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Potassium mediates *Escherichia coli* enzyme IIA^{Ntr}-dependent regulation of sigma factor selectivity

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Summary

An *Escherichia coli* mutant devoid of enzyme IIA^{Ntr} (EIIA^{Ntr}) of the nitrogen PTS is extremely sensitive to leucine-containing peptides due to decreased expression of acetohydroxy acid synthase. This decreased expression is due to defective potassium homeostasis. We further elucidate here the mechanism for regulation of gene expression by the intracellular level of K^+. The leucine hypersensitivity of a *ptsN* (encoding EIIA^{Ntr}) mutant was suppressed by deleting *rpoS*, encoding the stationary phase σ factor. Despite intracellular levels of sigma factors comparable to the wild-type strain, most of the genes downregulated in a *ptsN* mutant are controlled by σ^{70}, while all the upregulated genes are controlled by σ^S, implying that the balance of sigma activities is modified by *ptsN* deletion. This change of sigma factor activities in the deletion mutant was found to be due to increased levels of K^+. *In vitro* transcription assays demonstrated that a σ^{70} controlled gene and a σ^S controlled gene were differentially affected by potassium concentration. Biochemical studies revealed that K^+ is responsible for sigma factor competition by differentially influencing the binding of σ^{70} and σ^S to core RNA polymerase. Taken together, the data suggest that EIIA^{Ntr} controls sigma factor selectivity by regulating the intracellular K^+ level.

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Introduction

The phosphoenolpyruvate-dependent carbohydrate phosphotransferase system (carbohydrate PTS) catalyses the uptake and conversion into phosphorylated forms of numerous sugars in bacteria (Deutscher *et al.*, 2006). This system consists of two general cytoplasmic components, enzyme I (EI) and HPr, common to all PTS sugars and various substrate-specific transporters termed enzymes II (EII). Enzymes II comprise three independently folding functional units: EIIA and EIIB are hydrophilic and each domain contains one phosphorylation site, while EIIC spans the membrane and contains the sugar binding site. In addition to sugar uptake, the carbohydrate PTS also carries out numerous regulatory functions such as chemoreception (Lux *et al.*, 1995), activation of glycogen phosphorylase by HPr (Seok *et al.*, 1997), inhibition of non-PTS sugar permeases (Deutscher *et al.*, 2006), regulation of the fermentation/respiration switch protein FrsA by EIIA^{Glc} (Koo *et al.*, 2004) and activation of adenylyl cyclase by phospho-EIIA^{Glc} (Park *et al.*, 2006). Furthermore, unphosphorylated EIICB^{Glc} was shown to sequester the global repressor Mlc to derepress its target genes in the presence of glucose (Lee *et al.*, 2000; Tanaka *et al.*, 2000; Nam *et al.*, 2001).

In addition to the carbohydrate PTS, many bacteria contain a parallel PTS that has been referred to as the nitrogen PTS (PTS^{Ntr}) (Powell *et al.*, 1995; Peterkofsky *et al.*, 2006; Pflüger-Grau and Görke, 2010). This system consists of enzyme I^{Ntr} (EI^{Ntr}) encoded by *ptsP*, NPr encoded by *ptsO* and enzyme IIA^{Ntr} (EIIA^{Ntr}) encoded by *ptsN*, which are homologues of EI, HPr and EIIA^{Mtl} of the carbohydrate PTS respectively. Because components homologous to EIIB and EIIC have not yet been identified for the PTS^{Ntr}, it has been suggested that the major function of this system is in regulation (Reizer *et al.*, 1996). The PTS^{Ntr} has been implicated in poly-β-hydroxybutyrate accumulation and nitrogen fixation in *Azotobacter vinelandii* (Segura and Espin, 1998), virulence in *Legionella pneumophila* (Higa and Edelstein, 2001), melanin synthesis and nitrogen fixation in *Rhizobium etli* (Michiels *et al.*, 1998) and pulmonary infection (Zhang *et al.*, 2005) and carbon source-mediated inhibition of the σ^{54}-dependent Pu promoter of the TOL plasmid (Cases *et al.*, 2001) in *Pseudomonas* species. In *Escherichia coli*, EIIA^{Ntr} was

shown to be involved in the regulation of the essential GTPase, Era, which appears to function in cell cycle progression and the initiation of cell division (Powell *et al.*, 1995), although the mechanism has not yet been defined.

Recently, we reported that growth of a *ptsN* mutant was hypersensitive to leucine and leucine-containing peptides (LCP), and demonstrated that the dephosphorylated form of EIIA^{Ntr} is required for the derepression of *ilvBN* encoding acetohydroxy acid synthase I (AHAS I) catalysing the first step common to biosynthesis of branched chain amino acids (Lee *et al.*, 2005). Although we showed that this regulation is achieved by the phosphorylation state-dependent interaction of EIIA^{Ntr} with TrkA, an essential component of the major potassium transporter in *E. coli* (Lee *et al.*, 2007), the mechanism by which TrkA activity regulates *ilvBN* derepression was not elucidated. In this study, we employed random mutagenesis to search for mutants suppressing the leucine-sensitive phenotype of a *ptsN* mutant. The data presented herein demonstrate that the previously demonstrated regulation of cellular K^+ levels by the phosphorylation state of EIIA^{Ntr} plays an important role in transcription; cellular K^+ levels influence the selectivity of RNA polymerase for complex formation with σ^{70} or σ^S. This regulation has global implications for gene regulation including the observed effect on branched chain amino acid synthesis.

Results

Mutation in the rpoS *gene suppresses LCP sensitivity of a* ptsN *mutant*

As we previously found that a *trkA* mutation suppressed leucine sensitivity of a *ptsN* mutant by reducing the intracellular K^+ concentration (Lee *et al.*, 2007), we examined the possibility that a transcription factor(s) was directly involved in the K^+-dependent regulation of *ilvBN* derepression. To search for suppressors, other than *trkA*, of the LCP sensitivity of the *ptsN* mutant, we generated Tn5-*kan* gene disruption mutations in the *E. coli* chromosome by employing the EZ-Tn5^{TM}<KAN-2> transposon system (EPICENTRE Biotechnologies). Kanamycin-resistant transposon insertions were screened for growth in M9 minimal medium containing glucose as the carbon source and supplemented with 0.5 mM Ala-Leu. Out of eight independent candidate suppressors (Table S1), three transposon insertions, named *pns1–3*, were mapped within the *rpoS* gene encoding the stationary phase sigma factor (Lange and Hengge-Aronis, 1991). Junctions of the *pns1*, *pns2* and *pns3* transposons appeared at codons 315, 146 and 5 of the *rpoS* gene respectively (Fig. 1A). To confirm the resistance of these suppressor mutants to Ala-Leu, their growth curves were compared with those of wild-type (MG1655) and the parental *ptsN* mutant (Fig. 1B). While the *ptsN* mutant did not show detectable growth for 48 h, as reported previously (Lee *et al.*, 2005), the three Tn insertion mutants grew to the level of the wild type. To exclude the possibility of more than one Tn insertion in the these suppressor mutants and prove that resistance of these mutants to Ala-Leu is due solely to elimination of *rpoS* gene function, in frame deletion mutants of *rpoS* (*rpoS::neo*) were constructed in MG1655 and the *ptsN* mutant. The *ptsN rpoS* double mutant exhibited resistance to Ala-Leu similar to that of wild-type and the three suppressor mutants (Fig. 1B), indicating that loss of *rpoS* gene function is directly involved in suppression of the leucine-sensitive phenotype of the *ptsN* mutant.

As we previously showed that the toxicity of LCPs was due to the failure of derepression of the *ilvBN* operon encoding AHAS I (Lee *et al.*, 2005), we examined the effects of deletion of *rpoS* on expression of the *ilvBN* operon. Measurements of the cellular AHAS activity in crude extracts showed that introduction of the *rpoS* mutation restored activity in the *ptsN* mutant to close to that of wild-type cells (Fig. 1C). The transcription pattern of *ilvBN* deduced from β-galactosidase activity assays in strains harbouring an *ilvB–lacZ* fusion construct is essentially the same as that of AHAS activity (Fig. 1D). Thus, growth restoration by deletion of the *rpoS* gene in the *ptsN* mutant is closely associated with derepression of the *ilvBN* operon.

