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Growth Inhibition by External Potassium of *Escherichia coli* Lacking PtsN (EIIA^{Ntr}) Is Caused by Potassium Limitation Mediated by YcgO

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ABSTRACT

The absence of PtsN, the terminal phosphoacceptor of the phosphotransferase system comprising PtsP-PtsO-PtsN, in *Escherichia coli* confers a potassium-sensitive (K^s) phenotype as the external K$^+$ concentration ([K$^+$]$_e$) is increased above 5 mM. A growth-inhibitory increase in intracellular K$^+$ content, resulting from hyperactivated Trk-mediated K$^+$ uptake, is thought to cause this K^s. We provide evidence that the K^s of the Δ*ptsN* mutant is associated with K$^+$ limitation. Accordingly, the moderate K^s displayed by the Δ*ptsN* mutant was exacerbated in the absence of the Trk and Kup K$^+$ uptake transporters and was associated with reduced cellular K$^+$ content. Conversely, overproduction of multiple K$^+$ uptake proteins suppressed the K^s. Expression of PtsN variants bearing the H73A, H73D, and H73E substitutions of the phosphorylation site histidine of PtsN complemented the K^s. Absence of the predicted inner membrane protein YcgO (also called CvrA) suppressed the K^s, which was correlated with elevated cellular K$^+$ content in the Δ*ptsN* mutant, but the Δ*ptsN* mutation did not alter YcgO levels. Heterologous overexpression of *ycgO* also led to K^s that was associated with reduced cellular K$^+$ content, exacerbated by the absence of Trk and Kup and alleviated by overproduction of Kup. Our findings are compatible with a model that postulates that K^s in the Δ*ptsN* mutant occurs due to K$^+$ limitation resulting from activation of K$^+$ efflux mediated by YcgO, which may be additionally stimulated by [K$^+$]$_e$, implicating a role for PtsN (possibly its dephosphorylated form) as an inhibitor of YcgO activity.

IMPORTANCE

This study examines the physiological link between the phosphotransferase system comprising PtsP-PtsO-PtsN and K$^+$ ion metabolism in *E. coli*. Studies on the physiological defect that renders an *E. coli* mutant lacking PtsN to be growth inhibited by external K$^+$ indicate that growth impairment results from cellular K$^+$ limitation that is mediated by YcgO, a predicted inner membrane protein. Additional observations suggest that dephospho-PtsN may inhibit and external K$^+$ may stimulate K$^+$ limitation mediated by YcgO. It is speculated that YcgO-mediated K$^+$ limitation may be an output of a response to certain stresses, which by modulating the phosphotransfer capacity of the PtsP-PtsO-PtsN phosphorelay leads to growth cessation and stress tolerance.

All living cells possess mechanisms to accumulate potassium (K$^+$), and bacteria such as *Escherichia coli* maintain an intracellular K$^+$ concentration ([K$^+$]$_i$) of 200 to 400 mM that is thought to be required for optimal functioning of several metabolic processes (reviewed in references 1 and 2). In addition, K$^+$ is a major determinant in the maintenance of cell turgor, so that an increase in osmolarity of the medium is associated with increased [K$^+$]$_i$ (3–6). K$^+$ has also been proposed to act as a second messenger (2, 7).

The maintenance of cytoplasmic K$^+$ pools in *E. coli* is achieved through the balance of activities of K$^+$ uptake systems Kdp, Trk, and Kup (formerly known as TrkD) on the one hand and of an as yet unidentified K$^+$ efflux system or systems on the other (1, 2). The well-studied K$^+$ efflux systems KefG/B and KefF/C are known to act as K$^+$/H$^+$ antiporters, with K$^+$ efflux and concomitant H$^+$ influx leading to cytoplasmic acidification, serving to mitigate the detrimental effects of endogenous or exogenous electrophiles (reviewed in reference 8). A residual K$^+$ transport activity, TrkF, present in a *kdp kup trk* triple K$^+$ transporter-defective mutant is thought to represent a mode of K$^+$ uptake occurring through systems that do not normally transport K$^+$ (9), and an as yet uncharacterized turgor-activated efflux system is also believed to exist (10).

The Kdp transporter, encoded by genes of the *kdpFABC* operon, is a high-affinity K$^+$ uptake system (11, 12) that is transcriptionally induced when the external K$^+$ concentration ([K$^+$]$_e$) becomes limiting for growth (13, 14). More recent studies have shown that expression and/or activity of the Kdp transporter is also inhibited by [K$^+$]$_e$s above 5 mM (15, 16). The Trk and Kup systems, in contrast, are low-affinity K$^+$ uptake systems that are constitutively expressed (1, 2). Of these systems, Kup is a standalone K$^+$ transporter, whereas the Trk system is a multicomponent system, and a null mutation in *trkA*, coding for the regulatory subunit, disables the Trk system (2). The presence of multiple transport systems for K$^+$ allow, within the limits of its osmoregulatory capacity, robust growth of *E. coli* in media with a wide range of [K$^+$]$_e$s.

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In *E. coli*, components of the phosphotransferase system (PTS) mediate uptake of carbohydrates, wherein transport of the incoming sugar is coupled to its phosphorylation (reviewed in references 17, 18, 19, and 20). In each of these systems, a phosphate moiety is transferred from phosphoenolpyruvate (PEP) to the particular sugar via a multiprotein phosphorelay mechanism. *E. coli* also possesses a PTS comprising PtsP-PtsO-PtsN, with a PEP-dependent phosphorelay operating in the same sequence (21–23). However, the phosphorylation substrate of PtsN is unknown. Given that *ptsN* and *ptsO* are member genes of the *rpoN* operon (21), PtsP, PtsO, and PtsN have also been referred to as EI^{Ntr}, Npr, and $EIIA^{Ntr}$, respectively. A recent study has shown that the phosphorylation state of PtsN can be modulated, depending upon the quality of the nitrogen source in the medium, implicating a role for the PtsP-PtsO-PtsN phosphorelay in sensing cellular nitrogen stress (24).

Previously, an intriguing connection between K^+ metabolism and the PtsP-PtsO-PtsN phosphorelay was identified by Lee et al. (25), who showed that (i) a Δ*ptsN* mutant exhibits a K^+-sensitive (K^s) phenotype associated with elevated cellular K^+ content, and (ii) the PtsN-H73A variant (which lacks the site of phosphorylation on PtsN and hence is constitutively in the dephospho-PtsN form), but not PtsN, forms a complex with TrkA. Lee et al. have proposed that in the absence of dephospho-PtsN, TrkA activity is unfettered, leading to cellular K^+ overload due to enhanced Trk-mediated K^+ uptake, which causes the K^s (25).

