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Dephosphorylated NPr of the nitrogen PTS regulates lipid A biosynthesis by direct interaction with LpxD

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ABSTRACT

Bacterial phosphoenolpyruvate-dependent phosphotransferase systems (PTS) play multiple roles in addition to sugar transport. Recent studies revealed that enzyme IIA^{Ntr} of the nitrogen PTS regulates the intracellular concentration of K^+ by direct interaction with TrkA and KdpD. In this study, we show that dephosphorylated NPr of the nitrogen PTS interacts with *Escherichia coli* LpxD which catalyzes biosynthesis of lipid A of the lipopolysaccharide (LPS) layer. Mutations in lipid A biosynthetic genes such as *lpxD* are known to confer hypersensitivity to hydrophobic antibiotics such as rifampin; a *ptsO* (encoding NPr) deletion mutant showed increased resistance to rifampin and increased LPS biosynthesis. Taken together, our data suggest that unphosphorylated NPr decreases lipid A biosynthesis by inhibiting LpxD activity.

1. Introduction

The phosphoenolpyruvate (PEP):carbohydrate phosphotransferase system (carbohydrate PTS) plays an important role in the uptake and concomitant phosphorylation of carbohydrates [1]. The carbohydrate PTS consists of two general proteins (enzyme I (EI) and histidine phosphocarrier protein (HPr)) and a large number of sugar-specific proteins, collectively known as enzymes II (EIIs). The *Escherichia coli* glucose specific enzyme II consists of an amphipathic (IIA^{Glc}) and a transmembrane subunit ($IICB^{Glc}$). The glucose PTS occupies a central position in *E. coli* physiology as a result of the identification of multiple regulatory functions superimposed on the transport functions, such as: regulation by EI of chemotaxis [2]; regulation by HPr of glycogen breakdown [3]; regulation by $EIIA^{Glc}$ of carbohydrate transport and metabolism [1], the metabolic flux between fermentation and respiration [4], and adenylyl cyclase activity [5]; and regulation by the membrane-bound glucose transporter $EIICB^{Glc}$ of the global repressor Mlc [6–8]. These regulatory functions of the carbohydrate PTS depend on the phosphorylation state of the involved components.

The nitrogen PTS consists of EI^{Ntr} (an EI paralog encoded by *ptsP*), NPr (an HPr paralog encoded by *ptsO*), and $EIIA^{Ntr}$ (an $EIIA^{Mtl}$ paralog encoded by *ptsN*) [9]. The cascade of phosphoryl-transfer reactions is as follows: PEP → EI^{Ntr} → NPr → IIA^{Ntr}. NPr and $EIIA^{Ntr}$ are encoded in the *rpoN* operon (Fig. 1) [10], which also contains the genes coding for YhbH (a predicted ribosome-associated protein) and YhbJ (a predicted P-loop containing ATPase). Because no phosphate acceptor from $EIIA^{Ntr}$ has yet been discovered, it has been suggested that the role of this pathway is in regulation [9]. Although numerous regulatory activities are associated with the carbohydrate PTS, substantially less is known about regulatory mechanisms connected with the nitrogen PTS. Dephosphorylated $EIIA^{Ntr}$ is required for derepression of the *ilvBN* operon in *E. coli*, which encodes acetohydroxy acid synthase I [11]; the mechanism of the derepression involves regulation of sigma factor selectivity by regulating the intracellular K^+ level through interaction with TrkA (Fig. 1) [12,13]. Dephosphorylated $EIIA^{Ntr}$ was also shown to interact with and stimulate the potassium sensor kinase KdpD to maintain potassium homeostasis at limiting K^+ concentrations [14].

The three-dimensional structure of *E. coli* NPr (M_r = 9810) has been determined by solution nuclear magnetic resonance [15]. Phosphoryl transfer from P-EI^{Ntr} (His-356) to NPr is at His-16 [9]. In this study, we explored the possibility of intracellular functions of NPr not directly connected to the phosphoryl shuttle between EI^{Ntr} and $EIIA^{Ntr}$. Ligand fishing led to the isolation of a protein interacting with NPr. The binding partner was identified as LpxD (also known as FirA), an enzyme required for lipid A synthesis [16] and therefore essential for growth. Further studies support the proposal that dephosphorylated NPr inhibits lipid A biosynthesis by interaction with LpxD.