Intracellular levels of σ^S affect derepression of the ilvBN *operon*

Leucine-containing peptides inhibited the growth of a wild-type strain (MG1655) of *E. coli* K-12 in minimal medium by increasing the lag period by up to 10 h (Tavori *et al.*, 1981) and the *ptsN* mutation increases the lag time more than five times in a medium containing the same concentration of an LCP (Lee *et al.*, 2005). As deletion of *rpoS* suppresses the extreme LCP sensitivity of the *ptsN* mutant (Fig. 1B), we assumed that an increased level of RpoS might result in an opposite effect on LCP sensitivity. To test this idea, we generated an *rssB* mutant in wild-type MG1655. RssB is known to bind directly to σ^S and the protease ClpXP to facilitate degradation of σ^S by the protease during normal growth conditions (Muffler *et al.*, 1996; Pratt and Silhavy, 1996; Becker *et al.*, 1999; Stüdemann *et al.*, 2003). As expected, in frame deletion of *rssB* resulted in an increased σ^S level in *E. coli* MG1655 (Fig. 2A). While introduction of the *rpoS* mutation hardly influenced the Ala-Leu sensitivity of MG1655, the *rssB* mutant showed a greater Ala-Leu sensitivity than wild type (Fig. 2B). Furthermore, the AHAS activity of the *rssB* mutant was significantly reduced, while that of the *rpoS* mutant was slightly increased compared with that of wild

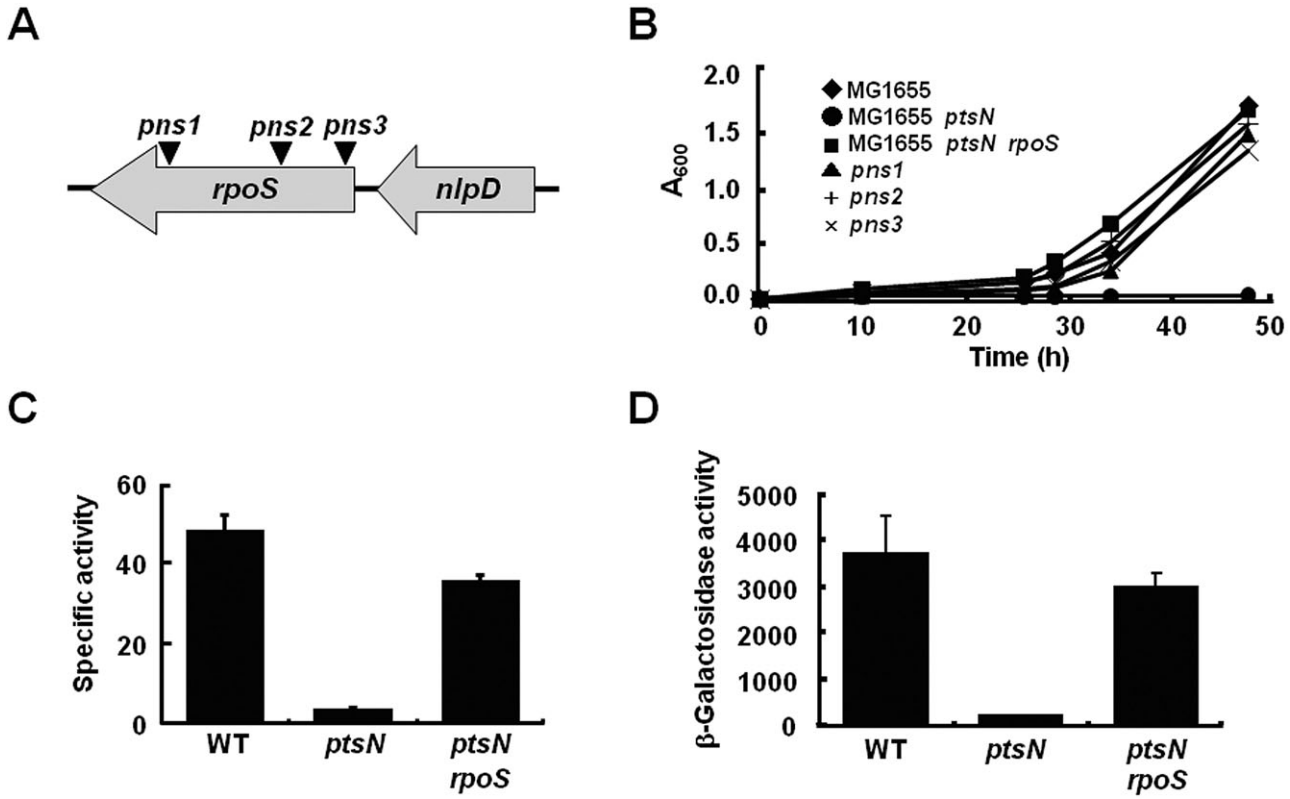

Fig. 1. Deletion of the *rpoS* gene suppresses LCP sensitivity of a *ptsN* mutant strain.
A. Tn5 insertions suppressing sensitivity of the *ptsN* mutant to LCP were mapped by sequencing as described in *Experimental procedures*.
B. Cells grown overnight in LB were washed with M9 medium, then inoculated into M9 medium containing 0.5% glucose supplemented with 0.5 mM Ala-Leu. Growth was measured as A_{600} versus time.
C. Strains MG1655 (WT), CR301 (*ptsN*) and CR305 (*ptsN rpoS*) grown in LB were harvested, washed and resuspended in M9 minimal medium containing 0.5% glucose. The cultures were grown to mid-logarithmic phase and the AHAS specific activities were measured.
D. β-Galactosidase activity was measured in CR400 (WT), CR401 (*ptsN*) and CR404 (*ptsN rpoS*) cells harbouring a single copy of the *ilvB–lacZ* fusion construct after growing to mid-logarithmic phase in M9 minimal medium containing 0.5% glucose.

type (Fig. 2C). Expression of *ilvBN* shows essentially the same pattern as that of AHAS activity (Fig. 2D). Therefore, these results implicate σ^S as another factor involved in the derepression of *ilvBN* operon.

Enzyme IIA^{Ntr} affects the activity of σ^S and σ^{70}

The involvement of both σ^S and EIIA^{Ntr} in derepression of the *ilvBN* operon suggested the possibility that EIIA^{Ntr} might regulate expression of σ^S and vice versa. This possibility was ruled out as neither *rpoS* deletion nor overexpression of σ^S by *rssB* deletion showed significant changes in the EIIA^{Ntr} level and *ptsN* deletion did not change the level of σ^S (Fig. 2A). Another possibility considered is that EIIA^{Ntr} might regulate the activity of σ^S. To check the activity of σ^S in the *ptsN* mutant, we compared the genome-wide transcription profile of wild-type and the *ptsN* mutant by DNA microarray analysis. The *E. coli* K-12 strain MG1655 and its isogenic *ptsN* mutant were grown in minimal medium containing glucose as the carbon source and total RNA was extracted at an A_{600} of 0.4 (during entry into exponential phase). Transcriptome analysis revealed that many genes were affected by *ptsN* deletion (Table S2). The most dramatic effect of *ptsN* deletion was upregulation of many genes under the control of σ^S (summarized in Tables 1 and S3). As the cellular level of σ^S was little affected in the *ptsN* mutant (Fig. 2A), the microarray data imply that deletion of the *ptsN* gene increases σ^S activity. The microarray results were confirmed by protein profile analysis and β-galactosidase activity assays (Fig. 3). Deletion of the *ptsN* gene caused superinduction of GadA, GadB and OsmY proteins of the σ^S regulon (Fig. 3A) and provoked a significant increase in β-galactosidase activity in strains harbouring *gadA–lacZ* and *gadB–lacZ* fusion constructs (Fig. 3B). Expression of other σ^S-dependent genes was also increased in the *ptsN* mutant (Fig. 3C). Intriguingly, in the *ptsN* mutant, the major fraction of downregulated genes (for example, LeuC as shown in Fig. 3A) were controlled by σ^{70} whereas all those upregulated were under the control of σ^S (Tables 1, S3 and S4). These results indicate that deletion of the *ptsN* gene leads to