In this study, we found that consistent with earlier reports (25, 26), a strain lacking PtsN was rendered K^s, as $[K^+]_e$ was raised above 5 mM in a synthetic minimal medium. However, genetic and physiological studies indicated that the K^s of the Δ*ptsN* mutant resulted from K^+ limitation and was not due to a K^+ overload as proposed previously (25). Absence of the predicted inner membrane protein CvrA (referred to throughout this study as YcgO) (27) suppressed the K^s, and its heterologous overexpression caused a K^s similar in many respects to that displayed by the Δ*ptsN* mutant, implicating YcgO to be the mediator of the K^s of the Δ*ptsN* mutant. An additional implication of this work is that YcgO activity in *E. coli* is probably rendered cryptic by dephospho-PtsN. A model for the occurrence of K^+ limitation in the Δ*ptsN* mutant is proposed, and finally, a probable physiological role for YcgO-mediated K^+ limitation is discussed.

MATERIALS AND METHODS

Bacterial strains, growth conditions, and plasmids. Genotypes of the *E. coli* K-12 strains used in this study are listed in Table 1. The Δ*ptsN*::Kan, Δ*ycgO*::Kan, Δ*kdpA*::Kan, and Δ*amtB*::Kan knockout mutations were obtained from appropriate strains of the Keio collection (28) and introduced into other strains by P1 transduction (29). Wherever required, the gene encoding the kanamycin resistance determinant was excised following treatment of strains with plasmid pCP20 (30). The *trkA405* and *trkD1* mutations representing loss-of-function alleles of *trkA* and *kup* were obtained from strain TL1105A (13) and introduced into other strains by P1 transduction in multiple steps. In this study, the *trkA405* and the *trkD1* alleles are referred to as *trkA* and *kup*, respectively. The routinely used rich growth media were LB and KML media. KML is a modified LB medium in which NaCl is substituted for with KCl (31), and it was used for the propagation of strains triply defective for K^+ transport (uptake) systems. The antibiotics ampicillin (Amp), chloramphenicol (Cm), kanamycin (Kan), and tetracycline (Tet) and the *lac* inducer isopropyl-β-D-thiogalactoside (IPTG) were used at appropriate concentrations, and the growth temperature was 37°C.

TABLE 1 *E. coli* strains and plasmids used in this study[a]

Strain or plasmid	Genotype or description
Strains	
MC4100	Δ(*argF-lac*)*U169 rpsL150 relA1 spoT1 araD139 flbB5301 deoC1 ptsF25*
JD17	MC4100 Δ*ptsN*::Kan
JD466	MC4100 Δ*ycgO*::Kan
JD509	MC4100 Δ*ptsN* Δ*ycgO*::Kan
JD624	MC4100 *kup trkA*
JD637	JD624 Δ*kdpA*
JD656	MC4100 Δ*kdpA*
JD657	JD637 *zha-203*::Tn*10*
JD658	JD657 Δ*ptsN*::Kan
JD660	JD624 Δ*ptsN*::Kan
JD662	JD656 Δ*ptsN*::Kan
JD693	JD637 Δ*ycgO*::Kan
JD694	JD656 Δ*ycgO*::Kan
JD696	JD624 Δ*ycgO*
JD710	JD624 Δ*ycgO* Δ*ptsN*::Kan
JD714	JD637 Δ*ycgO* Δ*ptsN*::Kan *zha-203*::Tn*10*
JD723	JD624 $ycgO_{FL}$ ΔKan
JD724	JD723 Δ*ptsN*::Kan
JD725	JD656 Δ*ycgO* Δ*ptsN*::Kan
JD726	MC4100 *att*λ::(P_{trc}99A *bla*)
JD727	MC4100 *att*λ::(P_{trc}*ycgO bla*)
JD728	JD624 *att*λ::(P_{trc}99A *bla*)
JD729	JD624 *att*λ::(P_{trc}*ycgO bla*)
JD732	JD624 *att*λ::(P_{trc}*ycgO bla*::Kan)
JD734	JD637 *att*λ::(P_{trc}99A *bla*)
JD735	JD637 *att*λ::(P_{trc}*ycgO bla*)
GJ14949	JD656 *att*λ::(P_{trc}99A *bla*)
GJ14950	JD656 *att*λ::(P_{trc}*ycgO bla*)
Plasmids	
pHYD3025	Derivative of plasmid pTrc99A, described in reference 41
pHYD724	pTrc99A in which *kdpA′*, which encodes N-terminal 135 amino acids of KdpA, is placed under expression control of P_{trc} promoter, described in reference 43
pHYD1852	pHYD3025 bearing *kup* expressed from P_{trc} promoter, present within EcoRI and HindIII sites
pHYD1858	pTrc99A containing *ycgO*, present within NcoI and HindIII sites, and expressed from P_{trc} promoter
pHYD5006	pHYD3025 containing *ptsN* appended at its 3′ end with DNA sequence encoding hexahistidine, placed under expression control of P_{trc} promoter, present within NdeI and HindIII sites
pHYD5007	Derivative of pHYD5006 encoding PtsN bearing H73A amino acid substitution
pHYD5008	Derivative of pHYD5006 encoding PtsN bearing H73D amino acid substitution
pHYD5009	Derivative of pHYD5006 encoding PtsN bearing H73E amino acid substitution

[a] All strains listed are *E. coli* K-12 strains, and MC4100 was from our laboratory collection. The Δ*ptsN*::Kan, Δ*ycgO*::Kan, and Δ*kdpA*::Kan deletion insertion mutations were obtained from strains JW3171, JW5184, and JW0686, respectively, of the Keio collection (28) and introduced into appropriate strains by P1 transduction. Removal of the antibiotic cassette was performed as described previously (30), and such mutations are indicated in the table by "Δ." *trkA* and *kup* represent the *trkA405* and *trkD1* alleles, respectively, which were obtained from strain TL1105A (13) and introduced into MC4100 by P1 transduction in multiple steps. *zha-203*::Tn*10* was obtained from CAG12072 (33) and introduced into appropriate strains by P1 transduction. $ycgO_{FL}$ encodes YcgO bearing a 3× FLAG epitope attached to the C terminus of YcgO, and the *att*λ::(P_{trc}*ycgO bla*), *att*λ::(P_{trc}99A *bla*), and *att*λ::(P_{trc}*ycgO bla*::Kan) chromosomal constructs were obtained by the plasmid-to-chromosome shuttling system (see the "Methods" section in the supplemental material for details). The ancestral plasmid pTrc99A is described in reference 34.

