Tyrosine Phosphorylation of the UDP-Glucose Dehydrogenase of Escherichia coli Is at the Crossroads of Colanic Acid Synthesis and Polymyxin Resistance

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Abstract

Background: In recent years, an idiosyncratic new class of bacterial enzymes, named BY-kinases, has been shown to catalyze protein-tyrosine phosphorylation. These enzymes share no structural and functional similarities with their eukaryotic counterparts and, to date, only few substrates of BY-kinases have been characterized. BY-kinases have been shown to participate in various physiological processes. Nevertheless, we are at a very early stage of defining their importance in the bacterial cell. In Escherichia coli, two BY-kinases, Wzc and Etk, have been characterized biochemically. Wzc has been shown to phosphorylate the UDP-glucose dehydrogenase Ugd in vitro. Not only is Ugd involved in the biosynthesis of extracellular polysaccharides, but also in the production of UDP-4-amino-4-deoxy-L-arabinose, a compound that renders E. coli resistant to cationic antimicrobial peptides.

Methodology/Principal Findings: Here, we studied the role of Ugd phosphorylation. We first confirmed in vivo the phosphorylation of Ugd by Wzc and we demonstrated that Ugd is also phosphorylated by Etk, the other BY-kinase identified in E. coli. Tyrosine 71 (Tyr71) was characterized as the Ugd site phosphorylated by both Wzc and Etk. The regulatory role of Tyr71 phosphorylation on Ugd activity was then assessed and Tyr71 mutation was found to prevent Ugd activation by phosphorylation. Further, Ugd phosphorylation by Wzc or Etk was shown to serve distinct physiological purposes. Phosphorylation of Ugd by Wzc was found to participate in the regulation of the amount of the exopolysaccharide colanic acid, whereas Etk-mediated Ugd phosphorylation appeared to participate in the resistance of E. coli to the antibiotic polymyxin.

Conclusions/Significance: Ugd phosphorylation seems to be at the junction between two distinct biosynthetic pathways, illustrating the regulatory potential of tyrosine phosphorylation in bacterial physiology.

Introduction

In bacteria, protein phosphorylation is catalyzed mainly by histidine-kinases which are key enzymes of the so-called “two component systems” [1], by proteins of the phosphotransferase system involved in sugar transport and phosphorylation as well as many regulatory functions and by Hanks-type Serine/Threonine Protein Kinases (STPKs) [2]. However, the presence of tyrosine-kinases has been proven in several bacterial species, and suggested in many more by homology-based gene annotation. These tyrosine-kinases share little structural similarities with their eukaryotic counterparts [3,4] and most of them have been recently unified in a new enzyme family called BY-kinase [5]. Until now, BY-kinases have been found only in bacteria and they seem to constitute an idiosyncratic class of enzymes. They have been shown to be involved in several physiological processes such as DNA metabolism or heat shock response [6,7]. In several bacteria, including both proteobacteria and firmicutes, they have also been established as co-polymerases involved in synthesis and export of extracellular polysaccharides [8,9]. However, their accurate functions remain poorly understood due to slow progress in structural characterization and to the fact that only few phosphorylation substrates have been detected. BY-kinases are autophosphorylating enzymes and they are also able to phosphorylate endogenous proteins. It was only recently that sugar-dehydrogenases or -transferases involved in polysaccharide production [10–13], RNA polymerase sigma factors [7] and single-stranded DNA binding proteins [14] were identified as BY-kinase substrates. Nevertheless, recent phosphoproteomic studies indicate that BY-kinases could phosphorylate a significant number of other proteins [15,16].

Escherichia coli produces two BY-kinases, Wzc and Etk. They are respectively encoded by genes located at approximately 46 min and 22 min on the E. coli chromosome in two gene-clusters both

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involved in the biosynthesis of extracellular polysaccharides [17,18]. Accordingly, Wzc and Etk have been characterized as polysaccharide co-polymers (PCP) belonging to multiprotein transmembrane machineries involved in synthesis and/or export of extracellular polysaccharides, and their autophosphorylation on several tyrosines has turned out to be a key feature in the production of these compounds [19]. In addition, it has been demonstrated that Wzc and Etk are involved in other processes. For instance, Wzc is able to phosphorylate and down regulate the activity of Asc, the integrase of coliphage HK022 [20]. In the same way, Etk has been found to be involved in heat shock response by phosphorylating sigma and anti-sigma factors [7].

Protein Ugd of *E. coli*, a UDP-glucose dehydrogenase, is one of the first identified substrates of a BY-kinase [10]. Indeed, it has been shown that Ugd is phosphorylated *in vitro* by the BY-kinase Wzc. Other types of sugar-dehydrogenases, including the UDP-glucose dehydrogenase of *Bacillus subtilis* Ugdhs [21] and the UDP-acetyl-mannosamine dehydrogenase CapO of *Staphylococcus aureus* [12], have recently been shown to be tyrosine-phosphorylated. Thus, it could be speculated that tyrosine phosphorylation of this class of enzymes is a common regulatory mechanism found in several bacteria. Ugd produces UDP-glucuronic acid (UDPGA) that is a precursor and an essential component in the biosynthesis of bacterial polysaccharides, notably of *E. coli* [19]. UDPGA is found in several capsular polysaccharides (K-antigens) and in colanic acid (M-antigen), an extracellular polysaccharide produced by many strains of *E. coli*. In addition, UDPGA participates in the production of a sugar derivative, UDP-4-amino-4-deoxy-L-arabinose (L-Ara4N) which is a crucial element in bacterial resistance to antibiotics such as polymyxin and cationic peptides of the innate immune system [22–25]. Thus, in the search of the role of tyrosine phosphorylation in bacteria, the question was raised whether Ugd phosphorylation could affect, on the one hand, the production of polysaccharides and, on the other hand, bacterial resistance to cationic peptides. In *E. coli* K-12, we had previously demonstrated that Wzc autophosphorylation influences the production of colanic acid, [26]. In addition, we had also shown that an etk knock-out mutant is much less resistant to polymyxin than a wild-type *E. coli* K-12 strain. [4,27]. However, Ugd has not been described as being phosphorylated by Etk. Therefore, it seemed particularly interesting to investigate if Ugd is also a phosphorylation substrate for Etk and if Ugd phosphorylation by either Wzc or Etk affects colanic acid production or polymyxin resistance, respectively.