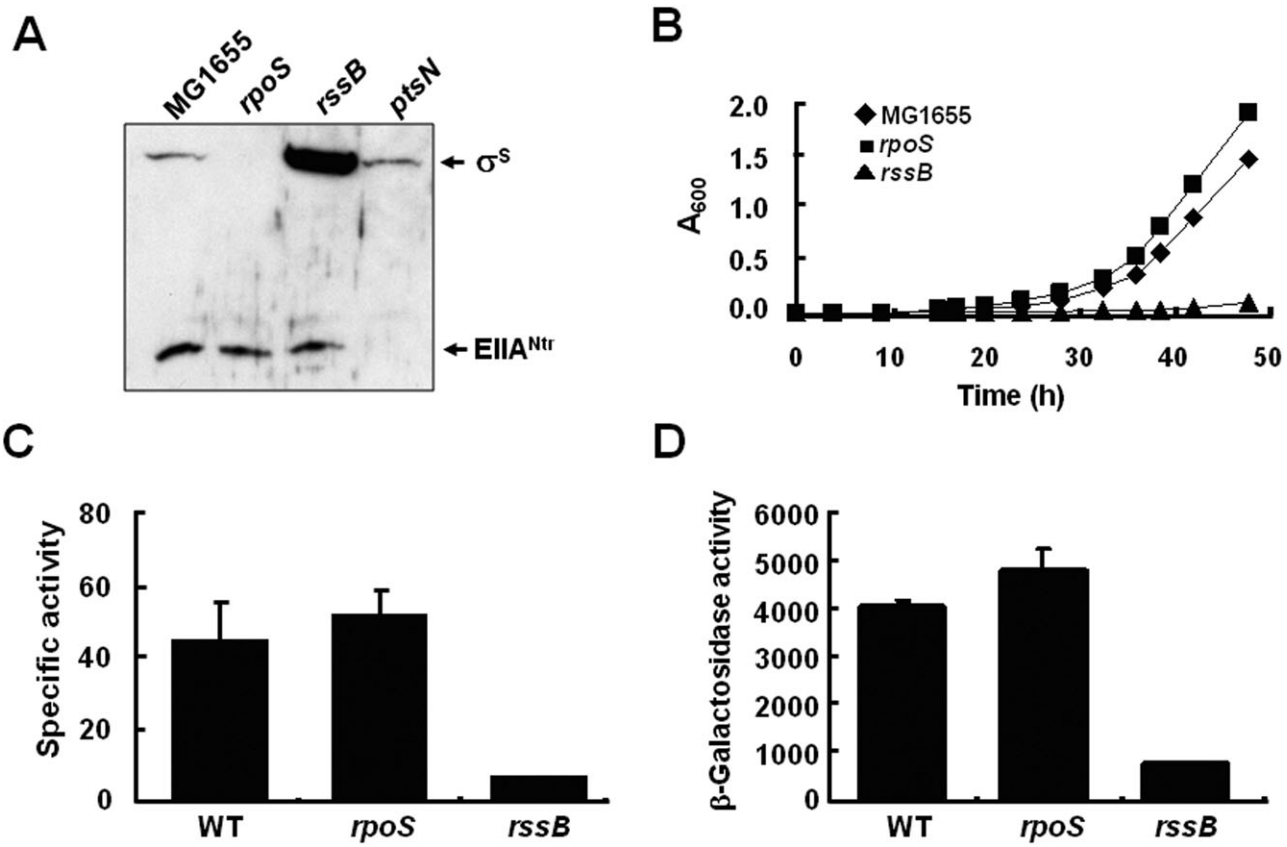

Fig. 2. Intracellular levels of σ^S affect derepression of the *ilvBN* operon.
A. Cells were grown to mid-logarithmic phase in M9 minimal medium containing 0.5% glucose. Western blot analysis was performed to determine intracellular levels of σ^S and EIIA^{Ntr}.
B. Growth of MG1655 and two otherwise isogenic *rpoS* (CR501) and *rssB* (CR601) mutants in M9 medium containing 0.5% glucose supplemented with 0.5 mM Ala-Leu.
C. AHAS activities of MG1655, CR501 and CR601 cells grown to mid-logarithmic phase in M9 minimal medium containing 0.5% glucose.
D. β-Galactosidase activity was measured in CR400 (WT), CR402 (*rpoS*) and CR403 (*rssB*) cells harbouring a single copy of the *ilvB–lacZ* fusion construct after growing to mid-logarithmic phase in M9 minimal medium containing 0.5% glucose.

increased expression of σ^S regulon genes while expression of σ^{70} regulon genes is mainly decreased. As expression levels of σ^S and σ^{70} themselves were not significantly (<2-fold) affected by *ptsN* deletion (Table S5 and Fig. S1), our data suggest that EIIA^{Ntr} affects some activity downstream of σ^S and σ^{70} expression.

K^+ regulates σ^S and σ^{70} selectivity

Although protein–protein interaction is the general mechanism for regulation mediated by PTS components, we could not detect an interaction between EIIA^{Ntr} and either σ^S or σ^{70} in the absence or presence of RNA polymerase core enzyme (data not shown), implying that EIIA^{Ntr} controls sigma activity by another mechanism.

It was previously shown that the level of one sigma factor can affect expression of genes dependent on other sigma factors (Farewell *et al.*, 1998) and this was concluded to be due to competition between sigma factors for a common ligand (Jishage *et al.*, 2002). Therefore, our results showing differential effects of a *ptsN* deletion on the σ^S and σ^{70} regulons suggested that such sigma factor competition might be involved.

A series of proteins and small molecules such as ppGpp, DksA and Crl are involved in sigma factor competition (Laurie *et al.*, 2003; Paul *et al.*, 2004; Typas *et al.*, 2007a). Transcription levels of *dksA* and *crl* genes showed no significant change in the *ptsN* mutant compared with those in the wild-type strain (Table S5). In addition, PTS^{Ntr} deletion strains accumulated (p)ppGpp at levels similar to those of the wild type after amino acid starvation (Fig. S2).

Preferential utilization of σ^S holoenzyme has been shown *in vitro* using various σ^S-dependent promoters such as *osmY*, *proU* in the presence of high concentrations of K-glutamate (Prince and Villarejo, 1990; Ding *et al.*, 1995; Ballesteros *et al.*, 1998; Lee and Gralla, 2004). Furthermore, it has been reported that the

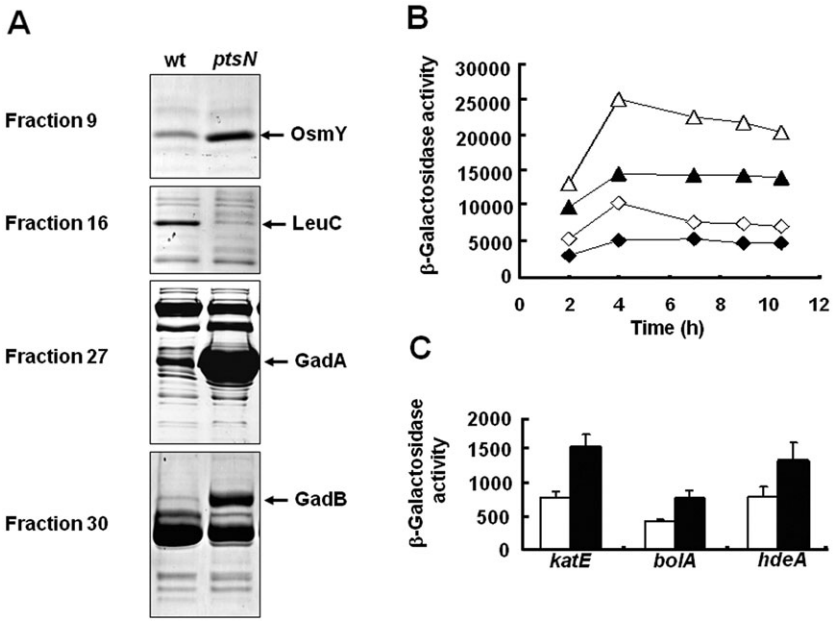

Fig. 3. Gene expression pattern changes in the *ptsN* mutant.
A. Cultures (200 ml each) of MG1655 and its *ptsN* mutant (CR301) grown to mid-logarithmic phase (A_{600} = 1) in M9 minimal medium containing 0.5% glucose were harvested. After disruption and centrifugation, the two supernatant solutions were fractionated using a MonoQ 10/10 column with a linear NaCl gradient and aliquots (20 μl) of eluted fractions were analysed side by side by SDS-PAGE. Proteins showing significantly different band intensities between wild-type and the *ptsN* mutant were identified by tryptic in-gel digestion followed by MALDI-TOF mass spectrometry (Lee *et al*., 2007).
B. Expression of single-copy *gadA–lacZ* (shaded symbols) and *gadB–lacZ* (open symbols) fusions was determined in wild-type (diamonds) and *ptsN* mutant (triangles) strains. Cells were grown in M9 minimal medium containing 0.5% glucose and β-galactosidase activities were measured.
C. β-Galactosidase activity was measured in wild-type (open bars) and *ptsN* mutant (shaded bars) cells harbouring a single copy of *katE–*, *bolA–* or *hdeA–lacZ* fusion construct grown to mid-logarithmic phase in M9 minimal medium containing 0.5% glucose.

C-terminal segment of σ^S is important for its activity under high K-glutamate concentrations (Ohnuma *et al*., 2000).

K^+ concentrations are known to be maintained relatively constant (300–500 mM) in *E. coli* by the activities of K^+ uptake and efflux systems (Bossemeyer *et al*., 1989; Roe *et al*., 2000). Based on the findings that the K^+ uptake rate increases in the *ptsN* mutant by relieving $EIIA^{Ntr}$-dependent inhibition of the major K^+ transport system Trk (Lee *et al*., 2007) and that deletion of the *ptsN* gene differentially affects σ^S-dependent and σ^{70}-dependent genes (Tables S3 and S4), we considered the possibility that K^+ level variations can influence competition between σ^S and σ^{70} for core RNA polymerase. To evaluate this idea, we first measured the intracellular K^+ concentrations at various growth phases (Fig. 4A). In wild-type cells, the cellular K^+ concentration gradually increased (197–367 mM) until A_{600} reached 1.2, followed by a plateau at higher cell densities. The intracellular level of K^+ in the *ptsN* mutant was determined to be about 200 mM higher than that in wild-type cells regardless of growth phase.

To confirm whether this increase in the K^+ level affects competition between the two sigma factors, *in vitro* transcription was performed with plasmids containing the *ilvB* (σ^{70} regulon) and *gadA* (σ^S regulon) promoters. We tested transcription of the *ilvB* gene in the presence of K^+ concentrations in the range of 100–500 mM. *ilvB* transcription, dependent on σ^{70}, was maximum at 100 mM K^+ but significantly decreased as the K^+ concentration increased (Fig. 4B). To examine the effect of K^+ concentration on the competition between the two sigma factors, the effect of σ^S on *ilvB* transcript synthesis was tested in the presence of two fixed concentrations of K^+. At 400 mM K^+, transcription of the *ilvB* gene decreased as the concentration of σ^S was increased; at 200 mM K^+, however, *ilvB* transcription was not significantly affected by changes in σ^S (Fig. 4C and D). Interestingly, as the K^+ concentration was increased, the σ^S-dependent *gadA* promoter was progressively stimulated and the transcript level was three to fourfold higher at 400 mM than at 100 mM (Fig. 5A). Furthermore, transcription of *gadA* was inhibited by an increase in σ^{70} concentration at 200 mM but not at 400 mM K^+ (Fig. 5B). These results support the premise that K^+ affects the competition between σ^{70} and σ^S.

In vivo glutamate levels increase substantially through *de novo* synthesis to counterbalance the K^+ charge (Dinnbier *et al*., 1988), and acetate also becomes rapidly concentrated in the cytoplasm when acetate is added to the medium (Roe *et al*., 1998). To determine the specificity of

Table 1. Effect of loss of EIIA^{Ntr} on gene expression.