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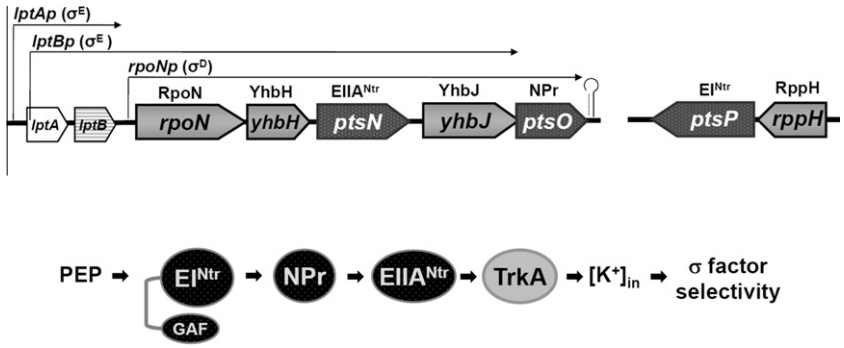

Fig. 1. Gene organization of the nitrogen PTS in *Escherichia coli*. NPr and EIIA^{Ntr} are encoded in the same operon with the genes coding for RpoN, the ribosome-associated protein YhbH, and a regulator of small RNAs (YhbJ). EI^{Ntr} is encoded elsewhere on the chromosome in the *rppH-ptsP* operon. In the DNA region near the *rpoN* gene, three transcription units have been reported as indicated: an *lptA* operon covering *lptA* and *lptB* driven by σ^E [24], an *lptB* operon spanning *lptB* to *yhbJ* driven by σ^E [25], and an *rpoN* operon from *rpoN* to *ptsO* driven by σ^D [10].

2. Materials and methods

2.1. Bacterial strains, plasmids and growth conditions

Construction of *E. coli* strains MG1655 Δ*ptsP* (CR101), MG1655 Δ*ptsO* (CR201), and MG1655 Δ*ptsN* (CR301) was described previously [11].

A *yhbJ* deletion mutant of *E. coli* MG1655 was constructed using *E. coli* DY330 as described previously [17]. The *yhbJ* gene (from the start codon to the stop codon) was replaced by the chloramphenicol acetyltransferase gene (*cat*). The *cat* gene was amplified by PCR from the CR101 strain [11]. The PCR product was electroporated into *E. coli* DY330 to make the strain CR400 (DY330 *yhbJ*::Cm^r). MG1655 Δ*yhbJ* (CR401) was constructed by P1 transduction of the Cm^r region of CR400.

To construct the pBR-NPr vector, the pCR2 vector (a pREI-based multicopy vector for NPr expression [13] was digested with HindIII and SalI. The digested fragment was cloned into pBR322. pET28a-NPr (which encodes NPr tagged with six histidines at the N-terminus, His-NPr) was constructed by cloning the NdeI–SalI fragment from pCR2 into pET28a (Novagen). Bacterial strains were grown in Luria–Bertani (LB) or Rich medium with the appropriate antibiotics (ampicillin, 100 μg/ml; kanamycin, 20 μg/ml; chloramphenicol, 30 μg/ml; tetracycline, 20 μg/ml).

2.2. Protein purification

To purify His-NPr, *E. coli* BL21 harboring pET28a-NPr was grown in 500 ml LB medium at 37 °C. IPTG was added to 1 mM when the culture reached an A_{600} of 0.5 and cells were harvested 3 h after induction. The pelleted cells were resuspended in 5 ml of Buffer A (20 mM Tris–Cl, 300 mM NaCl, pH 8), disrupted by two passages through a French pressure cell then centrifuged at 100,000*g* for 20 min at 4 °C. The pellet was resuspended in 5 ml of 6 M urea and recentrifuged. The supernatant solution was mixed with 500 μl TALON metal affinity resin (BD Biosciences Clontech) and agitated for 20 min at 4 °C. The resin was then centrifuged and washed sequentially with 20 volumes of 3, 1.5, 0.75, and 0 M urea-containing Buffer A. His-NPr was then eluted with 1 volume of Elution Buffer (20 mM Tris–Cl, 300 mM NaCl, 100 mM imidazole, pH 8) 5 times and analyzed by SDS-PAGE followed by staining with Coomassie Blue. The fractions containing substantial amounts of protein were dialyzed using Slide-A-lyzers (Pierce). Protein concentrations were determined by the Bradford assay (Bio-Rad).