For tests of the K^+-sensitive (K^s) phenotype, the medium of Epstein and Kim (32) was used, which is made with reciprocally varied concentrations of Na^+ and K^+ by mixing together suitable proportions of 115 mM K^+ phosphate-buffered medium and 115 mM Na^+ phosphate-buffered medium, each of pH 7.2. In this study, K_1 medium with a $[K^+]_e$ of 1 mM refers to a 115 mM Na^+ phosphate medium containing 1 mM KCl. In K_{10}, K_{20}, and K_{40} media, the K^+ concentrations are 10, 20, and 40 mM, respectively, and the Na^+ concentrations are 105, 95, and 75 mM, respectively. K_{115} medium contains 115 mM K^+. The minimal media mentioned above were supplemented with glucose (0.2%), $MgSO_4$ (1 mM), and B1 (0.0001%). Phosphate-buffered media of reciprocally varied Na^+ and K^+ concentrations at pHs of 6.0 and 7.8 were made after appropriate adjustments of the ratios of monobasic and dibasic phosphates. Glucose and other medium supplements were used as described above.

Δ*ptsN*::Kan derivatives of MC4100 or of MC4100 lacking the Kdp transporter were obtained by isolating Kan-resistant (Kan^r) transductants on LB agar plates containing Kan using an appropriate donor P1 lysate. A Δ*ptsN*::Kan derivative of a strain doubly defective for TrkA and Kup was obtained by isolating Kan^r transductants on Kan-supplemented glucose K_1 agar plates containing 0.2% Casamino Acids, and the strain was maintained on the aforementioned medium. Plasmids were introduced into this strain (and its $PtsN^+$ ancestor) by isolating transformants on antibiotic-supplemented glucose K_1 agar plates containing 0.2% Casamino Acids, and the transformants were maintained on the same medium. To generate the Δ*ptsN*::Kan derivative of the triple K^+ transporter-defective strain, the Δ*ptsN*::Kan allele was introduced via cotransduction with the *zha-203*::Tn*10* insertion derived from strain CAG12072 (33), with a primary selection for Tet-resistant transductants using a P1 lysate prepared on strain JD650 (MC4100 Δ*ptsN*::Kan *zha-203*::Tn*10*) on KML agar plates. *zha-203*::Tn*10* derivatives of MC4100 or of the triple K^+ transporter-defective strain JD637 grew at rates identical to those of their respective parental strains on media of various $[K^+]_e$s. Furthermore, the *zha-203*::Tn*10* marker did not affect the K^s of the Δ*ptsN*::Kan mutant.

The plasmids used in this study are listed in Table 1 (also see Table S2 in the supplemental material) and are derivatives of plasmids pTrc99A (34) and pACYC184 (35). Additional details regarding their construction are provided in the supplemental material. The oligonucleotide primers used in this study are listed in Table S1 in the supplemental material. Procedures for PCR, cloning, and overlap extension PCR-based site-directed mutagenesis were followed as described in reference 36, and the integrity of cloned genes was confirmed by DNA sequencing.

Testing for the K^s phenotype. For scoring of potassium-sensitivity (K^s), strains were streaked onto K_1, K_{20}, K_{40}, and K_{115} glucose agar plates, and their growth proficiency was gauged. To assess the influence of the Δ*ptsN*::Kan mutation on growth rates in different K^+ transporter-defective backgrounds, pairs of $ptsN^+$ and Δ*ptsN*::Kan strains were grown in K_1 medium to an A_{600} of 0.2 and were transferred to appropriate media at a starting A_{600} of 0.05, and the growth rates were determined in the exponential phase of growth. The aforementioned growth regimen was employed for pairs that contained an intact Kdp transporter. Pairs bearing a deficiency for the Kdp transporter were grown in K_{10} medium to early exponential phase, washed with K_1 medium, and subcultured in media of the indicated $[K^+]_e$ at an initial A_{600} of 0.05. A triple K^+ transporter-defective strain pair lacking the Kdp, Trk, and Kup K^+ uptake systems was initially grown in KML broth to A_{600} of 0.2, washed with K_1 medium, and inoculated as described above. For measurements of the effect of K^s caused by overexpression of *ycgO* on the growth rate in different K^+ transporter backgrounds, pairs of isogenic strains bearing either *ycgO* (expressed from the P_{trc} promoter, integrated at *attB*) or the vector equivalent (at *attB*) were grown in the absence of the inducer (IPTG) in K_1 medium (for Fig. 5A to C) and KML medium (for the strain pair in Fig. 5D) until early log phase. Cultures were then washed in K_1 medium and inoculated at an A_{600} of 0.05 in the media of the indicated K^+ concentration, and all media contained 0.1 mM IPTG. Values reported for growth rates are means ± standard errors (SE) of values obtained from three independent measurements.

Isolation of a transposon insertion in *ycgO*. Following transposon Tn*10*dCm mutagenesis (37) of the *hns trxA* double mutant GJ1495, which is a derivative of MC4100 and also exhibits a K^s similar to that displayed by the Δ*ptsN* mutant, Cm-resistant (Cm^r) suppressors of the K^s of GJ1495 were selected on K_{115} glucose Cm medium (i.e., double selection). P1 lysates prepared on the isolated suppressors were introduced into GJ1495 to establish that the Cm^r and the suppressor phenotypes were 100% linked. The site of Tn*10*dCm insertion in one of the suppressors wherein the Tn*10*dCm insertion caused the suppressor phenotype was determined by cloning the Tn*10*dCm element with the aid of the mini-Mu cloning procedure (38). The transposon junction site was sequenced with the primer 5′-TCCCTCCTGTTCAGCTACTGA-3′, which reads out from Tn*10*dCm, and the transposition event was located after nucleotide 1287 of *ycgO*.

Determination of cellular K^+ content. K^+ content of bacterial strains was determined by the method of Papp-Wallace and Maguire (39), with modifications. Cultures for K^+ content determination were grown until they reached an A_{600} of 0.2 in K_1 medium. The cells were pelleted and inoculated in K_1, K_{40}, and K_{115} media at an initial A_{600} of 0.05 for the parent. For the strain bearing the Δ*ptsN*::Kan mutation, the inoculum was adjusted to A_{600}s of 0.2 in K_{40} and K_{115} media and 0.05 in K_1 medium. A similar inoculum adjustment was performed for the strain in which the effect of overexpression of *ycgO* on the cellular K^+ pool was to be determined. Following growth for 2 h, 1 ml of culture was centrifuged at room temperature for 1 min at 13,000 rpm through a 0.3-ml layer of a 2:1 (vol/vol) mixture of dibutyl phthalate and dioctyl pthalate. The upper aqueous layer was discarded, and residual K^+ present over the organic phase was removed by repeated cycles of washing with 1 ml of Milli-Q water. After the organic layer had been thoroughly aspirated, the cell pellet was digested overnight in 0.1 ml of 1 N nitric acid, diluted with Milli-Q water to 5 ml, and clarified by centrifugation. K^+ in the nitric acid extract was quantified by inductively coupled plasma mass spectrometry (ICP-MS) (model ELAN DRC-e). ^{39}K was measured in conjunction with appropriate standards. K^+ content was expressed as nanomoles of K^+ per A_{600} of the culture at time of harvest. Results are reported as means ± standard deviations (SD) of single measurements conducted on three independent cultures. For cultures of a strain bearing the Δ*ptsN* mutation or cultures in which *ycgO* was overexpressed, a control experiment was included to verify that the proportion of spontaneous suppressors was $<10^{-5}$. Where the effect of overexpression of *ycgO* on cellular K^+ content was assessed, the growth medium at the time of the 2-h exposure contained 0.1 mM IPTG and the appropriate plasmid-specific antibiotic selection as required.