In this work, we provide *in vivo* evidence that, depending on the growth conditions, Ugd can be phosphorylated by either Wzc or Etk. We show that Ugd is phosphorylated on the same site by both BY-kinases, Wzc and Etk. We further show that Wzc-mediated phosphorylation of Ugd specifically affects the biosynthesis of colanic acid whereas resistance to the cationic antimicrobial peptide polymyxin is dependent upon Ugd phosphorylation by Etk. These data represent the first report of a bacterial protein phosphorylated by two distinct tyrosine-kinases. They contribute to define the role of tyrosine phosphorylation in bacteria and provide a basis for an emerging regulatory network in *E. coli*.

**Results**

**Ugd is phosphorylated *in vivo* both by Wzc and Etk**

We have previously reported that Ugd is monophosphorylated *in vitro* by the catalytic domain of Wzc, Wzc<sub>cat</sub> [10] (Table S1). The Wzc protein is not synthesized in standard growth conditions and to characterize *in vivo* phosphorylation of Ugd by Wzc, we used a strain that encodes the transcriptional regulator RcsA [26]. RcsA is known to enhance the expression of *wzc* and *ugd* [28,29] (Table S1) but not that of *etk*, which is not expressed in our culture conditions [30,31]. After growth in Luria Bertani medium, a total protein extract was prepared, analyzed by SDS-PAGE and immunoblotted against either a monoclonal anti-Wzc antibody, or a monoclonal anti-Ugd antibody, or an anti- phosphotyrosine antibody (Fig. 1A). Immunoreaction with the Wzc- and Ugd-specific antibodies showed signals at around 81-kDa and 45-kDa, which are respectively consistent with the expression of the two proteins. The anti-phosphotyrosine antibody revealed an expected signal corresponding to autophosphorylated Wzc. Also, we observed a 45-kDa signal that could correspond to the phosphorylated form of Ugd. To strengthen these observations, we similarly analyzed a *wzc* knock-out mutant [26]. We did not detect any signal for Wzc or phosphotyrosine around 45-kDa, whereas Ugd was still detected by the Ugd-specific antibody (Fig. 1A). In addition, the *ugd* gene was inactivated (Table S1) and the Δugd strain obtained was analyzed as described above (Fig. 1A). We still observed the expression and phosphorylation of Wzc, but no 45-kDa signal was detected by either the Ugd-specific antibody or the anti-phosphotyrosine antibody. These data confirmed that Ugd was phosphorylated *in vivo* on tyrosine by Wzc.

*E. coli* cells encode another BY-kinase, namely Etk, that is homologous to Wzc. We wondered whether Etk was also able to phosphorylate Ugd. Like Wzc, Etk is not produced by *E. coli* K-12 under standard laboratory growth conditions. In addition, *etk* expression is not dependent on protein RcsA. It has previously been shown that *etk*, but not *wzc*, is expressed when *E. coli* K-12 grows in a culture medium at low pH and low concentration of magnesium and iron ions. Such conditions induce resistance to cationic antimicrobial peptides [27]. We verified that *etk* was expressed during growth of *E. coli* K-12 in such medium. No 81-kDa signal was observed when detection was carried out with the Wzc-specific antibody whereas a signal appeared at this position when we used the anti-phosphotyrosine antibody (Fig. 1B). This observation confirmed that *wzc* is not expressed and strongly suggested that autophosphorylated Etk is produced under these culture conditions. To validate this point, we analyzed an *etk*-deficient strain. Immunoblot analysis showed no 81-kDa signal and supported the assumption that the signal detected with the anti-phosphotyrosine antibody in the wild-type strain corresponded to Etk autophosphorylation. In addition, RT-PCR experiments were performed and we observed that the *etk* gene was effectively expressed under these growth conditions (data not shown). To assess if Etk can phosphorylate Ugd, we also analyzed total protein extracts of wild-type, *ugd*-deficient and *etk*-deficient strains of *E. coli* by immunoblotting with the Ugd-specific antibody and the anti-phosphotyrosine antibody (Fig. 1B). We observed that Ugd was produced and phosphorylated in the wild-type strain. On the contrary, no 45-kDa phosphorylation signal was detected with *ugd* and *etk* knock-out mutants while Ugd was still detected in the *etk* mutant. These observations confirmed that Ugd was also phosphorylated *in vivo* by Etk.

