideoxy nucleotide sequencing (32).

**Purification of protein Wzc.** E. coli BL21(pREP4-groESL) cells were transformed with plasmid pGEX-wzc. Cells from this strain were used to inoculate 1 liter of 2YT medium supplemented with ampicillin and kanamycin and were incubated at 37°C under shaking until the $A_{660}$ reached 0.8. Isopropyl-$β$-D-thiogalactopyranoside (IPTG) was then added to a final concentration of 0.1 mM, and growth was continued for 2 h at 30°C under shaking. Cells were harvested by centrifugation at 3,000 × g for 10 min and suspended in 12 ml of buffer A (10 mM sodium phosphate [pH 7.4], 150 mM NaCl, 1 mM EDTA, 10% glycerol) containing 1 mM phenylmethylsulfonl fluoride plus DNAse I and Rnaase A, each at a final concentration of 100 μg/ml. Cells were disrupted in a French pressure cell at 16,000 lb/in². The resulting suspension was supplemented with Triton X-100 at a final concentration of 1% and centrifuged at 4°C for 20 min at 30,000 × g. The supernatant was incubated for 30 min at 4°C with 0.1% Triton X-100 and 5 mM glutathione. Eluted fractions were analyzed by SDS-PAGE (25).

**Purification of protein Wzb.** E. coli BL21(pREP4-groESL) cells were transformed with plasmid pQE30-wzb. Cells from this strain were also used to inoculate 100 ml of LB medium supplemented with ampicillin and kanamycin and were incubated at 37°C under shaking until the $A_{660}$ reached 0.7. IPTG was then added at a final concentration of 0.5 mM, and growth was continued for 5 min. One-dimensional gel electrophoresis was performed as previously described (25). In an alternative procedure used for two-dimensional gel analysis, after 10 min of incubation, the protein was precipitated with 5 vol of acetone for 30 min at -20°C and centrifuged for 5 min at 30,000 × g before dissolution in the loading buffer (29). After electrophoresis, gels were soaked in 16% trichloroacetic acid (TCA) for 10 min at 90°C. They were stained with Coomasie blue, and radioactive proteins were visualized by autoradiography.

**Phosphatase assay.** Acid phosphatase activity was monitored at 37°C by using a continuous method based on the detection of p-nitrophenol formed from p-nitrophenyl phosphate (PNPP). Rates of phosphohydrolase were determined at 405 nm in a reaction buffer containing 100 mM sodium citrate (pH 6.5), 1 mM EDTA, 0.1% β-mercaptoethanol, and PNPP at a concentration varying from 0.5 to 40 mM. The amount of p-nitrophenol released was estimated by using a molar extinction coefficient of $A_{405}$ of 18,000 M⁻¹ cm⁻¹ (8). The assay was optimized with respect to protein concentration, time, and pH.

Phosphotyrosine phosphatase (PTPase) activity was assayed at 37°C in a 50-μl reaction volume containing 10 mM O-phosphoseryl phosphate as the substrate, 1 mM EDTA, 100 mM sodium citrate (pH 6.5), and 1 μg of purified Wzb. After 15 min of incubation, the reaction was stopped by addition of 150 μl of 10 mM 50% of bovine serum albumin (10 mg/ml). The precipitated protein was removed by centrifugation, and the supernatant was used for measurement of released inorganic phosphate by using 1 volume of a mixture containing 1 M sulfuric acid, 0.5% ammonium molybdate, and 2% ascorbic acid. Samples were heated at 56°C for 15 min, and the $A_{660}$ was measured (7, 28).

**RESULTS**

The starting point of this study was the comparative analysis of the amino acid sequence deduced from the nucleotide sequence of the ptk gene of A. johnsonii (17) with the different amino acid sequences deduced from the E. coli genome (3). By using the Swissprot database, we detected a striking sequence similarity between protein Ptk and the previously described (33) E. coli protein Wzc. Indeed, the best-fit sequence alignments showed that these two proteins exhibit 36% identity and 61% similarity (Fig. 1). Since Ptk is known to autophosphorylate on multiple tyrosine residues (14), it was of interest to assay also Wzc for phosphorylation. For that purpose, it was first necessary to overproduce and purify this protein.