Other procedures. Procedures for transfer of *ycgO* from plasmid into the chromosomal *attB* site, construction of a strain encoding a C-terminal 3× FLAG epitope-tagged version of YcgO, transfer of $ilvG^+$ into *E. coli* K-12, and immunoblotting are described in the supplemental material.

RESULTS

Absence of PtsN yields a K^s that is exacerbated by the absence of constitutive K^+ uptake systems and is associated with reduced cellular K^+ content. In the background of the widely used laboratory strain of *E. coli* K-12 MC4100, we found that absence of PtsN led to a modest K^+-sensitive (K^s) phenotype. Whereas MC4100 and its Δ*ptsN*::Kan derivative, JD17, grew at comparable rates in K_1 medium, the growth rate of JD17 decreased as the medium $[K^+]_e$ was increased above 5 mM, and beyond a $[K^+]_e$ of 40 mM, JD17 displayed a constant but a lower growth rate than MC4100 (Fig. 1A). In a strain lacking the Kdp transporter, the absence of PtsN also led to K^s, and the pattern of K^s with respect to the $[K^+]_e$ of strain JD662 (Δ*kdp* Δ*ptsN*::Kan) was similar to that displayed by JD17 in K_{20}, K_{40}, and K_{115} media. In the lower $[K^+]_e$

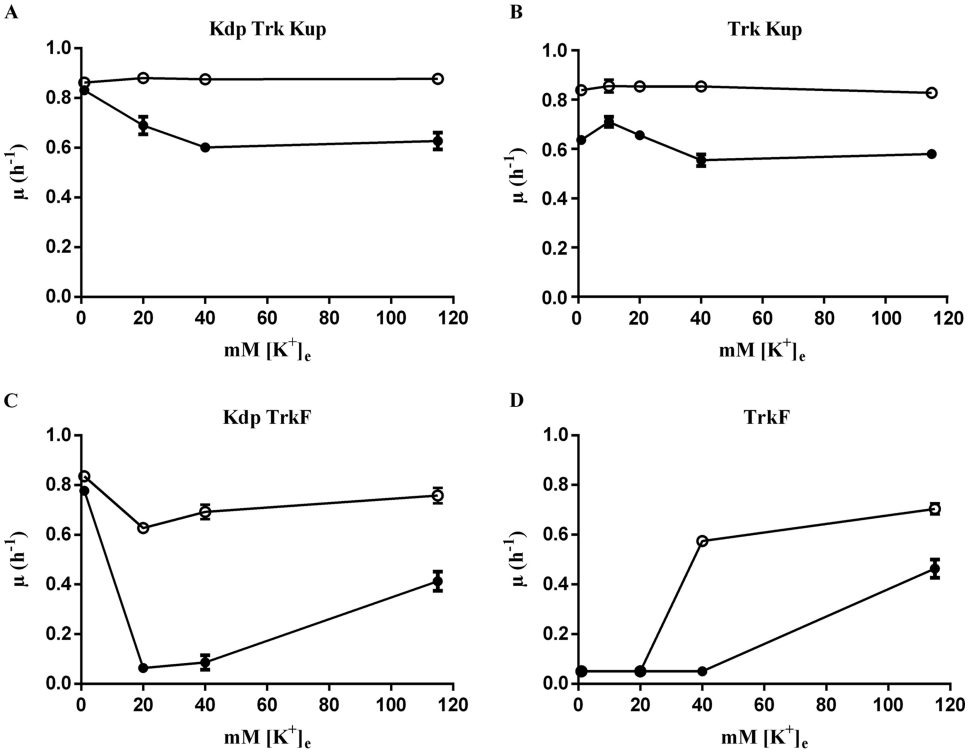

FIG 1 K^+-sensitive growth of the Δ*ptsN* mutant and its modulation by K^+ uptake systems. Shown are growth rates (μ) of pairs of isogenic *ptsN*$^+$ (open circles) and Δ*ptsN* (solid circles) strains bearing combinations of K^+ uptake systems with respect to $[K^+]_e$. The predominant K^+ transport systems present in each pair are indicated. Growth rates of all strain pairs were determined following their growth in K_1, K_{20}, K_{40}, and K_{115} media. The *ptsN*$^+$ and Δ*ptsN* strain pairs, respectively, are MC4100 and JD17 (A), JD656 and JD662 (B), JD624 and JD660 (C), and JD657 and JD658 (D).

range (10 mM and below), growth of JD662 was more retarded than that of its parent, JD656 (Fig. 1B). Absence of the constitutive K^+ uptake systems Trk and Kup increased the severity of K^s. In a strain that bears Kdp as the only active K^+ uptake system, JD624, the absence of PtsN (strain JD660) led to severely diminished growth in K_{20} and K_{40} media in comparison to JD624, and in K_{115} medium, the growth rate of JD660 increased further but remained below that of JD624 (Fig. 1C).

Herein, a feature of the growth pattern exhibited by the *kdp*$^+$ *kup trkA* strain JD624 is worth noting (Fig. 1C). JD624 grew at lower rates in media of intermediate $[K^+]_e$, that is, in K_{20} and K_{40} media, with the reduced growth rate being more marked in K_{20} medium than in K_1 or K_{115} medium. This growth pattern can be accounted for based upon the previously reported and apparently anomalous growth pattern displayed by a *kdp*$^+$ *kup trkA* strain that is known to be growth inhibited in media of intermediate (10 to 40 mM) but not low (1 mM) or high (115 mM) $[K^+]_e$s (15, 16, 40). The inhibition of the expression and/or activity of the Kdp system by $[K^+]_e$ is thought to cause the reduction in growth rate, which is very prominent when the medium pH is 6.0 (15, 16) (Fig. 2A). Growth inhibition by intermediate $[K^+]_e$s of the aforementioned strain correlates with K^+ limitation, and activities of the inducible Kdp transporter and the low-affinity TrkF K^+ uptake (9) are thought to satisfy the K^+ needs of this strain in media of low (1 mM) and high (>40 mM) $[K^+]_e$s, respectively (15, 16). In the studies described above, we employed the *trkA* and the *kup* mutations, which are not precise deletions but represent loss-of-function alleles (13). We found that a *kdp*$^+$ strain bearing clean deletions of TrkA and Kup also displayed an exacerbation of the K^s in the absence of PtsN (data not shown).