**Ugd is activated by phosphorylation on Tyr71**

To decipher the role of Ugd phosphorylation, it was necessary to identify the site of phosphorylation of *E. coli* Ugd. We had already observed that Ugd seemed to be monophosphorylated [10]. Mass spectrometry analysis (MALDI-TOF) failed to identify the Ugd phosphorylated tyrosine probably because of the low occupancy of bacterial phosphorylation sites [32]. Therefore, sequence alignments were performed with protein Ugdhs, a UDP-sugar-dehydrogenase from *Bacillus subtilis* homologous to Ugd and also phosphorylated on tyrosine (Fig. S1A). Several tyrosines of
Ugd (Tyr10, Tyr150, Tyr249, Tyr335 and Tyr380) that seemed conserved in Ugd Bs were mutated to phenylalanine. However, when each mutant protein was purified and phosphorylated in vitro by the catalytic domain of Wzc, Wzc cyto, a radioactive signal corresponding to phosphorylated Ugd was still detected for each of them (Fig. S1B). While this work was in progress, a study of the *B. subtilis* phosphoproteome indicated that UgdBs was phosphorylated in vivo on tyrosine 70 [16]. According to sequence alignments, the closest tyrosine in the *E. coli* Ugd sequence is Tyrosine 71 (Tyr71) (Fig. S1A). We hypothesized that Tyr71 could be the phosphorylation site of Ugd and we constructed the Ugd mutant Tyr71Phe (UgdY71F).

To check this hypothesis, the phosphorylation signal of Ugd was compared with that of UgdY71F. When incubated for 2 min with Wzccyto (kinase/substrate ratio of 1/100), we observed that Ugd was phosphorylated whereas no radioactive signal was detected for UgdY71F (Fig. 2A). These data suggested that Tyr71 is the phosphorylation site of Ugd. To check that the folding of the Ugd protein was not affected by the Tyr to Phe mutation, dynamic light scattering (DLS) measurements were performed. The hydrodynamic radius measured for Ugd and UgdY71F were the same ($R_h = 62^{+/-}3 \ \text{Å}$) confirming that the overall structure of Ugd was most likely not affected by the Tyr to Phe mutation. To confirm Tyr71 as the phosphorylation site responsible for activating the enzyme, the dehydrogenase activity of Ugd and UgdY71F was measured with or without prior incubation with Wzccyto, and ATP (Fig. 2B). We have previously shown that Ugd phosphorylation increased its dehydrogenase activity [10]. As expected, Ugd activity was stimulated more than 10-fold upon phosphorylation. By contrast, UgdY71F activity remained unaffected by incubation with Wzccyto. In addition, UgdY71F was less active than Ugd which is in agreement with our previous observations showing that Ugd purified from *E. coli* is partially phosphorylated, and that its extensive dephosphorylation by the phosphotyrosine phosphatase Wzb reduces its activity [10]. These data indicated that UgdY71F was no longer activated by phosphorylation and we concluded that Tyr71 was the regulatory phosphorylation site of Ugd.

**Ugd phosphorylation by Wzc on Tyr71 influences the production of colanic acid**

The *cps* operon that includes *wzc*, and the *ugd* gene that is located elsewhere on the genome, are required for the synthesis of colanic acid, the extracellular polysaccharide of *E. coli* K-12. Expression of both *cps* operon and *ugd* are dependent on the Rcs
system [29] and, as previously shown, the strain producing the transcriptional regulator RcsA is able to synthesize colanic acid [26]. In this strain, we showed that Ugd is phosphorylated in vivo by Wzc (Fig. 1A). We therefore used this strain (referred to herein as the wild-type) to assess the importance of Ugd phosphorylation on colanic acid synthesis. First, we confirmed that Ugd Tyr71 was not phosphorylated in vivo by Wzc. For this, we complemented the ugdl-deficient strain (Dugd strain) with an episomal copy of either the ugdl gene (Dugd+ugd strain) or the ugdlY71F allele (Dugd+ugdY71F strain) (Table S1). Total protein extracts were prepared from these two strains and the protein synthesis and phosphorylation for both Wzc and Ugd were analyzed by antibodies as described above (Fig. 1A). We observed that proteins Wzc, Ugd, and UgdY71F, were produced. In contrast, Ugd was phosphorylated only in the strain Dugd+ugd. Indeed, no 45-kDa phosphorylation signal was detected for the strain producing UgdY71F. These data confirmed that in vivo phosphorylation of Ugd by Wzc occurs at Tyr71.

E. coli K-12 producing colanic acid exhibits a mucoid phenotype, and on plates, colonies have a fatty and shiny appearance. In contrast, smaller and duller colonies are observed when colanic acid is not produced. Therefore, we compared the colony morphology of strains producing Ugd, wild-type form or mutated on Tyr71 (Fig. 3A). We first observed that colony morphologies of the wild-type and Dugd+ugd strains were almost indistinguishable and they had characteristics of a mucoid phenotype due to the production of colanic acid (Fig. 3A). By contrast, colonies formed by the Δugd strain differed considerably and were not mucoid. When looking at the colony morphology of Δugd+ugdY71F strain, we also observed a non-mucoid phenotype similar to the Δugd strain (Fig. 3A). These data supported the hypothesis that the Wzc-mediated phosphorylation of Ugd on Tyr71 controls colanic acid production. To strengthen this observation, we prepared and quantified colanic acid produced by each strain used (Fig. 3B). Quantification was performed by measuring the amount of fucose, which is exclusively found in this polysaccharide [17]. As expected, the wild-type and Dugd+ugd strains produced comparable amounts of colanic acid, whereas the amount of colanic acid determined for the Δugd strain was up to 10-fold lower. Concerning the Δugd+ugdY71F strain, a 5-fold reduction was measured compared to Dugd+ugd strain. Colanic acid still produced by the Δugd+ugdY71F strain was likely due to the basal activity of UgdY71F (Fig. 2B). Accordingly, colony morphology of Δugd+ugdY71F strain began to be mucoid when