	The number of genes whose expression is changed ≥3-fold in a *ptsN* mutant	
	Upregulated genes	Downregulated genes
Genes recognized only by σ^{70}	17	72
Genes recognized only by σ^{S}	12	0
Genes recognized only by σ^{54}	5	0
Genes recognized only by σ^{32}	3	2
Genes recognized by both σ^{70} and σ^{S}	15	5
Genes recognized by both σ^{70} and σ^{32}	2	1
Genes recognized by both σ^{70} and σ^{54}	0	1
Genes recognized by both σ^{70} and σ^{24}	0	1
Genes whose σ factors have not been defined	38	35
Total	92	117

Escherichia coli K-12 strain MG1655 and its isogenic *ptsN* mutant were grown in M9 minimal medium containing 0.5% glucose as carbon source. Cells from duplicate experiments were harvested in exponential phase when cultures reached an A_{600} of 0.4. DNA microarray analyses were carried out with RNAs extracted from each sample (Table S2). Genes whose expression was affected more than threefold in a *ptsN* mutant are summarized in Tables S3 (for upregulated genes) and S4 (for downregulated genes). Promoter recognition identifications were from http://www.ecocyc.org. Among genes recognized by both σ^{70} and σ^{S}, those upregulated in a *ptsN* mutant are mainly σ^{S}-dependent while the others are mainly σ^{D}-dependent (http://www.ecocyc.org and references therein).

the K^+ effect, we used glutamate, acetate and chloride as counterions of K^+. Similar inhibitory effects on σ^{70}-dependent transcription of the *ilvB* gene were observed with all three anions (Figs 4B and 5D), but chloride exhibited a somewhat different pattern than glutamate and acetate for transcription of the *gadA* gene (Fig. 5A and D). Thus, it appears that potassium is an important factor regulating sigma factor competition.

The interaction between sigma factors and core RNA polymerase is affected by K^+

Because sigma factor competition can be regulated at various levels (Typas *et al.*, 2007b), there are several possible mechanisms by which K^+ might affect the phenomenon of sigma factor competition. In order to demonstrate a selective effect of K^+ on sigma factor competition, we measured the relative amounts of σ^{70} and σ^{S} bound to core RNA polymerase (RNAP) at various potassium levels. YR300 cells carrying a modified *rpoC* gene encoding the β′ subunit of RNAP with six His residues at the C-terminus were harvested in early stationary phase, and then resuspended in lysis buffers containing different concentrations of potassium glutamate. After disruption, His-tagged β′ was purified using TALON™ metal affinity resin and the amounts of σ^{70} and σ^{S} were quantified by immunoblot analysis. Increasing potassium levels decreased the amount of σ^{70} bound to core RNAP, whereas the opposite tendency was observed for σ^{S} (Fig. 6). This result supports the idea that the cellular K^+ level can influence the competition between σ^{S} and σ^{70} for interaction with core RNAP *in vivo*.

To further study the effect of potassium on the interaction of sigma factors with core RNAP, we used surface plasmon resonance spectroscopy to monitor complex formation in real time. Each sigma factor was modified to have a His_6 tag that allowed its attachment to a Biacore nickel chip. Similar levels of σ^{70} and σ^{S} were bound to each flow cell; then, in the presence of varying concentrations of potassium glutamate, core RNAP was exposed to each immobilized sigma factor (Fig. 7). At 100 mM potassium glutamate, the dissociation constant (K_D) for the interaction between σ^{70} and core RNAP was lower than that of σ^{S} (4 versus 23 nM), indicating that σ^{70} binds more strongly to core RNAP than does σ^{S} (Fig. 8). It was found, as expected, that σ^{70} interaction with core RNAP was highly sensitive to potassium glutamate with the K_D increasing from 4 to 158 nM when the concentration of potassium glutamate was increased from 100 to 500 mM (Figs 7 and 8A). In contrast, σ^{S} exhibited a different response to potassium glutamate; the K_D decreased from 23 to 3 nM as the concentration of K-glutamate increased from 100 to 300 mM; at higher concentrations, the K_D increased back to 23 nM. Acetate showed a similar pattern as glutamate, but chloride inhibited the binding of both σ^{S} and σ^{70} to core RNAP, although the binding affinity of σ^{70} dropped more sharply than σ^{S} as the KCl concentration increased (Fig. 8B and C). These data provide further confirmation that σ^{70} and σ^{S} have different potassium sensitivities in their interaction with core RNAP although there may be some minor contributions by the nature of the anion.

Discussion

The present work demonstrates that the mechanism by which EIIA^{Ntr} controls expression of many genes is by regulating the concentration of K^+, which influences sigma factor selectivity for core RNAP.

Suppressor mutant screening and growth studies provided evidence that deletion of σ^{S}, the stationary phase alternative sigma factor, can restore derepression of *ilvBN* and suppress LCP sensitivity of a *ptsN* mutant (Figs 1 and 2). Several lines of evidence support the involvement of σ^{S} in derepression of *ilvBN* and thus sensitivity of *E. coli* K-12 cells to LCPs. First, expression of the *ilvBN* operon, controlled by σ^{70}, was previously shown to increase in an *rpoS* mutant (Farewell *et al.*, 1998). Second, a recent

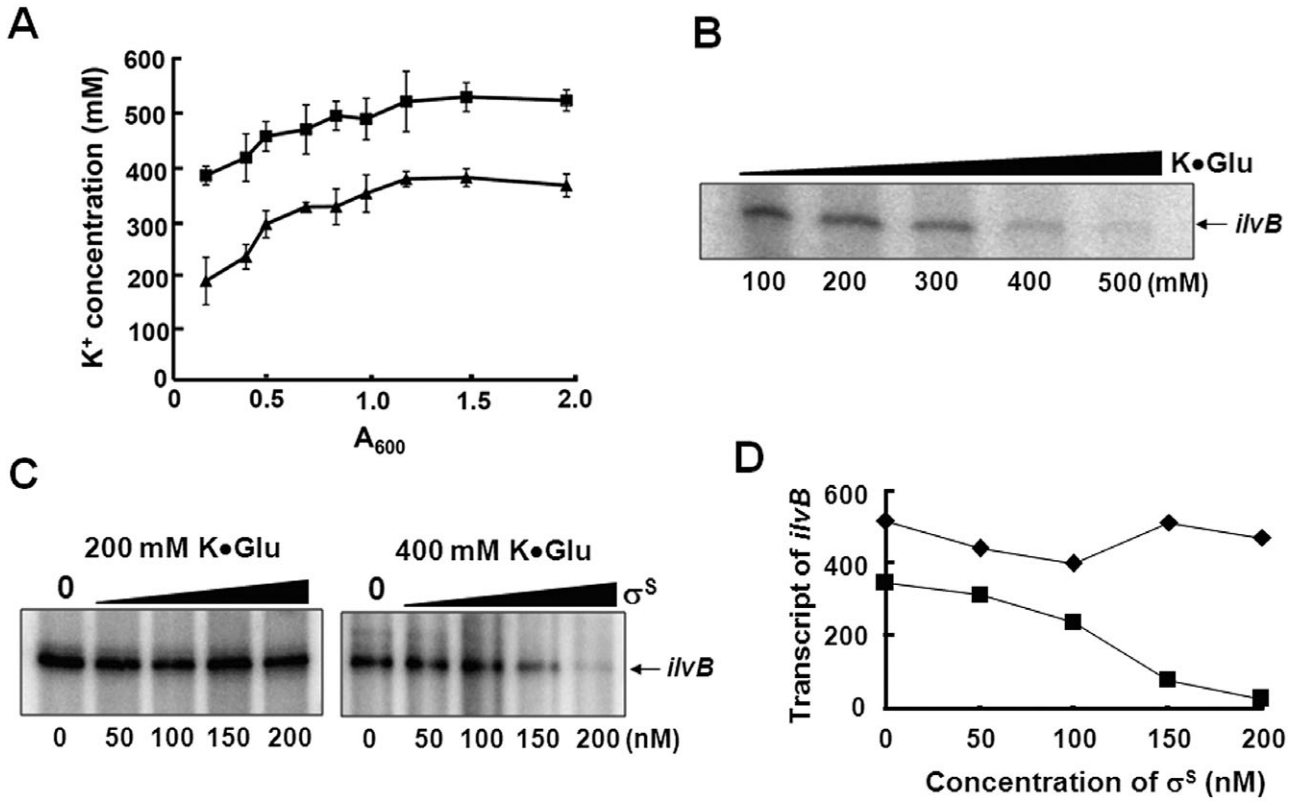

Fig. 4. Effects of K^+ on *ilvB* transcription *in vitro*.
A. Strain MG1655 (triangles) and its *ptsN* mutant CR301 (squares) were grown in M9 minimal medium containing 0.5% glucose. Cells were harvested by filtration at the indicated growth points and K^+ concentrations were measured (mean ± SD, $n = 2$) as described previously (Lee *et al*., 2007).
B. *ilvB* promoter activity, dependent on σ^{70}, was determined in the presence of varying amounts of potassium glutamate (100–500 mM) as described in *Experimental procedures*.
C. *In vitro* sigma competition assays were performed in the presence of 200 and 400 mM potassium glutamate. Transcription assays were performed with fixed amounts of σ^{70} (250 nM) and increasing levels of σ^S (0–200 nM).
D. Transcripts in (C) were quantified using the Multi Gauge V3.0 software and plotted as relative units: diamonds, 200 mM potassium glutamate; squares, 400 mM potassium glutamate.

phenotype microarray study revealed that the most prominent phenotype of an *E. coli* K-12 *rssB* mutant overexpressing σ^S was significantly slower growth than wild type in media containing LCPs (Zhou *et al*., 2003). Therefore, increasing the cellular level of σ^S negatively affects a gene controlled by σ^{70}.