K^s imparted by the Δ*ptsN*::Kan mutation persisted in the absence of the three active K^+ uptake systems. Both JD657, which is a triple K^+ transporter-defective strain, and its Δ*ptsN*::Kan derivative, JD658, did not grow in K_1 or K_{20} medium. However, while the growth rate of JD657 increased as the $[K^+]_e$ was raised above 20 mM, JD658 displayed a moderate increase in growth rate only in K_{115} medium, which was lower than that displayed by JD657 (Fig. 1D) and was also well below that displayed by MC4100, which contains all active K^+ uptake systems. Overall, these observations indicated that to some extent the constitutive K^+ uptake systems and the low-affinity TrkF K^+ uptake aid in mitigating the K^s caused by the Δ*ptsN* mutation.

PtsN is the terminal phosphoacceptor protein of the PTS pathway comprising PtsP-PtsO-PtsN (18, 19, 20, 21), and the K^s of the Δ*ptsN* mutant was unaffected by the absence of PtsP (data not shown). In MG1655, we found as seen for MC4100 absence of PtsN caused a modest K^s that was exacerbated by the absence of TrkA and Kup, and the K^s of the Δ*ptsN* mutant also persisted in a derivative of MG1655 lacking all K^+ uptake systems (data not shown).

We measured cellular K^+ content in the parent JD624 and its Δ*ptsN*::Kan derivative, JD660, after their growth in K_1 medium followed by a shift for 2 h into K_1, K_{40}, and K_{115} media (Table 2). Compared to JD624, JD660 displayed an approximately 30 to 40% reduction in cellular K^+ content in K_{40} and K_{115} media, whereas in K_1 medium, the K^+ contents of JD624 and JD660 remained com-

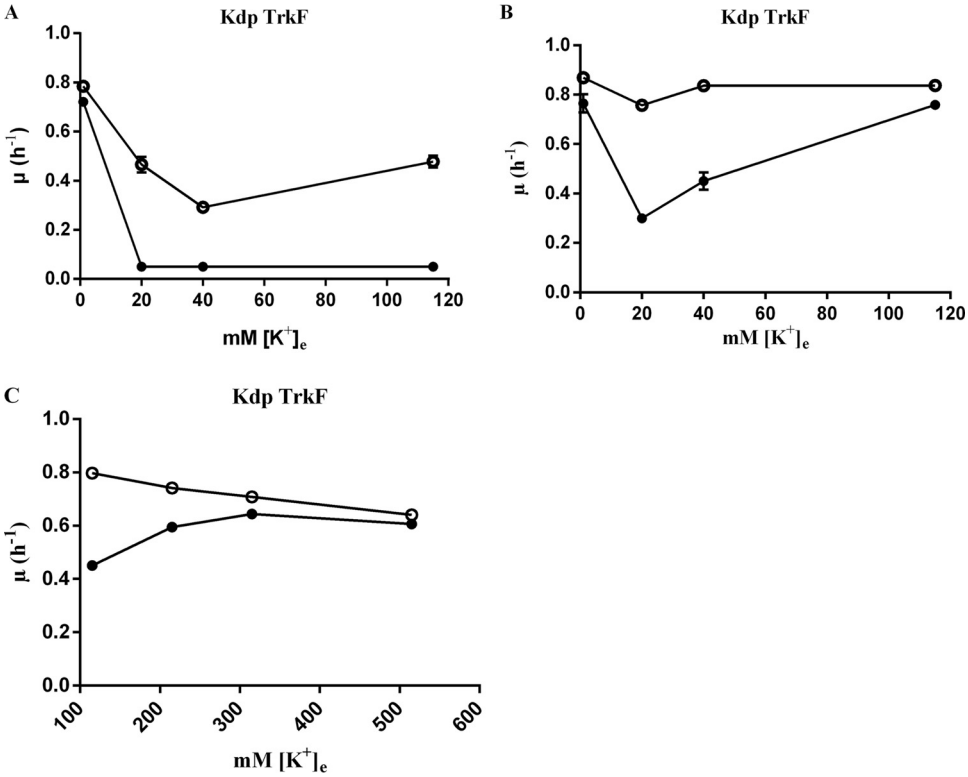

FIG 2 Modulation of the K^+-sensitive growth of the Δ*ptsN* mutant by alterations in external pH and its alleviation by elevated $[K^+]_e$. Shown are the growth rates (μ) of JD624 (open circles) and its Δ*ptsN*::Kan derivative, JD660 (solid circles), with respect to $[K^+]_e$. The K^+ transporters present in JD624 and JD660 are indicated. Growth rates were determined for JD624 and JD660 following their growth in K_1, K_{20}, K_{40}, and K_{115} media whose pH was maintained at 6.0 (A) and 7.8 (B). (C) Growth rates of JD624 (open circles) and JD660 (solid circles) with respect to $[K^+]_e$ in K_{115} medium and in K_{115} medium containing 100, 200, and 400 mM KCl. All growth media in panel C also contained 1 mM betaine, and their pH was 7.2.

parable. These observations indicate that the K^s of JD660 in K_{40} and K_{115} media was associated with a decrease in the cellular K^+ pool.

Modulation of K^s by alterations in external pH and its rescue by elevated $[K^+]_e$. In JD624, which bears Kdp as the sole active K^+ uptake system, the K^s imparted by the Δ*ptsN*::Kan mutation could be modulated by alterations in the pH of the medium. Growth of the Δ*ptsN*::Kan derivative of JD624, JD660, was severely restricted in K_{20}, K_{40}, and K_{115} media of pH 6.0 (Fig. 2A), whereas considerable improvement of its growth in K_{40} and K_{115} media occurred when the medium pH was raised to 7.8 (Fig. 2B). In K_1 medium under the two different external pH conditions, JD660 grew at rates comparable to those of JD624. It may be noted that alteration in medium pH itself affected the growth of the *ptsN*$^+$ parent of JD660, JD624. The growth rate of JD624 decreased quite sharply as the $[K^+]_e$ was raised above 1 mM with the external pH held at 6.0 and increased marginally in pH 6.0 media with $[K^+]_e$s of >20 mM (Fig. 2A). In media of pH 7.8, the growth rate of JD624 decreased marginally as the $[K^+]_e$ was raised above 1 mM, and the growth rates of JD624 in media with $[K^+]_e$s of >20 mM were

TABLE 2 Reduced cellular K^+ content in the Δ*ptsN* mutant and its restoration by the Δ*ycgO* mutation and by overexpression of *kup*