**Overproduction and purification of Wzc.** The wzc gene lacking the start codon was synthesized by PCR, by using oligonucleotide primers deduced from the wzc gene sequence (33). The amplified DNA was cloned in plasmid pGEX-KT previously digested with restriction enzymes BamHI and EcoRI. The resulting plasmid, termed pGEX-wzc, expressed a fusion protein consisting of Wzc with GST at its N terminus (Fig. 2). This construct was used to transform competent cells from 50 mM Tris-HCl (pH 7.4), 100 mM NaCl, 1 mM EDTA, 20% glycerol, and 5 mM dithiothreitol (DTT) at -20°C.

In vitro phosphorylation assay. In vitro phosphorylation of about 3 μg of purified GST-Wzc protein was performed at 30°C in 10 μl of a buffer containing 25 mM Tris-HCl (pH 7.0), 1 mM DTT, 5 mM MgCl₂, 1 mM EDTA, and 10 μM ATP with 200 μCi of [γ-32P]ATP ml⁻¹. After 10 min of incubation, the reaction was stopped by addition of an equal volume of 2× sample buffer, and the mixture was heated at 100°C for 5 min. One-dimensional gel electrophoresis was performed as previously described (25). In an alternative procedure used for two-dimensional gel analysis, after 10 min of incubation, the protein was precipitated with 5 vol of acetone for 30 min at -20°C and centrifuged for 5 min at 30,000 × g before dissolution in the loading buffer (29). After electrophoresis, gels were soaked in 16% trichloroacetic acid (TCA) for 10 min at 90°C. They were stained with Coomasie blue, and radioactive proteins were visualized by autoradiography.
E. coli BL21(pREP4-groESL). This strain overproduces the two chaperone proteins GroES and GroEL and is suitable for the overproduction of proteins that possess a high degree of hydrophobicity and thus a tendency to aggregate, such as Wzc. Upon induction by IPTG, efficient overexpression of a 105-kDa protein, consistent with the calculated molecular mass of the fusion protein, was obtained in the soluble fraction of cells. The GST-Wzc fusion protein was then purified to homogeneity in a two-step chromatographic procedure consisting of an affinity chromatography on glutathione-Sepharose 4B matrix followed by an anion-exchange chromatography on a Q-Sepharose column. In these conditions, about 1 mg of GST-Wzc protein was obtained from 1 liter of bacterial culture.

Autophosphorylation of Wzc at tyrosine. For comparison with Ptk, the GST-Wzc protein was assayed for phosphorylation. It was observed that purified GST-Wzc was significantly labeled in vitro in the presence of \([\gamma-32P]ATP\) (Fig. 3A). The ability of GST-Wzc to phosphorylate in these conditions indicated that it contains an intrinsic protein kinase activity that catalyzes its autophosphorylation. As a control, the phosphorylated fusion protein was submitted to proteolysis by thrombin to cleave Wzc from the linked GST, and the location of the bound radioactivity was determined. It was observed that the radioactive labeling of the fusion protein was due exclusively to the phosphorylation of the Wzc protein, while no radioactivity was present on GST (Fig. 3A).

The phosphoamino acid content of the labeled protein was determined after acid hydrolysis and two-dimensional analysis. In these conditions, only acid-resistant phosphoamino acids were analyzed since a number of other phosphorylated compounds, such as phosphohistidine, phosphoarginine, or phosphoaspartate, are known to be labile in acid (13). Only phosphotyrosine was revealed on the corresponding autoradiogram (Fig. 3B), which indicated that GST-Wzc was modified exclusively at tyrosine residues. To obtain more information on the phosphorylation state of GST-Wzc, the purified protein was phosphorylated in vitro and then analyzed by two-dimensional gel electrophoresis. Interestingly, this gel, stained with Coomassie blue, and the corresponding autoradiogram revealed a series of spots with the same molecular mass and a different isoelectric point, which likely correspond to a varying degree of phosphorylation of the protein (data not shown) as previously observed for Ptk (14). Wzc, like Ptk and other Wzc homologs,
Wzc was incubated with \([-32P]ATP\). The protein was analyzed by SDS-PAGE; in the N-terminal parts of numerous low-M\(_r\) proteins, both appeared to contain the CX5R(S/T) motif which is found similar over their entire lengths (Fig. 4). In particular, they two proteins showed that they were 33% identical and 58% similarity between the phosphotyrosine-protein phosphatase Ptp of eukaryotic cells (8, 35).