Figure 2. Activation of the UDP-glucose dehydrogenase activity of Ugd by phosphorylation on Tyr71. (A) Autoradiography of SDS-PAGE on which reaction mixtures containing [γ-32P]ATP and either Ugd and Wzccyto (lane 1) or UgdY71F and Wzccyto (lane 2) or Ugd and Etkcyto (lane 3) or UgdY71F and Etkcyto (lane 4) were analyzed. (B) UDP-glucose dehydrogenase activity was monitored at 340 nm for 10 min by measuring NADH formation: Ugd (□), Ugd previously phosphorylated by Wzccyto (■), UgdY71F (○) and UgdY71F previously incubated with Wzccyto (▲). As a control, a reaction mixture without Ugd was used (*). Standard deviations are not indicated because of low variations.
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Figure 3. Influence of Ugd Tyr71 phosphorylation on colanic acid production. (A) Colony morphology of E. coli wt and mutants. Photographs were taken after growth on LB Agar plates for 24 h at 37 C. (B) Production of colanic acid by wild-type E. coli and mutants. The amount of colanic acid was determined in each strain by measuring fucose, and was expressed as μg/L of culture of A600 = 1. Standard deviations from four independent experiments are indicated with error bars.
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growth was allowed for more than 48 hours (data not shown). These data nevertheless confirm the two distinct phenotypes: mucoid for the wild-type and Δugd+ugd strains, and non-mucoid for the Δugd and Δugd+ugdY71F strains. They also confirm that phosphorylation of UgdTyr71 by Wzc affects colanic acid production.

Ugd phosphorylation by Etk on Tyr71 influences polymyxin resistance

In E. coli K-12, Ugd catalyzes the first step of the biosynthesis of L-Ara4N, which confers bacterial resistance to cationic antimicrobial peptides and antibiotics such as polymyxin [33]. It has been previously reported that E. coli resistance to polymyxin depends also on the expression of etk [4,27]. Here, we showed that Ugd was phosphorylated in vivo by Etk when E. coli was grown under culture conditions allowing resistance to polymyxin [34] (Fig. 1B). Therefore, it was tempting to assume that Etk could regulate polymyxin resistance by phosphorylating Ugd. We therefore tested whether Etk-catalyzed in vivo phosphorylation of Ugd would also occur on Tyr71. For this, the Δugd+ugd and the Δugd+ugdY71F strains were grown in the conditions allowing polymyxin resistance and total protein extracts were prepared and analyzed by SDS-PAGE and immunoblotting. As shown in Fig. 1B, detection with the Ugd-specific antibody indicated that Ugd was produced in both Δugd+ugd and Δugd+ugdY71F strains. However, when immunoblotted with the anti-phosphotyrosine antibody, a phosphorylation signal was detected only when the wild-type form of Ugd was produced. This observation confirmed that Ugd was specifically phosphorylated in vivo on Tyr71 by Etk and that Ugd Tyr71 phosphorylation could consequently influence polymyxin resistance.

To check this, each strain was assayed for polymyxin resistance by measuring the percentage of surviving cells (Fig. 4). E. coli strains were grown in the culture medium allowing resistance to polymyxin, incubated for 1 hour in the presence of varying concentrations of polymyxin and plated onto agar-LB plates. Survival rates are expressed as the percentage of the number of colonies formed with a strain grown in the presence of polymyxin with respect to the number of colonies obtained with the same strain grown in the absence of the antibiotic. Optimal resistance of the wild-type strain was 39% or 25% survival in the presence of 2.5 or 5 μg/ml polymyxin, respectively. As expected, the Δugd strain exhibited 7% to 1% survival, which represents only 17% to 3% of the wild-type strain resistance (Fig. 4). The Δugd+ugd strain survival values were not significantly different from those of the wild-type strain. By contrast, the strain expressing UgdY71F showed a survival level decreased over 2 and 3-fold depending on the polymyxin concentration (Fig. 4). We therefore conclude that Ugd phosphorylation by Etk on Tyr71 participates in polymyxin resistance.

Discussion

In this work, we showed that Ugd of E. coli is phosphorylated on tyrosine 71 by the BY-kinases Wzc and Etk. This finding constitutes the first example of a bacterial protein phosphorylated on tyrosine by two distinct BY-kinases (Fig. 5). In E. coli, so far only 3 proteins have been characterized as being phosphorylated by Etk or Wzc [7,20]. However, no less than 7 other proteins have recently been found to be tyrosine phosphorylated in E. coli cells grown under standard laboratory conditions [15]. These proteins carry out functions as diverse as tRNA synthesis, transport of amino acids, protein translation and stress response. Since, Wzc and Etk are the only tyrosine kinases characterized in E. coli, it is possible that they phosphorylate these proteins. Alternatively, presently unidentified tyrosine kinases could be present in E. coli. To support the first hypothesis, it has been demonstrated that the colanic acid operon (ugs), which includes 19 genes, contains a stem-loop transcriptional attenuator which is located immediately after the third gene, namely wze [35]. wze might therefore be expressed independently of the other ugs genes and be involved in the phosphorylation of other proteins so as to regulate other cellular functions. There are also arguments for the second hypothesis, namely that other tyrosine kinases could be encoded by the genome of E. coli. Several proteins of unknown function harbor the Walker A and B motifs [36] that constitute the active sites of Wzc and Etk, and they might function as tyrosine kinases. Furthermore, Zheng and coworkers have recently reported that the protein of unknown function YihE indeed exhibits a eukaryotic-like kinase fold despite sharing no sequence homology with eukaryotic kinases [37]. This finding supports the existence of still unknown bacterial kinases that would harbor a new type of phosphorelay mechanism. Be that as it may, the role of BY-kinases in phosphorylating other proteins seems underestimated and new regulatory networks based on tyrosine phosphorylation are likely to exist not only in E. coli but also in a large number of proteobacteria and firmicutes in which BY-kinases have been identified [5].