Strain	Relevant genotype	Relative K^+ content after growth in medium[a]:		
		K_1	K_{40}	K_{115}
JD624	*kdp*$^+$ *kup trkA*	1 (0.10)	1 (0.04)	1 (0.15)
JD660	*kdp*$^+$ *kup trkA* Δ*ptsN*	0.98 (0.02)	0.58 (0.12)	0.63 (0.37)
JD696	*kdp*$^+$ *kup trkA* Δ*ycgO*	1.07 (0.07)	1.07 (0.29)	0.99 (0.02)
JD710	*kdp*$^+$ *kup trkA* Δ*ycgO* Δ*ptsN*	0.92 (0.05)	0.91 (0.01)	1.12 (0.25)
JD660/pHYD3025[b,c]	*kdp*$^+$ *kup trkA* Δ*ptsN*	0.99 (0.07)	0.6 (0.17)	0.68 (0.06)
JD660/pHYD1852[c,d]	*kdp*$^+$ *kup trkA* Δ*ptsN*	1.07 (0.02)	1.08 (0.05)	1 (0.03)

[a] Shown is the cellular K^+ content of strains JD660 (Δ*ptsN*), JD696 (Δ*ycgO*), and JD710 (Δ*ycgO* Δ*ptsN*) relative to parent strain JD624 following 2 h of growth in K_1, K_{40}, and K_{115} media. The K^+ content obtained for JD624 in K_1, K_{40}, and K_{115} media was set at 100% = 1, and the relative K^+ contents for the other strains are indicated. The relative standard deviations (SD) for the measurements are indicated in parentheses. The K^+ contents (mean ± SD) for JD624 following its growth in K_1, K_{40}, and K_{115} media were 165 ± 17.3, 169 ± 7, and 241 ± 38.1 nmol K^+/A_{600}, respectively.

[b] Vector.

[c] K^+ content was obtained following growth in K_1, K_{40}, and K_{115} media containing 0.1 mM IPTG.

[d] Plasmid bearing *kup* under the expression control of the P_{trc} promoter.

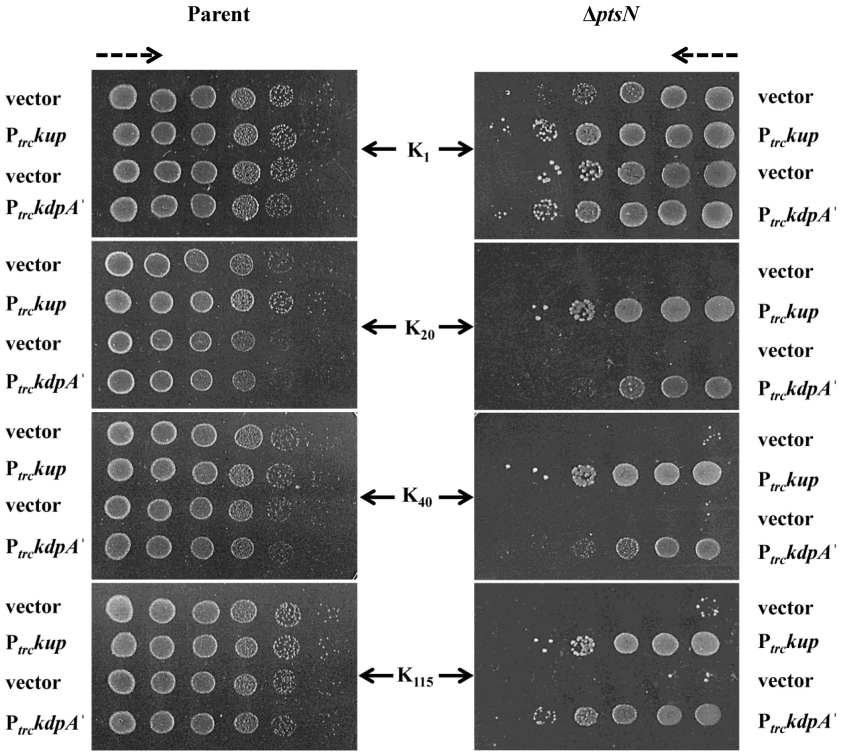

FIG 3 Alleviation of the K^s of the Δ*ptsN* mutant by overproduction of K^+ uptake proteins Kup and KdpA′. Tenfold serial dilutions of cultures of the parent strain (JD624 [*kdp*$^+$ *trkA kup*]) and its Δ*ptsN*::Kan derivative, JD660 (Δ*ptsN*), bearing the plasmids pHYD3025 (vector), pHYD1852 (P_{trc}*kup*), pTrc99A (vector), and pHYD724 (P_{trc}*kdpA′*), were spotted on the surface of K_1, K_{20}, K_{40}, and K_{115} glucose agar plates containing 0.1 mM IPTG. pHYD3025 is the vector control for pHYD1852. The dashed arrows indicate the direction of increasing order of cell dilution.

comparable to its growth rate in K_1 medium (Fig. 2B). In JD624, the inhibition by external K^+ of Kdp expression and/or activity renders its growth dependent on K^+ uptake via TrkF in media with $[K^+]_e$s of >20 mM (15, 16). K^+ uptake via TrkF is known to be modulated by alterations in medium pH, with acidification leading to reduction and alkalinization to enhancement of its K^+ uptake activity (9, 16). Thus, the modulation of the growth of JD624 by alterations in the pH of media with $[K^+]_e$s of >20 mM represents the modulation of the K^+ uptake via the TrkF pathway. It may be noted that medium alkalinization led to marked improvement in the growth rate of the Δ*ptsN*::Kan mutant JD660 in K_{40} and K_{115} media but not in K_{20} medium (Fig. 2B). A probable explanation for this and also for the K^s in K_{20} medium imparted by the Δ*ptsN* mutation in a strain that bears Kdp as the only active K^+ transporter is provided in the section describing the effect of the Δ*ycgO* mutation on K^s.

Increasing the $[K^+]_e$ of the K_{115} medium by supplementation with KCl led to a noticeable alleviation of K^s (Fig. 2C). To minimize osmotic effects arising due to high external concentrations of K^+, 1 mM betaine, an osmoprotectant (1), was added to all media. As the $[K^+]_e$ was raised to 515 mM, the growth rate of the parent strain, JD624, decreased. The growth rate of the Δ*ptsN*::Kan derivative of JD624, JD660, increased as the $[K^+]_e$ was raised above 115 mM until it reached a concentration of 215 mM (Fig. 2C). Further elevation of the medium $[K^+]_e$ led to a modest decrease in the growth rate of JD660. Addition of equimolar amounts of NaCl to K_{115} medium did not alleviate the K^s of JD660 (data not shown), indicating that the enhancement in the growth rate of JD660 was mediated by excess K^+ in the medium.

Overproduction of multiple K^+ uptake proteins alleviates the K^s. Since the severity of the K^s was found to increase with the absence of the Trk and the Kup K^+ transporters, we tested the effects of overproduction of K^+ uptake proteins on K^s. P_{trc}-directed overproduction of Kup from plasmid pHYD1852 with 0.1 mM IPTG suppressed the K^s of JD660, whereas JD660 bearing plasmid pHYD3025 (vector) (41) displayed K^s (Fig. 3). Suppression of K^s by overproduction of Kup was also associated with elevation in K^+ content of JD660 bearing plasmid pHYD1852 in comparison to its vector-bearing counterpart in K_{40} and K_{115} media (Table 2).