To purify LpxD, *E. coli* Rosetta (DE3) cells harboring pDC015-1 [18] were grown in 200 ml LB medium at 37 °C and 1 mM IPTG was added when the culture reached an A_{600} of 0.5. After 3.5 h of induction, the cells were harvested, resuspended in 20 mM Tris–Cl (pH 7.5) containing 50 mM NaCl, and disrupted by two passages through a French pressure cell. After centrifugation at 100,000*g* for 45 min at 4 °C, the supernatant solution was loaded onto a MonoQ 10/100 anion exchange column (GE Healthcare), equilibrated with 20 mM Tris–Cl (pH 7.5) containing 50 mM NaCl. LpxD was eluted with a 50 ml gradient, from 50 to 500 mM NaCl, prepared in the same buffer. LpxD containing fractions, identified by SDS-PAGE, were concentrated and run through a Superose 12 10/300 column equilibrated with Buffer P (10 mM potassium phosphate, 200 mM NaCl, pH 7.0).

EI^{Ntr} was purified as described previously [13].

2.3. Ligand fishing to search for proteins interacting with His-tagged NPr

E. coli MG1655 cells grown overnight in 1 L of LB medium were harvested and washed with Buffer A, then resuspended in 10 ml of buffer A. Cells were disrupted by two passages through a French pressure cell and centrifuged at 100,000*g* for 45 min at 4 °C. The supernatant solution was mixed with 1 mg of His-NPr then added to 500 μl of TALON metal affinity resin. After incubation for 20 min at 4 °C, the mixture was dumped into a Poly-Prep column (8 × 40 mm, Bio-Rad). The column was washed three times with 10 volumes of buffer A, then the bound proteins were eluted three times with 1 volume of Elution Buffer. The eluted proteins were analyzed by SDS-PAGE, followed by staining with Coomassie Blue. The protein band specifically bound to His-NPr was excised from the gel, and then in-gel tryptic digestion and MALDI-TOF mass analysis were carried out as described previously [13].

2.4. Confirmation of specific binding

Fifty-microliter aliquots of TALON metal affinity resin were mixed with various amounts of His-NPr or His-HPr, then agitated for 20 min at 4 °C. The protein bound resin was washed with 20 volumes of Buffer A, then purified LpxD in Buffer P was added to each tube. After agitation for 30 min at 4 °C, the resin was washed with 20 volumes of Buffer P and bound protein was eluted with 50 μl of 5 × SDS sample buffer. Aliquots (20 μl) of the eluates were analyzed by 4–20% SDS-PAGE; the Coomassie Blue stained gel was quantitated with an image scanner (GE Healthcare).

2.5. Rifampin sensitivity test

Overnight cultures were serially diluted and 2 μl aliquots were spotted onto LB agar plates with or without 6 μg/ml rifampin. After incubation at 30 °C for 18–20 h, the plates were scanned.

2.6. Isolation of lipopolysaccharide

Lipopolysaccharide (LPS) was isolated as described previously [19] with some modifications. Briefly, cells were grown to stationary phase in 10 ml LB medium at 30 °C and harvested by centrifugation. After a wash with distilled water, the cells were collected in a 1.5 ml tube by centrifugation. LPS was recovered in an insoluble form together with denatured protein by boiling for 15 min in a solution containing 10 mM Tris–Cl (pH 8), 50 mM $MgCl_2$ and 2% Triton X-100. After centrifugation, LPS in the pellet was solubilized by incubating with shaking in a solution containing 50 mM EDTA and 2% Triton X-100 at 37 °C for 1 h. The suspension was then centrifuged at 16,000*g* and the supernatant solution was transferred to a fresh tube. To reprecipitate LPS, the supernatant was mixed with $MgCl_2$ (100 mM), incubated at 37 °C for 1 h and centrifuged at 100,000*g* for 45 min. The LPS pellet was resuspended in 5 × SDS sample buffer and boiled for 15 min. Aliquots of the samples were analyzed by SDS-PAGE and visualized by silver staining.