We have previously shown that dephospho-$EIIA^{Ntr}$ derepresses the *ilvBN* operon by inhibiting TrkA, an essential component of the major potassium transport system in *E. coli* and consequently a *trkA* mutant suppresses the LCP sensitivity of the *ptsN* mutant (Lee *et al*., 2007). Regulation of potassium transporters by $EIIA^{Ntr}$ was further extended by the discovery that $EIIA^{Ntr}$ interacts with the potassium sensor kinase KdpD and increases KdpFABC production (Lüttmann *et al*., 2009). However, a phenotype microarray study with an *E. coli* K-12 mutant deleted for the entire *kdpFABCDE* operon revealed that no phenotypic changes directly attributable to a potassium transport defect could be detected and the only phenotypic changes were increased resistance to novobiocin and increased sensitivity to hygromycin (Zhou *et al*., 2003). Furthermore, we could not detect a transposon insertion in the Kdp K^+ transport system as a suppressor of the leucine sensitivity phenotype. The different effects between the Trk system and the Kdp system on the leucine sensitivity phenotype are probably because the *kdp* genes are expressed only when growth is limited by the availability of potassium ion while *trkA* is constitutively expressed (Siebers and Altendorf, 1988).

Transcriptome analysis of cells in exponential phase revealed that all of the significantly upregulated genes in the *ptsN* mutant are regulated by σ^S while most of the downregulated genes belong to the σ^{70} regulon (Tables 1, S3 and S4). Both transcriptome and proteome analyses showing differential effects of *ptsN* deletion on σ^{70} and σ^S regulon genes (Table 1; Fig. 3A) led us to focus on competition between the two sigma factors *in vivo*.

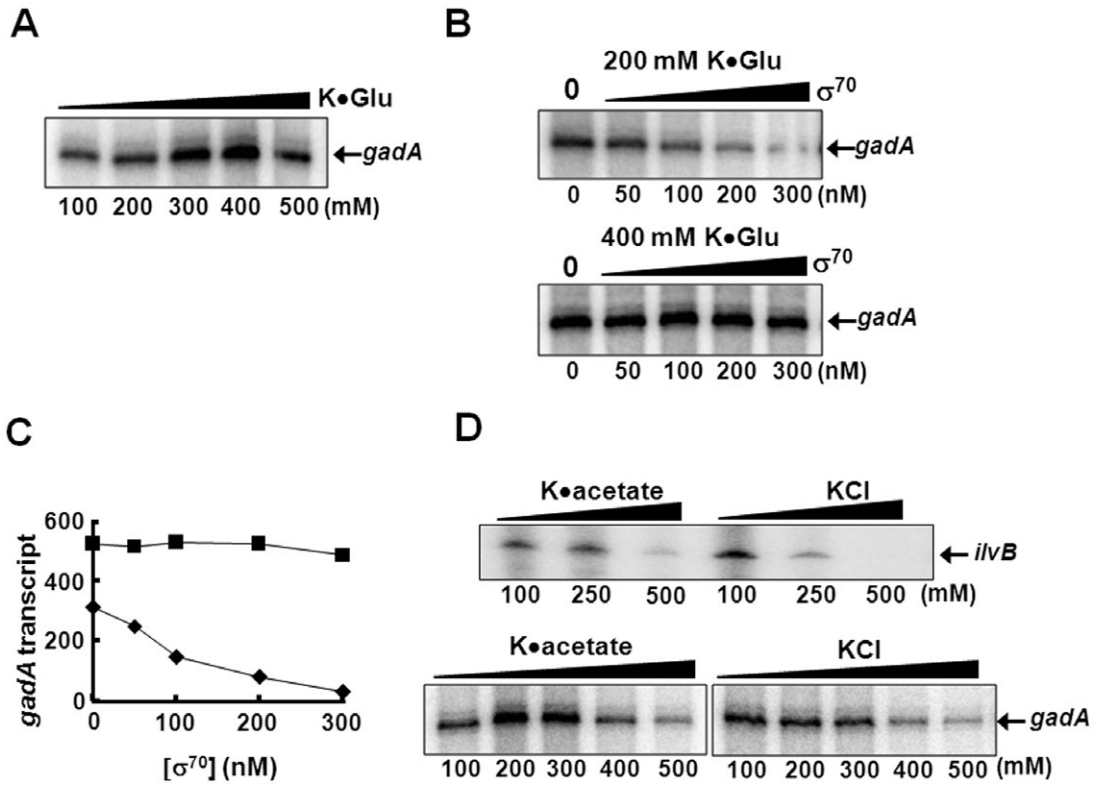

Fig. 5. Effects of potassium on *gadA* transcription *in vitro*.
A. The *gadA* promoter was transcribed by Eσ^S in the presence of varying amounts of potassium glutamate (100–500 mM).
B. *In vitro* sigma competition assays were performed in the presence of 200 and 400 mM potassium glutamate. Transcription assays were performed with fixed amounts of σ^S (250 nM) and increasing levels of σ^{70} (0–300 nM).
C. Quantification of the transcripts in (B) plotted as relative units: diamonds, 200 mM potassium glutamate; squares, 400 mM potassium glutamate.
D. *In vitro* transcriptions of *ilvB* and *gadA* promoters were carried out in the presence of the indicated concentrations of potassium acetate and potassium chloride by using σ^{70} and σ^S respectively.

We established that the effect of *ptsN* deletion on global gene expression was not due to changes in the levels of the sigma factors themselves (Table S5 and Fig. S1). Genes under the control of Eσ^S are efficiently expressed under certain conditions, such as in stationary phase and upon stress, even though the σ^S concentration never exceeds that of σ^{70}. Furthermore, when σ^S was ectopically expressed in exponential phase, it showed only basal activity (Kvint *et al*., 2000), implying that the activity of σ^S is controlled by other factors as well as its cellular level. Although *relA* mutants are as sensitive to leucine as the *ptsN* mutant (data not shown) and ppGpp is known to affect σ^S effectiveness by a mechanism not connected to regulation of the sigma factor level (Jishage *et al*., 2002), the ppGpp level was not changed in the *ptsN* mutant (Fig. S2). A recent report showed that Crl also supports competition of σ^S with σ^{70} for core RNAP by increasing the formation of σ^S-containing RNAP holoenzyme (Typas *et al*., 2007a), but the *crl* transcript level was slightly decreased in the *ptsN* mutant (Table S5).

We previously reported the effect of EIIA^{Ntr} on derepression of the *ilvBN* operon (Lee *et al*., 2005) and subsequently showed that dephospho-EIIA^{Ntr} inhibits TrkA, an essential component of the major potassium transport system (Lee *et al*., 2007). Our focus on EIIA^{Ntr}-dependent regulation therefore turned to K$^+$-dependent regulatory events. Consequently, we studied the effect of K$^+$ concentration on sigma factor activities. A variety of studies, *in vivo* and *in vitro*, indicated that, at the appropriate concentrations, K$^+$ can inhibit Eσ^{70} but stimulate Eσ^S (Figs 4 and 5). Further analysis revealed that K$^+$ affects the interaction of σ^{70} and σ^S with core RNAP (Figs 6–8). All in all, the data suggest that K$^+$ concentration can affect sigma factor selectivity. The biological picture that emerges is that EIIA^{Ntr}, dependent on its phosphorylation state, interacts directly with TrkA resulting in a regulation of the intracellular concentration of K$^+$; the resultant variation in cellular K$^+$ levels controls transcription of global genes by controlling sigma factor selectivity. Intriguingly, the intracellular K$^+$ level in wild-type cells gradually increased until a plateau was reached at stationary phase (Fig. 4A). This

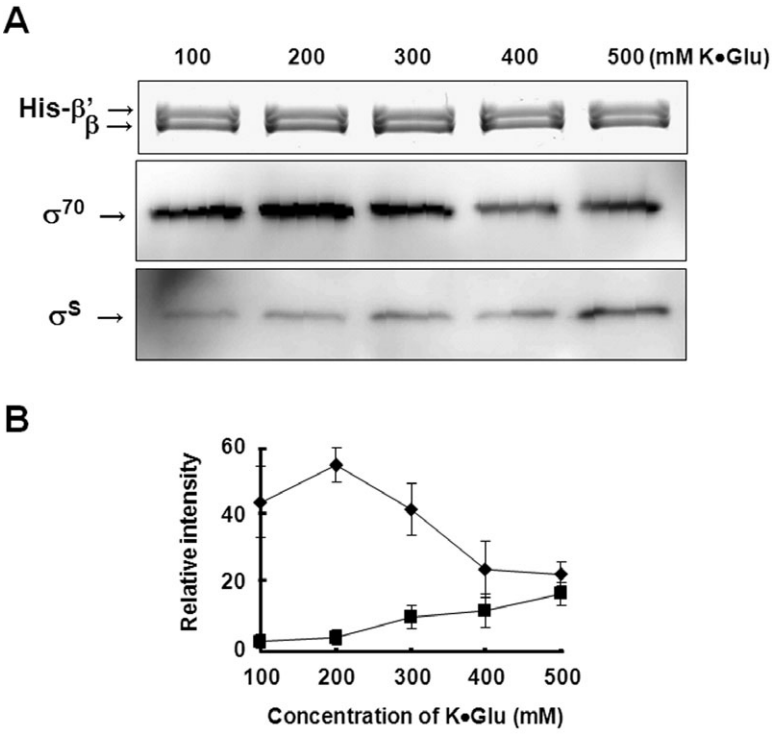

Fig. 6. Measurement of the concentrations of σ^{70} and σ^S bound to core RNAP in the presence of varying amounts of potassium glutamate.
A. YR300 cells were grown to early-stationary phase in M9 minimal medium containing 0.5% glucose and harvested. Cells were lysed at the indicated K^+ concentrations in order to obtain whole-cell extracts, which were loaded onto BD TALON™ metal affinity resin. Eluted proteins were analysed by SDS-PAGE and visualized by immunoblots using antibodies against σ^{70} and σ^S. A representative from two independent experiments is shown.
B. Quantification of the band intensities in (A) using the Multi Gauge V3.0 software plotted as relative units: diamonds, σ^{70}; squares, σ^S.

might be one of the reasons why σ^S-dependent genes are efficiently expressed even though the σ^S concentration never exceeds that of σ^{70} in stationary growth phase.