The TetA polypeptide specifying tetracycline resistance encoded on plasmid pBR322 (also present on plasmid pACYC184) and the N-terminal 135 amino acids of the KdpA K^+-translocating subunit of the Kdp complex (KdpA′) are thought to be examples of K^+ carrier proteins as their expression confers upon a strain that lacks all K^+ uptake systems a K^+-sparing phenotype (42, 43), which is the ability of a strain to grow proficiently in media when $[K^+]_e$ becomes limiting for its growth. P_{trc}-directed overproduction of KdpA′ from plasmid pHYD724 with 0.1 mM IPTG in JD660 alleviated its K^s, whereas JD660 containing the vector pTrc99A displayed the K^s (Fig. 3). Furthermore, the presence of the plasmid pACYC184 but not its derivative, pHYD586, which lacks *tetA*, in JD660 alleviated its K^s (see Fig. S1 in the supplemental material). Transformants of JD624 (parent) bearing all of the plasmids described above did not display any overt alterations of their growth in the indicated media (Fig. 3; see Fig. S1).

A variant of the ammonium transporter AmtB bearing the H168D and H318E substitutions ($AmtB^K$) has recently been

shown to mediate selective uptake of K^+ in place of its natural substrate, the ammonium ion (44). Expression of this variant (*amtB*^{K}) in an otherwise wild-type strain leads to a K^s that correlates with elevated cellular K^+ content in synthetic media of $[K^+]_e$s of 100 mM and above (44). In addition, in a triple K^+ transporter-defective strain, *amtB*^{K} expression causes a K^s and K^+-sparing phenotype such that this strain additionally displays an enhanced ability to grow in media containing less than 20 mM K^+ (44). The aforementioned two growth phenotypes pertaining to *amtB*^{K} expression were recapitulated in this study using plasmid pHYD1855 (see Table S2 in the supplemental material), which expresses *amtB*^{K} from the P_{trc} promoter (data not shown). We found that expression of *amtB*^{K} alleviated the K^s of the Δ*ptsN*::Kan Δ*amtB* double mutant JD767, whereas JD767 containing the vector or the plasmid expressing *amtB* displayed K^s (see Fig. S2 and Table S2 in the supplemental material).

In addition, expression of all of the K^+ uptake proteins described above in the Δ*ptsN*::Kan derivative of MC4100, JD17, alleviated its K^s, and overexpression of *kup* in the Δ*ptsN*::Kan derivative of MG1655 also alleviated its K^s (data not shown). Taken together, these observations lend additional support to the notion that the K^s of the Δ*ptsN* mutant correlates with K^+ limitation.

YcgO and K^s. In work to be described elsewhere, we have found that MC4100 doubly defective for the nucleoid protein H-NS and either thioredoxin 1 (TrxA) or thioredoxin reductase (TrxB) also displays a K^s similar to that displayed by the Δ*ptsN* mutant. H-NS functions as a regulator of global gene expression in *E. coli*, with a well-documented role as a repressor (45), and TrxB along with thioredoxin 1 (TrxA) mediates cytoplasmic protein reduction (46). The K^s in both instances is exacerbated by removal of TrkA and Kup and is suppressed by expression of *kdpA′*, *kup*, *tetA*, and *amtB*^{K} (data not shown). We isolated a suppressor mutant of the K^s of an *hns trxA* double mutant after Tn*10*dCm transposon mutagenesis. Further studies showed that a transposon insertion located after nucleotide 1287 of *ycgO* that is predicted to encode an inner membrane protein implicated in cytoplasmic alkali cation homeostasis (27) caused the suppressor phenotype. Suppression resulted from a deficiency of YcgO, as the presence of a clean deletion of *ycgO* (Δ*ycgO*::Kan) also suppressed the K^s of the *hns trxA* double mutant (data not shown). We found that the K^s of the Δ*ptsN* mutant was also suppressed by the Δ*ycgO*::Kan mutation (Fig. 4).

In Fig. 4, the influence of single and double deficiency of PtsN and YcgO on growth rates in media with various $[K^+]_e$s is presented for a quartet of strains bearing Kdp as the sole active K^+ uptake system. Among the quartet, the strain lacking YcgO, JD696, grew at rates comparable to those of the parent in all the K^+-containing media employed. The Δ*ptsN*::Kan mutant JD660 displayed a K^s that was prominent in K_{10}, K_{20}, and K_{40} media, whereas its Δ*ycgO* derivative, JD710, grew at rates equivalent to those of the parent only in K_1, K_{40}, and K_{115} media but not in K_{10} and K_{20} media. A recent study has implicated a requirement for dephospho-PtsN in the optimal expression of the *kdp* operon in *E. coli* (47), which may provide a basis to explain why the Δ*ycgO* mutation does not suppress the K^s imparted by the Δ*ptsN* mutation in K_{10} and K_{20} media. As mentioned earlier, a *kdp*$^+$ *trkA kup* strain experiences partial K^+ limitation in media with intermediate $[K^+]_e$s, due to the repression of the Kdp transporter by $[K^+]_e$, and growth of this strain in media of low (below 40 mM) $[K^+]_e$s is dependent on the Kdp transporter (15, 16). If one assumes that the

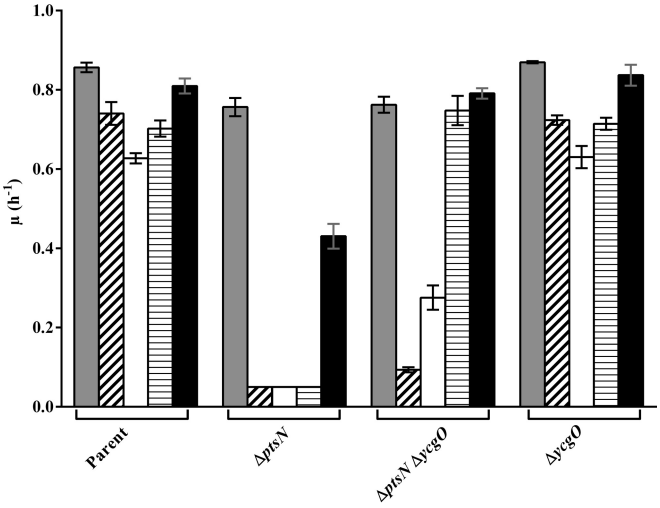

FIG 4 Involvement of YcgO in the K^s of the Δ*ptsN* mutant. Shown are the growth rates (μ) of the parent strain (JD624 [*kdp*$^+$ *trkA kup*]) and its Δ*ptsN* (JD660), Δ*ptsN* Δ*ycgO* (JD710), and Δ*ycgO* (JD696) derivatives in media of various $[K^+]_e$s. The indicated strains were grown in K_1 (gray bars), K_{10} (bars with diagonal stripes), K_{20} (white bars), K_{40} (bars with horizontal stripes), and K_{115} (black bars) media, and their growth rates were determined.