Further searches in the Swissprot database revealed, on the other hand, a high similarity of Wzb, especially its enzymatic activity on dephosphorylatable substrates, in more detail. For this, it was first necessary, as previously done for Wzc, to overproduce and purify the protein. The oligonucleotide primers corresponding to the 5' and 3' ends of the wzb gene (33) were prepared with the appropriate restriction sites at both ends. The wzb gene lacking the start codon ATG was then synthesized by PCR and cloned in the expression vector pQE30 from E. coli, previously digested with the restriction enzymes BamHI and KpnI. The resulting plasmid pQE30-wzb allowed production of the Wzb protein with an N-terminal addition of 11 amino acids, including 6 histidines (Fig. 5). It was used to transform competent cells of E. coli BL21(pREP4-groESL). Upon induction with 0.5 mM IPTG, a relatively high level of a 19-kDa protein, consistent with the calculated molecular mass of the fusion protein His\(_6\)-Wzb, was obtained in the soluble fraction of cells. The fusion protein was then purified to homogeneity in a single-step chromatographic procedure by using a Zn\(^{2+}\)-immobilized matrix generally used for purifying His-tagged proteins. In these conditions, about 1 mg of pure protein was obtained from 100 ml of bacterial culture.

**Phosphotyrosine-protein phosphatase activity of Wzb.** The phosphatase activity of His\(_6\)-Wzb was first assayed for its ability to cleave NPPIP. It was observed that the protein could efficiently hydrolyze this synthetic substrate at an optimum pH value of 6.5. The corresponding kinetic constants, \(K_m\) and \(V_{max}\) measured at 37°C, were 1 mM and 4.6 \(\mu\)mol min\(^{-1}\) mg\(^{-1}\), respectively. These values are in the same range as those previously reported for eukaryotic low-\(M_r\) PTPases such as bovine heart phosphatase (34).

Further experiments were performed to measure the in vitro activity of Wzb on individual phosphorylated amino acids. Wzb was shown to quantitatively release inorganic phosphate from phosphotyrosine but had no effect on either phosphoserine or phosphothreonine. This result is consistent with a strict specificity of the dephosphorylating activity of Wzb for phosphotyrosine, which is a general property of the low-\(M_r\) PTPases of eukaryotic cells (8, 35).

**Endogenous substrate for Wzb.** At this stage, two proteins of E. coli harboring opposing activities had been identified: the Wzc protein, which is able to autophosphorylate on tyrosine residues, and the Wzb protein, which possesses the characteristics of a phosphotyrosine-protein phosphatase. In view of a possible regulation of bacterial physiology by reversible protein phosphorylation, further studies were made to obtain the Wzc protein in its native state, i.e., without GST at its N terminus, after cleavage by thrombin. The protein thus obtained had no more autophosphorylating activity. This loss of activity might be related to the aggregation of the protein, due to its high degree of hydrophobicity. The oligonucleotide primers corresponding to the 5' and 3' ends of the wzb gene (33) were prepared with the appropriate restriction sites at both ends. The wzb gene lacking the start codon ATG was then synthesized by PCR and cloned in the expression vector pQE30 from A. johnsonii. Identification of amino acids are indicated by asterisks, and high similarity is indicated by double dots. The phosphatase specific with the calculated molecular mass of the fusion protein His\(_6\)-Wzb, was obtained in the soluble fraction of cells. The fusion protein was then purified to homogeneity in a single-step chromatographic procedure by using a Zn\(^{2+}\)-immobilized matrix generally used for purifying His-tagged proteins. In these conditions, about 1 mg of pure protein was obtained from 100 ml of bacterial culture.

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phosphorylation on tyrosine, it was then of special interest to check whether Wzb could utilize Wzc as an endogenous substrate and catalyze its dephosphorylation. For this, the purified Wzc protein was first radioactively labeled in the presence of $[^{32}\text{P}]{\text{ATP}}$ and then incubated in the presence of Wzb. The results presented in Fig. 6 clearly indicate that in these conditions, Wzc was rapidly and extensively dephosphorylated by Wzb. These data provide evidence that Wzb can use Wzc as an endogenous substrate and support the concept that the enzymatic activity of the phosphorylatable kinase Wzc is regulated by the dephosphorylating activity of Wzb.