There are several lines of evidence that K^+ concentration regulates both the activity and selectivity of sigma factors. First, the intracellular accumulation of high potassium levels associated with a high-salt environment reduced the fraction of total cellular RNA, which was associated with the σ^{70} regulon (Afflerbach *et al.*, 1998; Weber and Jung, 2002). Second, it was recently reported

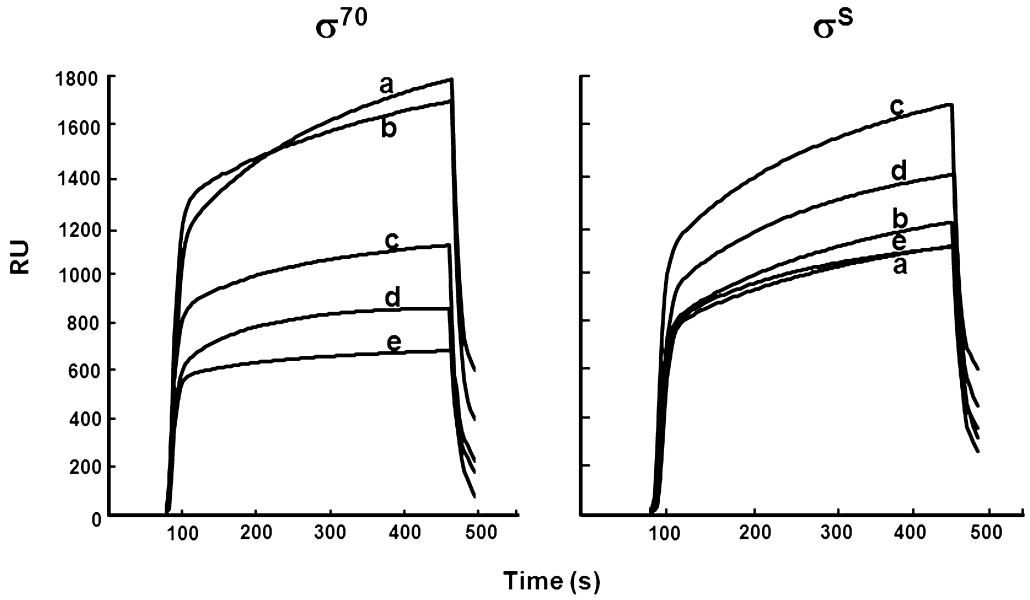

Fig. 7. SPR analysis of σ–core RNAP interactions. All SPR binding assays were conducted on a Biacore 3000 (BIAcore AB, Uppsala, Sweden). Purified N-terminal His_6-tagged σ^{70} and σ^S were immobilized to surfaces of a NTA sensor chip according to the manufacturer's instructions. Another surface was left unliganded and used as a reference flow cell. Core RNAP (300 nM) in buffers containing varying concentrations of potassium glutamate was then injected for 7 min at a flow rate of 5 μl min^{-1}, and the dissociation was followed for 2 min: a, 100 mM; b, 200 mM; c, 300 mM; d, 400 mM; e, 500 mM potassium glutamate. RU, resonance unit.

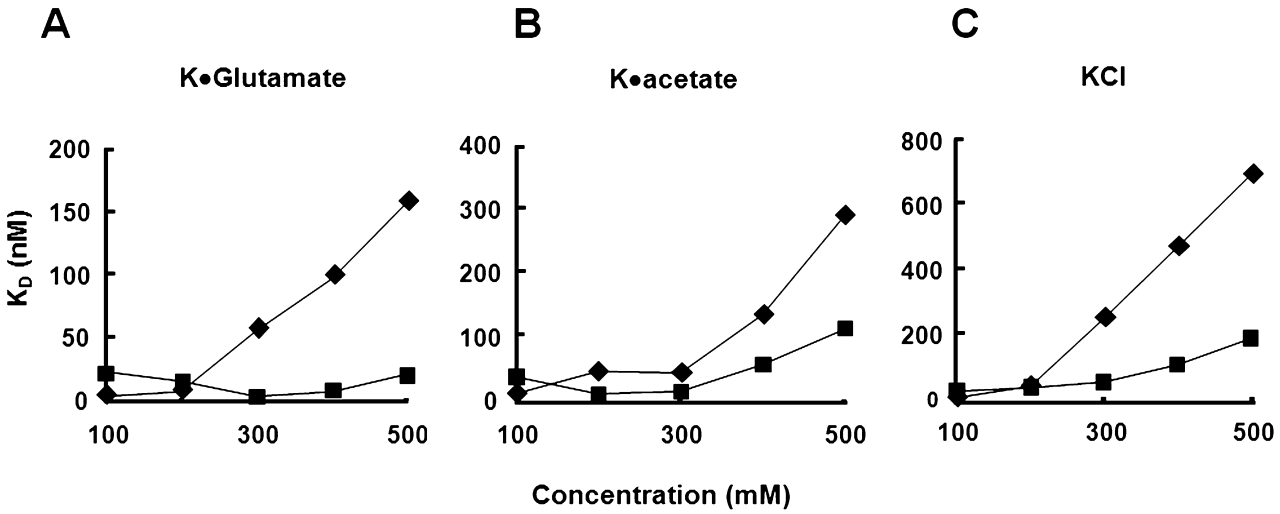

Fig. 8. Effect of potassium on the interaction between sigma factors and core RNAP. σ–core RNAP interactions were assayed using BIAcore in the presence of the indicated concentrations of potassium glutamate (A), potassium acetate (B) and potassium chloride (C). The binding curves were globally analysed with a non-linear least squares algorithm implemented in the BIAevaluation 4.1 software (Biacore). Dissociation constants (K_D) were determined based on at least two experiments. Diamonds, σ^{70}; squares, σ^S.

that transcription of ribosomal promoters was inhibited by potassium *in vitro* (Gralla and Vargas, 2006). Third, most prominent among the induced transcripts upon up-shift of intracellular potassium were those made by σ^S (Hengge-Aronis, 1996; Weber and Jung, 2002). σ^S-dependent promoters such as *osmY*, *proU* and *osmB* were stimulated by K^+ *in vitro* (Prince and Villarejo, 1990; Ding *et al.*, 1995; Lee and Gralla, 2004); these studies are in accordance with the idea that K^+ promotes σ^S-dependent transcription, but decreases σ^{70} activity. Furthermore, luminescence resonance energy transfer assays showed that the interaction of σ^{70} with core RNAP is quite sensitive to potassium glutamate, whereas the interaction of σ^{32} with core RNAP is insensitive to potassium glutamate even at concentrations higher than 500 mM (Glaser *et al.*, 2009). It should be noted that the physiological concentration of K^+ is maintained between 300 and 500 mM in *E. coli* by the K^+ transport systems, while that of Na^+ does not exceed 20 mM (Bossemeyer *et al.*, 1989; Roe *et al.*, 2000; Lo *et al.*, 2006). A high cytoplasmic K^+ concentration is required for processes such as maintenance of cell turgor and adaptation of cells to osmotic conditions (Epstein, 2003). Furthermore, many genes related to osmoregulation, including the trehalose biosynthetic genes (*otsAB*), are under the control of σ^S (Hengge-Aronis *et al.*, 1991; Lee and Gralla, 2004). Therefore, sigma factor selectivity might be one of the reasons why *E. coli* must accumulate such high cytosolic concentrations of K^+. It should be noted, however, that transcription of a particular gene may be affected by K^+ in various ways. Although we could not detect any σ^S regulon gene whose expression decreases in the *ptsN* mutant (Tables 1 and S4), effects of K^+ concentration on transcription vary significantly depending on the promoter (Tables S3 and S4). Furthermore, expression of some σ^{70}-dependent genes increased in the *ptsN* mutant (Table 1), even though the interaction of σ^{70} with core RNAP is weaker at high K^+ concentrations (Figs 7 and 8). This variation of K^+ effects might be due to differential effects on the interaction between promoter DNA and $E\sigma^S$ or $E\sigma^{70}$ or on the interaction of the transcription factors involved with their target promoters and RNAP holoenzymes. Therefore, many factors should be considered to delineate the overall effect of K^+ on transcription of a particular gene.