2.7. Biofilm formation assay

Overnight cultures of each strain were seeded into fresh LB medium in a 96-well microtiter plate (150 μl in each well) and incubated at 37 °C for 24 h without shaking. Biofilm formation was assayed by staining polystyrene-attached cells with crystal violet (CV). After removal of the medium and planktonic cells followed by two washes with 150 μl of distilled water, surface-attached cells were stained with 160 μl of 0.5% CV for 15 min. Following two subsequent washes with 170 μl of distilled water, surface-bound CV was extracted with 180 μl of 96% ethanol. The extracted CV was quantified by spectrophotometry at 590 nm and biofilm formation was normalized by dividing A_{590} by the planktonic cell density measured at 600 nm (A_{600}).

3. Results

3.1. Dephosphrylated NPr interacts specifically with LpxD

We used ligand fishing to search for a partner protein for NPr. MG1655 crude extract was mixed with His–NPr and subjected to a pull-down assay using TALON metal affinity resin. We found a protein of molecular mass ~36 kDa that co-eluted from the affinity resin with His-NPr. This protein was identified as LpxD by in-gel digestion and MALDI-TOF analysis (Fig. 2A). LpxD is an essential enzyme involved in lipid A synthesis and it catalyzes the R-3-hydroxymyristoyl-acyl carrier protein-dependent *N*-acylation of UDP-3-O-(R-3-hydroxymyristoyl)-glucosamine, the third step of lipid A biosynthesis [18].

To confirm the specificity of the interaction between NPr and LpxD, purified LpxD was mixed with increasing amounts of His-NPr or its homologous protein His-HPr. A concentration dependent interaction of LpxD was observed only with His-NPr (Fig. 2B).

Because the interaction with target proteins and regulatory functions of the PTS depend on the phosphorylation state of the involved component [1], the effect of phosphorylation of NPr on its interaction with LpxD was assessed. The phosphorylation state of NPr can be changed *in vitro* by incubation with EI^{Ntr} in the absence or presence of PEP. As confirmed by its mobility shift in a native gel, most of the NPr could be phosphorylated in the presence of EI^{Ntr} and PEP (Fig. 2C, lower panel). To test whether interaction with LpxD depends on the phosphorylation state of NPr, LpxD was mixed with either phospho- or dephospho-NPr. LpxD showed a preferential interaction with dephosphorylated NPr (Fig. 2C, upper panel). The trace binding of LpxD to His-NPr in the presence of EI^{Ntr} and PEP is most likely due to the residual amount of unphosphorylated NPr.

3.2. In vivo effect of NPr on resistance to rifampin and the level of LPS

The phenotype of a *lpxD* mutant includes an increased sensitivity to hydrophobic antibiotics such as rifampin [20]. If the observed interaction with NPr affects the function of LpxD, we expected that a *ptsO* mutant (deficient in NPr) would exhibit an altered sensitivity to rifampin. There was no significant difference in antibiotic sensitivity between a wild type and the mutant strain below the minimal inhibitory concentration (MIC) for rifampin and hydrophilic antibiotics such as bacitracin and kanamycin (data not shown). However, at concentrations slightly above the MIC, the *ptsO* mutant shows higher resistance to rifampin than does the wild-type strain (Fig. 3A, upper panel). The increased rifampin resistance could be reversed by episomal expression of the *ptsO* gene. Therefore, we conclude that resistance to rifampin is solely due to deletion of *ptsO*. We also tested a *yhbJ* mutant which is known to negatively regulate glucosamine-6-phosphate biosynthesis [21], a precursor for UDP-*N*-acetylglucosamine that is an essential building block for peptidoglycan and lipid A synthesis. In the case of the *yhbJ* mutant, there was a much smaller change in rifampin resistance compared to the *pts*O mutant.

As demonstrated above (Fig. 2C), LpxD binding affinity was affected by the phosphorylation state of NPr. We therefore explored the possibility that dephosphorylated NPr inhibits LpxD function *in vivo*. We expect dephosphorylated NPr to be increased in a *ptsP* (phosphate donor of NPr) mutant and the phosphorylated form to be increased in a *ptsN* (phosphate acceptor of NPr) mutant. Therefore, the sensitivity to rifampin was compared among wild type and such nitrogen PTS component mutant strains. As expected, the *ptsP* mutant was more sensitive to rifampin than the wild type and the *ptsN* mutant (Fig. 3A, lower panel). These results provide evidence that dephosphorylated NPr inhibits LpxD function *in vivo*.