Considering the high similarity between, on the one hand, the phosphorylatable proteins Ptk and Wzc and, on the other hand, the phosphotyrosine-protein phosphatases Ptp and Wzb, it was interesting to see whether these proteins could cross-react. For that purpose, Wzc from *E. coli* and Ptk from *A. johnsonii* were labeled in vitro in the presence of $[^{32}\text{P}]{\text{ATP}}$ and then assayed for dephosphorylation by using either Wzb from *E. coli* or Ptp from *A. johnsonii* as the protein phosphatase. It appeared that Wzb could dephosphorylate protein Ptk (Fig. 7, lane 5) with the same efficiency as Ptp (Fig. 7, lane 6). Conversely, Ptp protein could catalyze the extensive dephosphorylation of Wzc (Fig. 7, lane 3) as well as Wzb (Fig. 7, lane 2).

**DISCUSSION**

The main result of this study is the demonstration that two proteins of *E. coli*, Wzc and Wzb, carry an autophosphorylating protein-tyrosine kinase activity and a phosphotyrosine-protein phosphatase activity, respectively. The presence of a protein-tyrosine kinase activity in *E. coli* had been previously suggested by the original finding of phosphotyrosine in an acid hydrolysate prepared from the total protein fraction of this bacterium (27), and it was further documented by the detection of a phosphoprotein of unknown function, termed TypA, modified selectively at tyrosine (16). But no evidence had been adduced for the occurrence of a specific kinase responsible for such modification of proteins. Our results now show, for the first time, that a phosphorylating enzyme of this type, Wzc, is indeed present in *E. coli* cells. Similarly, our data show that *E. coli* harbors a phosphotyrosine-protein phosphatase, Wzb, with the same biochemical characteristics as those of several low-M$_r$ acid phosphotyrosine-protein phosphatases, namely, of eukaryotic origin, previously described by other authors (8, 35). Here again, this is the first evidence for an enzyme of this type in *E. coli* cells. Of particular interest is the further finding that Wzb can dephosphorylate in vitro Wzc, which thus appears as a specific endogenous substrate for Wzb. This observation supports the existence, to be tested, of a regulatory mechanism of bacterial physiology operating by reversible protein phosphorylation on tyrosine.

Interestingly, the same possibility was previously envisaged for *A. johnsonii*. Indeed, we have recently identified two genes,
monas solanacearum (21), and phosphorylation may be part of the cascade of reactions that many pathogens. On this basis, we suggest that protein tyrosine and non specific host immunity, and prevention of desiccation ridges. It has been widely demonstrated that cell surface poly-

The finding that the Wzc-Wzb pair of proteins of E. coli is a homolog of the Ptk-Ptp pair of A. johnsonii proteins confirms that similar activities can be predicted from sequence relationships. Therefore, one can expect that comparable pairs of proteins acting in the same dual manner would exist in other bacterial species as well. Indeed, some genes similar to wzc and wzb have been detected in various bacteria, including amxA and amxA1 in Erwinia amylovora (5, 6), epsB and epsP in Pseudomonas solanacearum (21), and orf6 and orf5 in Klebsiella pneumoniae (2). They all belong to gene clusters involved in the synthesis or transport of exopolysaccharides and are present only in these clusters, but their specific functions are unknown. It would be worthwhile to check for the protein-tyrosine kinase and phosphotyrosine-protein phosphatase activities of the proteins encoded by these different genes and thus to assess the general nature of the relationship between reversible tyrosine phosphorylation of proteins and production of polysaccharides. It has been widely demonstrated that cell surface polysaccharides play a critical role in a number of important biological processes, including adherence, resistance to specific and non specific host immunity, and prevention of desiccation (30). Exopolysaccharides also mediate direct interaction between bacteria and their immediate environment and, for that reason, are considered an important factor in the virulence of many pathogens. On this basis, we suggest that protein tyrosine phosphorylation may be part of the cascade of reactions that determine the pathogenicity of bacteria.

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