We established that Wzc-mediated phosphorylation of Ugd influences the production of colanic acid (Fig. 5). It has already been demonstrated that the phosphorylation-dephosphorylation of BY-kinases is involved in the biosynthesis of extracellular polysaccharides in various bacteria and more precisely in the assembly or the export of the nascent polysaccharide [26,38–42]. The function of BY-kinases has been mainly studied by performing mutations in their catalytic site. Therefore, one might assume that, in addition to an effect due to the phosphorylation-dephosphorylation process of a BY-kinase itself, an effect due to the altered phosphorylation of a potential substrate might also influence the biosynthesis of the polysaccharide. Ugd, that is involved in the biosynthesis of the repeat unit of colanic acid, is in line with this idea (Fig. 5) [17]. Therefore, colanic acid biosynthesis is controlled...
by phosphorylation at two levels: the assembly and export of the polysaccharide (Wzc phosphorylation) and the synthesis of the colanic acid repeat unit (Ugd phosphorylation). This analysis could be extended to other bacteria. For instance, Minic and co-workers have described the phosphorylation of the UDP-glycosyl-transferase EpsE, which is involved in the production of the expolysaccharide of *Streptococcus thermophilus*, by the BY-kinase EpsD [13].

In the particular case of *E. coli* K-12, the phosphorylation of Ugd could also have side effects on the production of other polysaccharidic compounds. A recent study has demonstrated that variation of polysaccharide chain length depends on the UDP-glucose (UDPG) concentration available in the bacterial cell [43]. In this work, we did not observe such a variation of the colanic acid polymer length (data not shown) even though the UDPG/UDPGA ratio might vary in accordance with the Ugd phosphorylation level. Also, UDPGA is involved in the biosynthesis of certain Group 4 capsules (G4C) produced by some pathogenic strains of *E. coli* [44]. G4C are well-established virulence factors and require Etk to be secreted [18,30]. Accordingly, Etk-mediated phosphorylation of Ugd could influence the production of G4C (Fig. 5). Similarly, Meredith and co-workers have reported that colanic acid repeats could modify the lipopolysaccharide (LPS) of *E. coli* K-12, forming thus a novel LPS glycoform henceforth called M_{LPS} [45]. The biosynthesis of M_{LPS} could also be affected by phosphorylation of Ugd (Fig. 5). Therefore, tyrosine phosphorylation of Ugd, and more generally of enzymes involved in polysaccharide biosynthesis, could provide keys to understand the biosynthesis of polysaccharide which might be far more complex than presently believed.

We also demonstrated that Etk-mediated phosphorylation of Ugd is connected with *E. coli* resistance to polymyxin (Fig. 5). The *etk* gene is located in the *ymc* operon which is thought not to be expressed in *E. coli* K-12 strain grown in LB medium, but only in pathogenic *E. coli* strains [18]. Indeed, an insertion sequence IS1 is found in the promoter of the *ymc* operon in *E. coli* K-12 but not in pathogenic *E. coli* strains. Therefore, our results raise the question of the regulation of *etk* expression in *E. coli* K-12. We have previously shown that an *etk*-deficient strain of *E. coli* is unable to resist to polymyxin [27]. This finding has recently been confirmed by a report indicating that the kinase activity of Etk is required per se for polymyxin resistance [4]. In addition, an *etk* knock-out mutant of an *E. coli* K-12 strain has been reported to be altered in its heat shock response [7]. These observations demonstrate that *etk* is expressed in *E. coli* K-12 in spite of the IS1 sequence, at least under some particular growth conditions. No data have been reported concerning the expression of the *ymc* operon (Fig. 5) but, as *etk* is the last gene of this operon, one might suggest that a cryptic promoter could be involved in *etk* specific expression. More likely, it could be speculated that the IS1 insertion could have a positive transcriptional effect under particular conditions. For example, genomic transposition within the regulatory locus *bgaR* constitutes the major class of activating mutations that enable transcription of the *bga* operon, which is silent in wild-type *E. coli* strains under laboratory conditions [46].