The biological importance of sigma factor regulation by EIIA^{Ntr} is worthy of discussion. A recent report suggests that the PTS^{Ntr} is implicated in maintenance of bacterial cell envelope integrity (Hayden and Ades, 2008). Many genes of the σ^S regulon assist in maintaining that integrity (Hengge-Aronis, 1996) and K^+ plays a key role in the global gene expression switch that increases expression of genes belonging to the σ^S regulon. In summary, the PTS^{Ntr} uses a sigma factor selectivity regulation mechanism to deal with stress conditions and the mediator of that selectivity is K^+. When the signal regulating the phosphorylation state of the PTS^{Ntr} proteins is discovered, the biological mechanism by which EIIA^{Ntr} accomplishes the regulation of sigma factor activity will be more thoroughly understood.

Experimental procedures

Bacterial strains, plasmids and culture conditions

The bacterial strains and plasmids used in this study are listed in Table 2. Luria–Bertani broth (LB) was used as the

Table 2. Bacterial strains and plasmids used in this study.

Strain or plasmid	Genotype or description	Source or reference
Strains		
DY330	W3110 Δ*lacU169 gal490* λ*CI857* Δ*(cro–bioA)*	Yu *et al.* (2000)
GI698	F⁻ λ⁻ *lacI*^{q} *lacPL8 ampC*::P_{trp} cI	LaVallie *et al.* (1993)
JM101	*glnV44 thi-1* Δ(*lac–proAB*) *F′* [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	Miller (1972)
MG1655	Wild-type *E. coli* K-12	Blattner *et al.* (1997)
CR101	MG1655 *ptsP*::Cm^R	Lee *et al.* (2005)
CR201	MG1655 *ptsO*::Tet^R	Lee *et al.* (2005)
CR301	MG1655 *ptsN*::Tet^R	Lee *et al.* (2005)
CR500	DY330 *rpoS*::Km^R	This study
CR501	MG1655 *rpoS*::Km^R	This study
CR600	DY330 *rssB*::Km^R	This study
CR601	MG1655 *rssB*::Km^R	This study
CR305	MG1655 *rpoS*::Km^R, *ptsN*::Tet^R	This study
CR400	JM101 λ [Φ(*ilvB′–′lacZ*)]	Lee *et al.* (2005)
CR401	CR400 *ptsN*::Tet^R	Lee *et al.* (2005)
CR402	CR400 *rpoS*::Km^R	This study
CR403	CR400 *rssB*::Km^R	This study
CR404	CR400 *rpoS*::Km^R, *ptsN*::Tet^R	This study
CR700	JM101 λ [Φ(*gadA′–′lacZ*)]	This study
CR701	CR700 *ptsN*::Tet^R	This study
CR800	JM101 λ [Φ(*gadB′–′lacZ*)]	This study
CR801	CR800 *ptsN*::Tet^R	This study
CR900	JM101 λ [Φ(*katE′–′lacZ*)]	This study
CR901	CR900 *ptsN*::Tet^R	This study
YR100	JM101 λ [Φ(*bolA′–′lacZ*)]	Gift from Dr. Choy
YR101	YR100 *ptsN*::Tet^R	This study
YR200	JM101 λ [Φ(*hdeA′–′lacZ*)]	Shin *et al.* (2005)
YR201	YR200 *ptsN*::Tet^R	This study
YR300	MG1655 β′–His	Gift from Dr. Choy
YR401	MG1655 *yhbH*::Cm^R	This study
YR501	MG1655 *yhbJ*::Cm^R	This study
YR601	MG1655 *rppH*::Km^R	This study
Plasmids		
pRE1	Expression vector under control of λP_L promoter, Amp^r	Reddy *et al.* (1989)
pRS415	*lacZ lacY*$^+$ *lacA*$^+$, Amp^r	Simons *et al.* (1987)
pCR3H	pRE1-based expression vector for EIIA^{Ntr} with N-terminal 6 histidines	Lee *et al.* (2005)
pYR1H	pRE1-based expression vector for σ^{70} with N-terminal 6 histidines	This study
pYR2H	pRE1-based expression vector for σ^S with N-terminal 6 histidines	This study
pCR6	pRS415[Φ(*gadA′–′lacZ*)], Amp^r	This study
pCR7	pRS415[Φ(*gadB′–′lacZ*)], Amp^r	This study
pCR8	pRS415[Φ(*katE′–′lacZ*)], Amp^r	This study
pCR9	pRS415[Φ(*bolA′–′lacZ*)], Amp^r	This study
pCR10	pRS415[Φ(*hdeA′–′lacZ*)], Amp^r	This study
pSA600	The template plasmid for *in vitro* transcription, contained the *bla* gene and the wild-type *lac* promoter region followed by a transcription terminator	Ryu and Garges (1994)
pYR3	pSA600 incorporated with *ilvB* promoter region –620 ~ +309 (EcoRI/PstI)	This study
pYR4	pSA600 incorporated with *gadA* promoter region –512 ~ +302 (EcoRI/PstI)	This study

complex culture medium for the routine growth of bacteria unless indicated otherwise. For synthetic minimal medium, M9 medium containing 0.5% glucose, supplemented with the indicated amounts of amino acids, as necessary, was used. For the growth of GI698 strains, M9 salts-based rich medium was used as described previously (LaVallie *et al.*, 1993). DY330, GI698 and their derivatives were grown at 30°C, while the other strains listed in Table 2 were grown at 37°C. The *rpoS* deletion mutant was constructed using *E. coli* DY330 as described previously (Yu *et al.*, 2000). The *rpoS* gene (from the start codon to the stop codon) was replaced by the *neo* gene. The *neo* gene was amplified by PCR from the TSDH02 strain (Nam *et al.*, 2005) with the following primers: forward primer, 5′-ACA GAA AAG GCC AGC CTC GCT TGA GAC TGG CCT TTC TGA CAG ATG CTT ACT CAG AAG AAC TCG TCA AGA A-3′ and reverse primer, 5′-AGG CTT TTG CTT GAA TGT TCC GTC AAG GGA TCA CGG GTA GGA GCC ACC TTA TGA TTG AAC AAG ATG GAT T-3′. The PCR product was electroporated into *E. coli* DY330 to make strain CR500. MG1655 Δ*rpoS* (CR501) was constructed by P1 transduction of the Km^R region of CR500. Strains CR600 (DY330 *rssB*::Km^R) and CR601 (MG1655 *rssB*::Km^R) were prepared in a similar way using primers 5′-AGG TGG CAA TAG CAT GCC ACT ATT GAG TAA AGC CAG TCA GGG GAG AGA ACA TGA TTG AAC AAG ATG GAT T-3′ and 5′-ACC AGC CGA CAT TAG CAG GTA ATG CAA ATT TAG CCC GCG TTA TCG TTT GCT CAG AAG AAC TCG TCA AGA A-3′ to amplify the Km^R region from the

chromosomal DNA of TSDH02 and replace the coding region of *rssB* with that of the Km^R gene.

Purification of EIIA^{Ntr}, σ^{70} and σ^S

To construct pYR1H, the vector for His$_6$–σ^{70} expression, primers possessing the synthetic restriction enzyme sites NdeI, located 3 bp upstream from the ATG start codon (in boldface type) (5′-AAGTGTGGATACCGTCAT**ATG** GAGCAAAAC-3′), and SalI, located 5 bp downstream from the TAA stop codon (5′-CGCCTGATCCGGTCGACCGAT TAATCGTCC-3′) (restriction sites underlined) of the *rpoD* gene were used to amplify, by PCR, the *rpoD* gene from MG1655 genomic DNA. After digestion, the NdeI–SalI fragment was inserted into the corresponding sites of pRE1 (Reddy *et al*., 1989).

The expression vector pYR2H for overproduction of His$_6$–σ^S was generated in a similar manner using a primer pair covering the *rpoS* gene: forward primer, 5′-CCAGCC TCGG TCGACACTGGCCTTTCTGAC-3′; reverse primer, 5′-AGCCACCATA TGAGTCAGAA TACGCTGAAA-3′.

Purification of proteins was carried out using BD TALON™ metal affinity resin as described previously (Lee *et al*., 2007). *E. coli* GI698 harbouring plasmids were grown and protein expression was induced as described previously for overproduction of other proteins (LaVallie *et al*., 1993). The cell pellet obtained from 500 ml of culture containing overexpressed proteins was resuspended in binding buffer (50 mM HEPES·KOH, pH 8.0, containing 300 mM NaCl and 10% glycerol) and then passed twice through a French pressure cell at 10 000 p.s.i. The lysate was cleared of cell debris by centrifugation at 100 000 *g* for 90 min. The soluble fraction was loaded onto the BD TALON™ metal affinity resin and bound proteins were eluted with binding buffer containing 200 mM imidazole. To obtain homogeneous protein and to remove imidazole, the eluted proteins were chromatographed on a HiLoad 16/60 Superdex 75 prepgrade column (Amersham Biosciences) equilibrated with buffer A (50 mM Tris-HCl, pH 8.0 containing 50 mM NaCl and 10% glycerol).

Acetohydroxy acid synthase assay

The specific activity of AHAS was measured using the standard colorimetric assay for acetoin, which is formed from acetolactate after acid quench (Epelbaum *et al*., 1990; Lee *et al*., 2005). Specific activity was expressed as micromoles acetoin formed per hour per milligram protein under the conditions stated, referenced to an acetoin standard curve. Total protein was determined colorimetrically, using bovine serum albumin as the standard.