Because lipid A is an essential component of the LPS layer, we also examined the amount of LPS in strains carrying mutations in the nitrogen PTS. We extracted the total LPS from stationary phase cells followed by analysis by SDS-PAGE and silver staining. The amount of LPS was dramatically increased in the *ptsO* mutant (Fig. 3B), which correlated with loss of rifampin sensitivity in that strain (Fig. 3A). In the *ptsO* mutant harboring the NPr expression plasmid, the level of LPS was restored close to that of the wild-type strain. Since it was previously reported that an *lpxD* mutant accumulates less LPS than wild type [16], the increased LPS amount in the *ptsO* mutant also implies that elimination of NPr is associated with increased LpxD activity.

3.3. Increased LPS in a ptsO mutant affects biofilm formation

LPS is an important factor in adhesion to a solid surface for Gram-negative bacteria at the initial step of biofilm formation [22] and the quantity of LPS has been correlated with biofilm formation in *Vibrio vulnificus* [23]. We therefore compared the extent of biofilm formation in wild type and mutants. The level of biofilm formation was increased more than 2-fold in the *ptsO* mutant compared to that in wild type and this phenotype could be complemented by episomal expression of NPr (Fig. 4). It is worthy of note that the level of biofilm formation was little affected in the *ptsP* and *ptsN* mutants. These results support the model that dephosphorylated NPr inhibits LPS synthesis and suggest that overproduced LPS can be translocated to the outer membrane surface in a *ptsO* mutant.