*Figure 5. Schematic model for Ugd phosphorylation by Wzc or Etk as control element for extracellular polysaccharides production and resistance to antimicrobial cationic peptide.* The two-component systems PhoP/PhoQ and PmrA/PmrB, or the RcsA/RcsB/RcsC system alone allow expression of genes involved in L-Ara4N production (*arn* and *ugd*) or colanic acid biosynthesis (*cps* operon and *ugd*), respectively. In both situations, phosphorylation of Ugd by Wzc and/or Etk influences UDP-glucuronic acid production and consequently the two phenotypes. Since, Ugd is involved in M_{LPS} and Group 4 capsule synthesis, it is assumed that Ugd phosphorylation could also influence their production (boxed question mark symbols). A question mark symbol is also used to indicate that the mechanisms governing the expression of the *ymc* operon are still unknown. doi:10.1371/journal.pone.0003053.g005
Colanic acid synthesis or polymyxin resistance depend each on two distinct sets of proteins, that include respectively Wzc or Etk, and that are synthesized under specific conditions (Fig. 5). Beside the influence of Ugd phosphorylation, one cannot preclude that other events would affect those biological processes. To illustrate this, it has been shown that Wzc expression does not complement polymyxin resistance of an etk-deficient strain [27]. Similarly, Wzc proteins from the E. coli K12 and K30 strains are also not interchangeable because of specific interactions between each Wzc proteins and their cognate capsule assembly complex [47]. Therefore, we assume that Wzc and Etk themselves are not likely only crucial in Ugd phosphorylation, but also in establishing interactions with other proteins involved in colanic acid synthesis and polymyxin resistance. Another possibility is that Etk and Wzc would likely phosphorylate other proteins [15]. Therefore, one cannot exclude that Wzc and/or Etk would specifically phosphorylate other endogenous proteins involved in colanic acid production or polymyxin resistance. For instance, WcaJ protein is involved in colanic acid synthesis in E. coli K12 [17] and its homolog in S. thermophilus, EpsE, is phosphorylated on tyrosine by the BY-kinase EpsD [13]. Therefore, Wzc-mediated phosphorylation of WcaJ would also influence colanic acid synthesis.

One can speculate that certain environments would induce simultaneous both expression of colanic acid and polymyxin resistance. At this moment, some factors could also participate in determining whether colanic acid or polymyxin resistance is expressed. The presence of these factors could depend on activation of the Rcs, PmrA/PmrB and PhoP/PhoQ two-components systems, that govern the expression of the eps and arn operons (Fig. 5), but that are also known to regulate other numerous cellular activities [48,49]. For instance, it has already been brought up that BY-kinases, namely Wzc and Etk, could act as membrane receptors, capable of sensing input signals, thereby affecting their kinase activity and controlling signal transduction [5,50]. In line with our hypothesis, some factors produced by either the Rcs, or PmrA/PmrB or PhoP/PhoQ systems could influence specifically Etk or Wzc kinase activity or their ability to function in colanic acid synthesis and polymyxin resistance.

Complicated as it may, both high-throughput phosphoproteomic studies [50] and structural characterization of Wzc and Etk [4] will be helpful to generate valuable data to understand further their biological role in E. coli. More generally, our data represent a step toward deciphering the regulatory role of tyrosine phosphorylation in bacteria and illustrate that bacterial protein phosphorylation networks could be more complex than initially expected.

Methods

Bacterial strains

Strains and plasmids used in this study are listed in Table S1. E. coli JM83, wild type or mutated, was used to perform experiments on colanic acid production. E. coli W3110, wild type or mutated, was used to perform polymyxin resistance assay. E. coli XL1-Blue strain was used to propagate plasmids in cloning experiments. Bacteria were grown in LB medium at 37°C or in the medium inducing polymyxin resistance (see below). Antibiotics were added at the following concentrations: 50 μg/ml ampicillin, 25 μg/ml kanamycin, 15 μg/ml tetracyclin.

Gene disruption, mutagenesis and cloning

Gene replacement of ugd in E. coli W3110 or JM83 strain was performed by one-step inactivation as previously described [51]. Here, ugd was replaced with the kanamycin resistance cassette (KanR) (Table S1). Two-way mutagenic PCR was performed to substitute Ugd tyrosines to phenylalanines. For in vivo analysis of colanic acid production and polymyxin resistance, ugd and ugdI71F were expressed after cloning either in the pUC-rcsA plasmid opened with SmaI and Acc65I, or in the pUC plasmid opened with Acc65I and BamHI, respectively. The 840-bp etk fragment encoding the cytoplasmic domain of Etk (amino acids 447-726) was cloned into the pQE30 vector previously opened with BamHI and HindIII. Constructs were checked by DNA sequencing. Primers used in this study are described in Table S2.

RNA manipulation

Total RNA was prepared from E. coli cells collected in the postexponential phase of growth. RNA was purified with the High Pure RNA isolation kit (Roche). Contaminating DNA was removed by additional treatment for 20 min at 37°C with 10 U DNase I (Roche). RT-PCR amplification was carried out with the SuperScript II reverse Transcriptase (Invitrogen) following the manufacturer’s recommendations. A control sample without reverse transcriptase was included to confirm the absence of contaminating DNA.

Protein purification, kinase assay and UDP-glucose dehydrogenase assay

WzcEtk, EtkEyc or Ugd or UgdY71F were expressed in E. coli XL1-Blue cells. The purification procedure and in vitro kinase assays were performed as previously described [10]. For kinase assays, 1 μg of protein Ugd was incubated for 30’ to 15 min at 37°C with varying amount of WzcEyc ranging from 0.002 μg to 1 μg. The reaction mixtures were then analyzed by SDS-PAGE. After electrophoresis, gels were soaked in 20% TCA for 10 min at 90°C, stained with Coomassie blue and dried. Radioactive proteins were visualized by autoradiography using direct exposure films. Ugd dehydrogenase activity measurement were performed in a thermostated cuvette at 37°C on a PowerWave 340 BIO-TEK spectrophotometer as described [52].