Construction of lacZ *fusions and β-galactosidase assays*

The DNA fragment covering the region from 646 bp upstream to 287 bp downstream of the *gadA* start codon was amplified by PCR using primers 5′-CAACGCAGGTCGAT GGATCCGGATTGCGGA-3′ (an engineered BamHI site underlined) and 5′-ATTAATTACGCCGAATTCCCCCATTT GATAA-3′ (an engineered EcoRI site underlined). The PCR product, digested with EcoRI and BamHI, was inserted into pRS415 (Simons *et al*., 1987) to make pCR6. The accuracy of the *gadA* insert was verified by sequencing, and the fusion was transferred to λRS45 by *in vivo* recombination (Simons *et al*., 1987). Bacteriophage λ carrying the fusion was used to lysogenize JM101 at low multiplicity, and several lysogens were analysed to obtain a monolysogen (CR700). CR701 (CR700 *ptsN*::Tet^R) was constructed by P1 transduction of the Tet^R region of CR700. Similarly, the DNA fragment covering the region from 538 bp upstream to 276 bp downstream of the *gadB* start codon was amplified by PCR using primers 5′-CCAACAGCGTAGAATTCAACAATTAGCGAA-3′ (an engineered EcoRI site underlined) and 5′-CGATG GCTGCGGATCCCGGATATTCTTCTT-3′ (an engineered BamHI site underlined) to make the *gadB–lacZ* fusion construct and strains CR800 and CR801 (see Table 2). The DNA fragment covering the region from 583 bp upstream to 262 bp downstream of the *katE* start codon was amplified by PCR using primers 5′-TGATTAGGAATTCTTAACCATTTTAAA ATA-3′ (an engineered EcoRI site underlined) and 5′-TGA TCGTCGGGGATCCGCACGCCCTGATTA-3′ (an engineered BamHI site underlined) to make the *katE–lacZ* fusion construct and strains CR900 and CR901 (see Table 2). After cells were grown in LB medium to the mid-logarithmic phase, cells were centrifuged, washed and resuspended in M9 minimal medium containing 0.5% glucose. After nutritional downshift, β-galactosidase activity was measured at mid-logarithmic growth phase (Miller, 1972).

Random mutagenesis

The EZ::Tn5<KAN-2>Tnp transposome kit was used as suggested by the manufacturer (Epicentre). The EZ::Tn5<KAN-2> transposon–EZ::Tn5 transposase complexes were introduced by electroporation into CR301 competent cells. The electroporated cells were plated on minimal medium agar containing 0.5% glucose, 40 μg ml^{-1} of kanamycin, 20 μg ml^{-1} of tetracycline and 0.25 mM Ala-Leu. The genomic regions harbouring the insertion of the EZ::Tn5<KAN-2> transposon from suppressor mutants were rescued by PCR with transposon inner primer and arbitrary primer: 5′-TCC GGC TTC CCA TAC AAT CGA TAG-3′(Kan R2), 5′-CCC ACA GGA AGG TAT TCT GGA AGA TAC GGC GGT-3′(SynArb1). The arbitrary primer was constructed by adding the GGCGGT sequence to the random primer sequence. There are about 2600 GGCGGT sequences and about 2500 ACCGCC sequences in the MG1655 genome. PCR was performed with primer SynArb1 and primer Kan R2 using genomic DNAs as templates. The PCR products were isolated and purified using a PCR purification kit (QIAGEN), and the automatic sequencing (ALFexpress, Amersham Phamacia Biotech, Upsala, Sweden) used primer ME (5′-AGATGTGTATAAGAGACAG-3′).

Surface plasmon resonance spectroscopy

Real-time interaction of core RNAP with σ^{70} or σ^S was monitored by SPR detection using a BIAcore 3000 (BIAcore AB) (Lee *et al*., 2007). σ^{70} and σ^S were separately immobilized onto the surface of a nitrilotriacetic acid sensor chip (NTA chip). All experiments were conducted at 25°C in modified running buffer [50 mM Tris-HCl (pH 8.0), 100 mM KCl, 3 mM $MgCl_2$, 50 μM EDTA]. N-terminal His$_6$-tagged sigma proteins were attached to an NTA chip as follows: 20 μl of 500 μM

$NiSO_4$ solution was injected over one flow cell at a flow rate of 10 μl min^{-1}. Then 100 μl of sigma factor was injected over the same flow cell. Assuming that 1000 resonance units correspond to a surface concentration of 1 ng mm^{-2}, σ^{70} and σ^S were immobilized to a surface concentration of 2.0 and 1.7 ng mm^{-2} respectively. After immobilization of sigma factor to the chip, 300 nM core RNAP in running buffer containing varying amounts of potassium was injected over the two flow cells. A third empty flow cell was used as a control for non-specific binding. The sensor surface was regenerated between assays by using the running buffer at a flow rate of 100 μl min^{-1} for 10 min to remove bound analytes. The chip was washed by injection of 150 mM imidazole at 10 μl min^{-1} followed by an injection of 0.25 M EDTA.

In vitro *transcription assay*

pYR3 and pYR4 used as templates were extracted from transformants using a Plasmid Purification Kit (Nucleogen) and a phenol-chloroform solution. Approximately 250 nM sigma and 50 nM core RNAP were incubated at 37°C for 10 min in binding buffer containing varying amounts of potassium glutamate [20 mM Tris-HCl (pH 8.0), 1 mM DTT, 3 mM $MgSO_4$, 100 μM cAMP, 100 μg ml^{-1} BSA]. After incubation of holoenzyme and supercoiled DNA template (2 nM) at 37°C for 10 min, transcription was initiated by adding NTP mix [1 mM ATP, 100 μM each GTP and UTP, 10 μM CTP, 0.75 μM of [α-^{32}P]CTP (800 Ci $mmol^{-1}$)] in a total volume of 20 μl. The synthesized transcripts were extracted with a phenol-chloroform solution and precipitated with 300 mM sodium acetate and 2 volumes of ethanol. The transcripts were resuspended in 10 μl of formamide loading buffer and analysed by electrophoresis on a 6% polyacrylamide gel containing 8 M urea.

Quantification of σ^{70} and σ^S associated with core RNAP

Cells expressing C-terminal His_6-tagged β′ protein were grown in LB medium to the early-stationary growth phase. Cultures (50 ml each) were harvested and resuspended in 3 ml reconstitution buffer containing varying concentrations of potassium glutamate [50 mM Tris-HCl (pH 8.0), 0.1 mM DTT, 0.1 mM EDTA, 50 mM NaCl and 5% glycerol] (Maeda *et al.*, 2000), and then passed twice through a French pressure cell at 10 000 p.s.i. The lysate was cleared of cell debris by centrifugation at 100 000 *g* for 30 min. The soluble fraction was loaded onto BD TALON™ metal affinity resin and bound proteins were eluted with binding buffer containing 200 mM imidazole. Eluted proteins were dot-blotted onto PVDF membranes and bound σ^{70} and σ^S were detected with specific antibodies (Santa Cruz Biotechnology).

Detection of intracellular σ^S and $EIIA^{Ntr}$

To determine the intracellular levels of σ^S and $EIIA^{Ntr}$, we purchased monoclonal antibody against σ^S (Santa Cruz Biotechnology) and made polyclonal antibodies against $EIIA^{Ntr}$ using female ICR mice. Cells were grown in M9-based minimal medium containing 0.5% glucose to mid-logarithmic phase and 0.4 ml of cell culture was collected. After boiling for 5 min, the samples were analysed with 15% SDS-polyacrylamide gels. Immunoblotting was performed according to standard procedures using specific antibodies.

DNA microarray analysis

For transcriptome analysis, the *E. coli* whole-genome TwinChip, manufactured and kindly provided by the 21C Frontier Microbial Genomics and Applications Center (Daejeon, South Korea), was used. The *E. coli* K-12 strain MG1655 and its isogenic *ptsN* mutant were grown in M9 minimal medium containing 0.5% glucose as carbon source. Cells from duplicate experiments were harvested in exponential growth phase when cultures reached an A_{600} of 0.4. RNA was extracted from each sample using a QIAGEN RNA extraction kit according to the manufacturer's instruction, and aminoallyl cDNA was synthesized using an aminoallyl cDNA-labelling kit according to the protocols of the manufacturer (Ambion, Austin, TX, USA). The aminoallyl cDNA from the *ptsN* mutant and that from the wild type were labelled with Cy3 and Cy5 (Ambion), respectively, and equal amounts of the labelled cDNA were combined and used to hybridize the microarray slides at 42°C for 16 h. After hybridization, the arrays were washed, dried and scanned using GenePix 4000B (Axon Instruments, Foster City, CA, USA). Data from two independent experiments were normalized and then analysed using the GenePix Pro 3.0 software (Axon Instruments). The full data have been deposited to GEO (http://www.ncbi.nlm.nih.gov/geo/) under accession number GSE24373.

Comparison of protein profiles

Cells were grown in M9-based minimal medium containing 0.5% glucose to exponential phase and were centrifuged at 11 000 *g* for 10 min. The cells were resuspended in buffer A [50 mM Tris-HCl (pH 8.0) containing 50 mM NaCl and 10% glycerol] and passed twice through a French pressure cell at 10 000 p.s.i. The lysate was cleared of cell debris by centrifugation at 11 000 *g* for 30 min. The supernatant solution was chromatographed through a MonoQ 10/10 column (Amersham Biosciences) equilibrated with buffer A using a gradient of 50–1000 mM NaCl. Elution was performed at a flow rate of 2 ml min^{-1} and fractions (3 ml) were collected. Aliquots (20 μl) of eluted fractions were analysed by SDS-PAGE and stained with Coomassie brilliant blue R.

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Supporting information

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