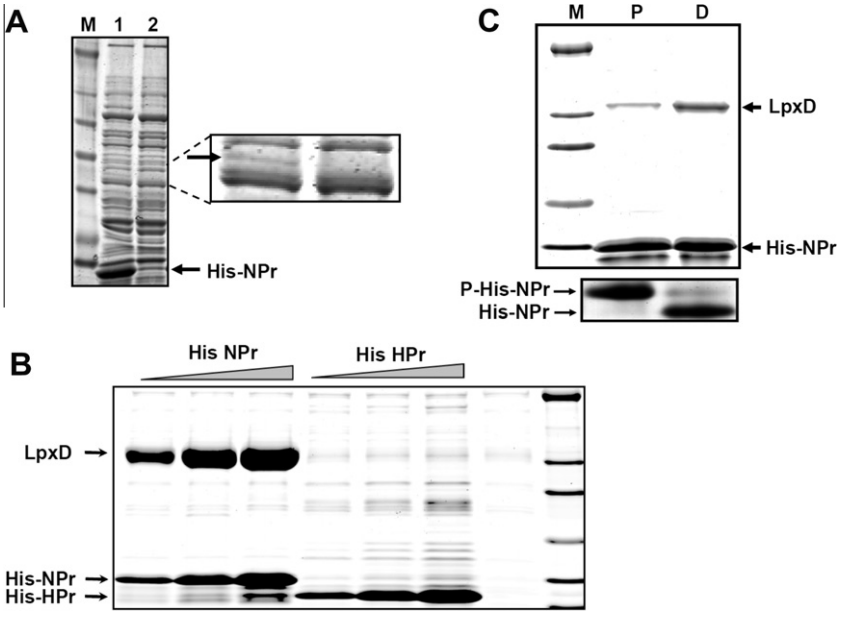

Fig. 2. Specific interaction between LpxD and dephosphorylated NPr. (A) Ligand fishing using purified His-NPr crude extract prepared from MG1655 grown in LB medium to stationary phase was mixed with TALON metal affinity resin in the presence (lane 1) or absence (lane 2) of His-NPr and processed as described in section 2. In-gel digestion followed by MALDI-TOF analysis identified the protein band bound specifically to His-NPr as LpxD (marked with an arrow in the right panel). EZ way protein-blue MW markers (KOMA biotech) were used as molecular mass markers (lane M). (B) LpxD (100 μg) was incubated with various amounts of His-NPr or His-HPr (100, 50, 25, 0 μg) in a total volume of 1 ml and subjected to a pull-down assay using TALON metal affinity resin. (C) His-NPr (100 μg) was incubated with 20 mM Tris–Cl (pH 8.0), 2 mM $MgCl_2$, and EI^{Ntr} (10 μg) in the presence (designated P) or absence (designated D) of 2 mM PEP in a total volume of 100 μl. After incubation for 10 min at 37 °C, the phosphorylation state of His-NPr was confirmed by running 5 μl aliquots on a 4–20% native polyacrylamide gel and staining with Coomassie blue (lower panel). The remainder of the reaction mixtures (95 μl each) were mixed with LpxD (200 μg) and TALON resin (35 μl) in 1 ml of Buffer P and subjected to metal affinity chromatography. After elution of bound proteins with 50 μl of 5 × SDS sample buffer, 15 μl aliquots were analyzed by 4–20% SDS-PAGE followed by Coomassie blue staining (upper panel).

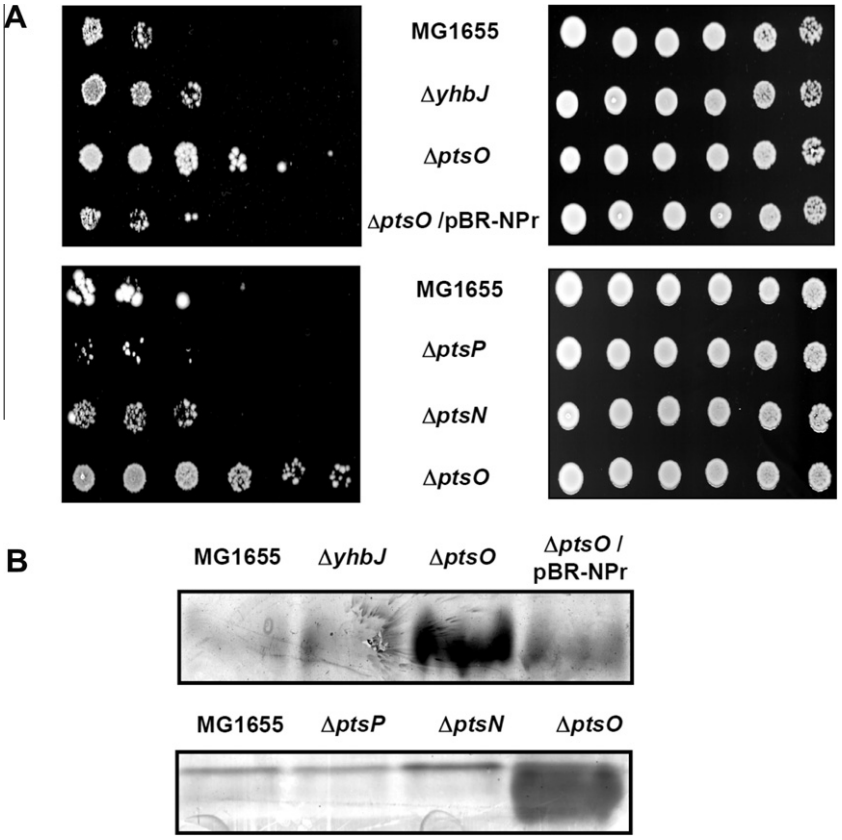

Fig. 3. *In vivo* effect of NPr on resistance to rifampin and the level of LPS. (A) Stationary phase cells of the indicated strains were serially 10-fold diluted from 10^8 to 10^3 cells/ml (upper panel) or 4-fold diluted from 10^8 to 10^5 cells/ml (lower panel) and spotted onto LB agar plates with (left) and without (right) the addition of rifampin (6 μg/ml). (B) LPS was extracted from cells of the indicated strains grown overnight in LB, purified as described in section 2 and analyzed by SDS-PAGE followed by silver staining.