Dynamic light scattering (DLS) measurements

DLS measurements were performed at 18°C in a buffer containing 50 mM NaH2PO4 pH 7.8, 300 mM NaCl, 10% glycerol and 150 mM imidazol with a Zetasizer Nano series Malvern instrument. A light path of 3 mm was used. Protein concentration was of 14 mg/mL and 8 mg/mL for Ugd and UgdY71F, respectively.

Immunoblot analysis

Bacterial cell extracts were analyzed by Western blotting after SDS-PAGE. Proteins Wzc and Ugd were detected using specific monoclonal antibodies prepared in our laboratory according to the procedure described [33] and a goat-anti-mouse secondary antibody HRP conjugate (Biorad). Phosphorylation was detected using PY20 monoclonal anti-phosphotyrosine-HRP conjugate antibody (Sigma).

Colanic acid purification

The method used was based on the procedure previously described [54]. 50 ml cell culture were heated for 15 min at 100°C to denature EPS-degrading enzymes. After cooling, they were centrifuged at 13,200 xg at 4°C for 30 min. 40 ml of the supernatant fraction were then precipitated by addition of three volumes of ethanol. The mixture was maintained at 4°C overnight and centrifuged in the same conditions as above. The resulting pellet was dissolved in 5 ml of distilled water, dialyzed for 48 h against distilled water (Membrane MWCO 3,500 Da) and dried.
Residual polypeptides were removed by precipitation with 5 ml of 10\% (v/v) trichloroacetic acid and centrifuged again at 13,200 ×g at 4 °C for 30 min. The supernatant was dialyzed again for 5 days against distilled water and dried. The resulting preparation was suspended in 1 ml of distilled water and stored until quantification.

Quantification of colanic acid

Quantification of colanic acid was carried out according to [55] by measuring the amount of non-dialyzable methlypentose (6-deoxy-hexose), namely fucose which is a specific component of this exopolysaccharide. 10 to 100 μl of the colanic acid preparation were diluted to 1 ml with distilled water, and mixed with 4.5 ml of H2SO4/H2O (6:1 v/v). The mixture was prepared at room temperature, then heated at 100 °C for 20 min, and finally cooled down to room temperature. For each sample, absorbance at 396 nm and 427 nm was measured either directly (control sample (Aco)) or after addition of 100 μl of cystine hydrochloride (cystine sample (Acy)). Indeed, biological extracts often contain compounds which under heating with H2SO4 yield brown absorption of the sample: A 396-co and A 427-co were respectively due to this unspecific reaction was subtracted from the total products absorbing between 396 and 427 nm. The absorption values of (DA 396–DA427) were directly correlated to methylpentose concentration by using a standard curve obtained with a fucose concentration ranging from 5 to 100 mg/ml.

Polymyxin resistance assay

Tested strains were grown for 12 h in LB medium at 37 °C, diluted 100 fold and grown again overnight in N-minimal medium at pH 7.7, in the presence of 0.2% glucose and 10 mM MgCl2. Cultures were then harvested and washed three times with a N-100,000 bacteria were thus recovered and incubated for one hour, 1:100 into the same medium and incubated for 4 h at 37 °C. For each sample, absorbance at 427 nm at 4°C for 30 min. The supernatant was dialyzed again for 5 days down to room temperature. For each sample, absorbance at 396 nm and 427 nm was measured either directly (control sample (Aco)) or after addition of 100 μl of cystine hydrochloride (cystine sample (Acy)). Indeed, biological extracts often contain compounds which under heating with H2SO4 yield brown absorption of the sample: A 396-co and A 427-co were respectively subtracted from A396cy and A427cy to obtain DA 396 and DA 427. Values of (DA396–DA427) were directly correlated to methylpentose concentration by using a standard curve obtained with a fucose concentration ranging from 5 to 100 mg/ml.

Supporting Information

Figure S1 Analysis of the Ugd amino acids sequence to characterize the phosphorylated tyrosine. (A) Comparison of both the amino-acid sequences and the predicted secondary structure of Ugd and YwqF. β, α, and η indicate β-sheet, α-helices and 3.10 helices, respectively. Secondary structure elements of Ugd and YwqF have been predicted using Streptococcus pyogenes UDP-glucose dehydrogenases (PDB code 1DL1) and Pseudomonas aeruginosa GDP-mannose dehydrogenase (PDB code 1MV8) as templates respectively (Gouet et al., 2003; Rost and Liu, 2003)

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Author Contributions

Conceived and designed the experiments: SL, CG. Performed the experiments: SL, EB. Analyzed the data: SL, EB AC IM CG. Wrote the paper: IM CG.

References

1. Stock AM, Robinson VL, Goudreau PN (2000) Two-component signal transduction. Annu Rev Biochem 69: 183–215.
2. Bakal C, Davies JE (2000) No longer an exclusive club: eukaryotic signalling domains in bacteria. Trends Cell Biol 10: 32–38.
3. Oliveira-Ellena V, Meyer P, Becher E, Greger-Chaignon V, Lazere-Riquier S, et al. (2008) Structural basis for the regulation mechanism of the tyrosine kinase CapiP from Staphylococcus aureus. PLoS Biol.
4. Lee DC, Zheng J, She YM, Jia Z (2008) Structure of Escherichia coli tyrosine phosphatase YpII reveals a novel activation mechanism. Emeo.
5. Grangeasse C, Cozzone AJ, Deutscher J, Cozzone AJ, et al. (2007) Tyr-Phosphorylation of Ugd, a UDP-glucose dehydrogenase. J Biol Chem 282: 3320–3323. Rost, B., and Liu, J. (2003) The PredictProtein server. Nucleic Acids Res 31: 3300–3304.

Table S1 Bacterial strains and plasmids used in this study

Table S2 Primers used in this study

Figure S1 Analysis of the Ugd amino acids sequence to characterize the phosphorylated tyrosine. (A) Comparison of both the amino-acid sequences and the predicted secondary structure of Ugd and YwqF. β, α, and η indicate β-sheet, α-helices and 3.10 helices, respectively. Secondary structure elements of Ugd and YwqF have been predicted using Streptococcus pyogenes UDP-glucose dehydrogenases (PDB code 1DL1) and Pseudomonas aeruginosa GDP-mannose dehydrogenase (PDB code 1MV8) as templates respectively (Gouet et al., 2003; Rost and Liu, 2003)

Conserved tyrosines are indicated in cyan. Tyr70 of Ugd and Tyr71 of Ugd are highlighted in green. (B) Autoradiography of SDS-PAGE on reaction mixtures containing [γ-32P]ATP and either Ugd and Wzcyto (lane 1), or UgdY10F and Wzcyto (lane 2), or UgdY150F and Wzcyto (lane 3), or UgdY249F and Wzcyto (lane 4), or UgdY333F and Wzcyto (lane 5), or UgdY380F and Wzcyto (lane 6) were analyzed. References 1.Gouet, P., Robert, X., and Courcelle, E. (2003) ESPript/ENDscript: Extracting and rendering sequence and 3D information from atomic structures of proteins. Nucleic Acids Res 31: 3320–3323. 2.Rost, B., and Liu, J. (2003) The PredictProtein server. Nucleic Acids Res 31: 3300–3304. Found at: doi:10.1371/journal.pone.0003053.s001 (21.88 MB TIF)
17. Stevenson G, Andrianopoulos K, Hobbs M, Reeves PR (1996) Organization of the Escherichia coli K-12 gene cluster responsible for production of the extracellular polysaccharide colanic acid. J Bacteriol 178: 4885–4893.
18. Peleg A, Shafir Y, Ilan O, Nadler-Yona C, Nov S, et al. (2005) Identification of an Escherichia coli operon required for formation of the O-antigen capsule. J Bacteriol 187: 5259–5266.
19. Whithfield C (2006) Biosynthesis and assembly of capsular polysaccharides in Escherichia coli. Annu Rev Biochem 75: 39–60.
20. Kollor M, Gorovits R, Silverstein N, Fichtman B, Yapil E (2008) Phosphorylation of the integrate protein of coliphage HK022. Virology 373: 383–390.
21. Mijakovic I, Petranovic D, Deutscher J (2004) How tyrosine phosphorylation affects the UDP-glc dehydrogenase activity of Bacillus subtilis YwqF. J Mol Microbiol Biotechnol 8: 19–25.
22. Raetz CR, Reynolds CM, Trent MS, Bishop RE (2007) Lipid A modification systems in gram-negative bacteria. Annu Rev Biochem 76: 295–329.
23. Gao L, Liu KB, Podjaje CM, Daniel M, Gunn JS, et al. (1999) Lipid A acylation and bacterial resistance against vertebrate antimicrobial peptides. Cell 95: 189–198.
24. Trent MS, Ribeiro AA, Lin S, Cotter RJ, Raetz CR (2001) An inner membrane enzyme in Salmonella and Escherichia coli that transfers 4-amino-4-deoxy-L-arabinose to lipid A induction on polymyxin-resistant mutants and role of a novel lipid-linked donor. J Biol Chem 276: 43122–43131.
25. Breazeale SD, Ribeiro AA, Raetz CR (2003) Origin of lipid A species modified with 4-amino-4-deoxy-L-arabinose in polymyxin-resistant mutants of Escherichia coli: An aminotransferase (AmT) that generates UDP-4-amino-4-deoxy-L-arabinose. J Biol Chem 278: 24731–24739.
26. Ohiada B, Lacour S, Douhet P, Baubichon-Cortay H, Cozzone AJ, et al. (2007) Influence of Tyrosine-Kinase-Wae Activity on Colanic Acid Production in Escherichia coli K12 Cells. J Mol Biol 367: 42–53.
27. Lacour S, Douhet P, Ohiada B, Cozzone AJ, Grangeasse C (2006) A novel role for protein-tyrosine kinase Ezk from Escherichia coli K-12 related to polymyxin resistance. Res Microbiol 157: 637–641.
28. Gottsman A, Traider P, Torres-Cabassa A (1985) Regulation of capsular polysaccharide synthesis in Escherichia coli K-12: characterization of three regulatory genes. J Bacteriol 162: 1111–1119.
29. Mouslim C, Grossman EA (2005) Control of the Salmonella ugd gene by three two-component regulatory systems. Mol Microbiol 54: 335–344.
30. Iban O, Bloch Y, Frankel G, Ullrich H, Geider K, et al. (1999) Protein tyrosine kinases in bacterial pathogens are associated with virulence and production of exopolysaccharides. Embo J 18: 3241–3248.
31. Vincent C, Duclos B, Grangeasse C, Vaganay E, Riberty M, et al. (2000) Tyrosine Kinase Required for Capsule Biosynthesis in Streptococcus pneumoniae. J Bacteriol 182: 3089–3097.
32. Mijakovic I, Petranovic D, Deutscher J (2004) How tyrosine phosphorylation affects the UDP-glc dehydrogenase activity of Bacillus subtilis YwqF. J Mol Microbiol Biotechnol 8: 19–25.