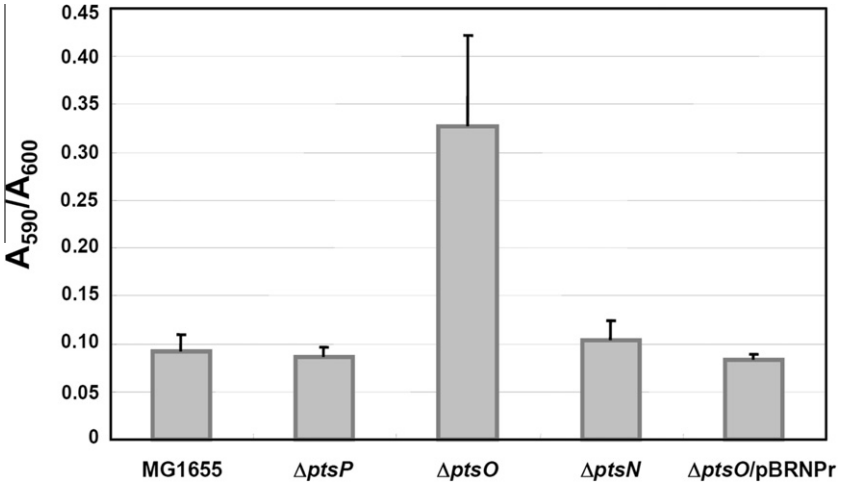

Fig. 4. Effect of NPr on biofilm formation. MG1655, MG1655 Δ*ptsP*, MG1655 Δ*ptsO*, MG1655 Δ*ptsN*, and MG1655 Δ*ptsO* harboring the pBR-NPr plasmid were grown in 96-well plates for 24 h. Biofilm formation was measured as described in section 2. Each bar shows the average and standard error of four independent experiments ($P = 0.005$).

4. Discussion

In this study, we discovered LpxD as a binding partner for NPr. LpxD interaction depends on the phosphorylation state of NPr; LpxD interacts preferentially with the dephosphorylated form of NPr. A *ptsO* deletion mutant was more resistant to rifampin and had a dramatically increased amount of LPS while a *ptsP* mutant showed the opposite phenotype. These observations suggest that dephosphorylated NPr inhibits LpxD activity *in vivo* by a direct interaction.

Together with our results, several lines of evidences support regulation of LPS biosynthesis by the nitrogen PTS. The *lptB* gene has been identified as a component of an ABC transporter for LPS translocation [24]. *lptB*, *ptsN*, and *ptsO* genes were recently demonstrated to reside in the same operon as *rpoN* under the control of σ^E (Fig. 1) by developing an accurate promoter prediction model; it was shown that genes from *lptB* to *yhbJ* were significantly up-regulated upon overexpression of *rpoE* in *E. coli* [25], indicating a possible connection in their function. Interestingly, the gene organization from *lptB* to *ptsO* shown in Fig. 1 is conserved in all bacterial species thus far known to have the *ptsN* and *ptsO* genes of the nitrogen PTS in the same operon as *rpoN*. Furthermore, the *E. coli yhbJ* gene product was recently shown to regulate production of glucosamine-6-phosphate which is an essential precursor for biosynthesis of peptidoglycan and LPS [21]. A possible role of the nitrogen PTS in maintenance of bacterial cell envelope integrity is also suggested by a recent study showing that overexpression of $EIIA^{Ntr}$ suppresses the essentiality of the extracytoplasmic stress factor σ^E [26].

In addition to regulation of K^+ homeostasis by $EIIA^{Ntr}$ [13,14], EI^{Ntr} of the nitrogen PTS has been implicated in virulence of some bacteria. Mutations in the *ptsP* gene, encoding EI^{Ntr}, dramatically reduced the ability of *Legionella pneumophila* to multiply in guinea pig lungs and eliminated the extrapulmonary invasiveness of the bacterium [27]. Similar observations were made in *Pseudomonas aeruginosa*, in which a *ptsP* mutant showed increased susceptibility to opsonization mediated by pulmonary collectin (surfactant protein A) in mouse lung [28]. It was suggested that bacterial gene functions that are required to maintain membrane integrity play crucial roles in resistance of *P. aeruginosa* to the permeabilizing effects of the surfactant protein. Lipid A is a bacterial endotoxin and LPS is necessary to maintain the integrity of the bacterial cell envelope as well as to form the biofilm that is an important virulence factor in pathogenic bacteria. Since we observed that an *E. coli ptsP* mutant accumulated somewhat less LPS and was more sensitive to rifampin than wild type (Fig. 3), examination of the amount of lipid A or LPS in *ptsP* mutants of pathogens might elucidate the underlying mechanism for the reduced infectivity of such mutants.

Acknowledgments

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