Δ*ptsN* mutation in such a strain yields two distinct types of K^s, one occurring in the intermediate range of $[K^+]_e$s and the other occurring (maximally) in the 40 to 115 mM range, then the absence of alleviation of the K^s of the Δ*ptsN* mutant by the Δ*ycgO* mutation in K_{10} and K_{20} media may be explained. The K^s caused by the Δ*ptsN* mutation in K_{10} and K_{20} media could represent K^+ limitation, arising mainly due to reduced *kdp* expression, and that occurring in the 40 to 115 mM range of $[K^+]_e$s could represent K^+ limitation related to the activity of YcgO. Furthermore, we have observed that the Δ*ycgO* mutation does not affect the expression of a *kdp-lac* transcriptional fusion (data not shown), a finding that is compatible with the observation that the Δ*ycgO* mutation does not suppress the K^s of a Δ*ptsN*::Kan derivative of the *kdp*$^+$ *trkA kup* strain in the 10 to 20 mM range of $[K^+]_e$s.

If the aforementioned rationale is valid, then the minimal alleviation of the K^s of JD660 by alkalinization of K_{20} medium may also be explained (Fig. 2B), the basis being that the effect of medium alkalinization is restricted to enhancement of TrkF activity, whose magnitude is very low in the $[K^+]_e$ range, below 40 mM (9, 16). The additional effect of the Δ*ptsN*::Kan mutation in reducing *kdp* expression may not be subject to modulation by medium alkalinization. Thus, the poor growth of JD660 in a medium with a $[K^+]_e$ of 20 mM and pH 7.8 is due to impaired *kdp* expression and the absence of significant TrkF activity. Overall though, the two K^s phenotypes displayed by the Δ*ptsN* derivative of the *kdp*$^+$ *trkA kup* strain correlate with K^+ limitation because they were suppressed by overexpression of multiple K^+ uptake proteins (Fig. 3; see Fig. S1 and S2B in the supplemental material).

We measured cellular K^+ content in strain JD710, which is a Δ*ptsN*::Kan Δ*ycgO* double mutant, and strain JD696, which bears the Δ*ycgO* mutation, after their growth in K_1 medium followed by a shift for 2 h into K_1, K_{40}, and K_{115} media. A comparison of their K^+ contents with respect to those obtained for the parent strain, JD624, and its Δ*ptsN*::Kan derivative, JD660, permitted the following conclusions: (i) the absence of YcgO in a strain bearing the

TABLE 3 K^s phenotype of the Δ*ptsN* mutation and its suppression by absence of YcgO in K^+ transporter-defective backgrounds

Strain	Relevant genotype	Growth rate (h^{-1}) on medium[a]:			
		K_1	K_{20}	K_{40}	K_{115}
MC4100[b]	*kdp*$^+$ *kup*$^+$ *trkA*$^+$	0.86	0.88 ± 0.01	0.88 ± 0.01	0.88 ± 0.01
JD17[b]	*kdp*$^+$ *kup*$^+$ *trkA*$^+$ Δ*ptsN*	0.83 ± 0.01	0.69 ± 0.01	0.60 ± 0.02	0.63 ± 0.03
JD466	*kdp*$^+$ *kup*$^+$ *trkA*$^+$ Δ*ycgO*	0.91 ± 0.01	0.89 ± 0.02	0.91 ± 0.02	0.90 ± 0.03
JD509	*kdp*$^+$ *kup*$^+$ *trkA*$^+$ Δ*ptsN* Δ*ycgO*	0.83 ± 0.02	0.80 ± 0.02	0.82	0.84
JD656[b]	Δ*kdpA kup*$^+$ *trkA*$^+$	0.84 ± 0.02	0.85 ± 0.02	0.85 ± 0.02	0.83 ± 0.02
JD662[b]	Δ*kdpA kup*$^+$ *trkA*$^+$ Δ*ptsN*	0.64 ± 0.02	0.66 ± 0.01	0.55 ± 0.02	0.58 ± 0.01
JD694	Δ*kdpA kup*$^+$ *trkA*$^+$ Δ*ycgO*	0.85 ± 0.01	0.89 ± 0.02	0.89 ± 0.01	0.89
JD725	Δ*kdpA kup*$^+$ *trkA*$^+$ Δ*ptsN* Δ*ycgO*	0.70 ± 0.06	0.81 ± 0.02	0.82	0.82
JD657[b,c]	Δ*kdpA kup trkA*	<0.05	<0.05	0.67 ± 0.01	0.83 ± 0.01
JD658[b,c]	Δ*kdpA kup trkA* Δ*ptsN*	<0.05	<0.05	<0.05	0.46 ± 0.02
JD693	Δ*kdpA kup trkA* Δ*ycgO*	<0.05	<0.05	0.72 ± 0.03	0.86 ± 0.01
JD714[c]	Δ*kdpA kup trkA* Δ*ptsN* Δ*ycgO*	<0.05	<0.05	0.76 ± 0.24	0.88 ± 0.01

[a] Shown are growth rates (means ± standard errors) of three quartets of strains in K_1, K_{20}, K_{40}, and K_{115} media. The first quartet, MC4100 to JD590, bears all K^+ uptake systems, the second, JD656 to JD725, lacks the Kdp transporter, and the third, JD657 to JD714, lacks all K^+ uptake systems. Growth rates for the quartet with Kdp as the sole K^+ uptake system are displayed in Fig. 4. The PtsN, YcgO, and K^+ transporter status with respect to Kdp, Trk, and Kup in each quartet is indicated.
[b] Values for growth rates from Fig. 1.
[c] Contains the *zha-203*::Tn*10* marker.

Δ*ptsN*::Kan mutation led to restoration of cellular K^+ content to a level approaching that of the parent following exposure to K_{40} and K_{115} media, and (ii) the absence of YcgO in the parent did not lead to significant alterations in cellular K^+ content (Table 2).

We also documented the extent to which the Δ*ycgO* mutation suppressed the K^s of the Δ*ptsN* mutant in backgrounds bearing mutations in K^+ uptake systems (Table 3). In the background bearing all K^+ uptake systems, the Δ*ycgO* mutation suppressed the K^s of the Δ*ptsN* mutant with the outcome that the Δ*ptsN* Δ*ycgO* derivative of MC4100, JD509, grew at rates comparable to those of MC4100 in K_1, K_{20}, K_{40}, and K_{115} media. In the background devoid of the Kdp transporter, the Δ*ycgO* mutation suppressed the K^s of the Δ*ptsN* mutant in K_{20}, K_{40}, and K_{115} media. In K_1 medium, the slow growth associated with the presence of the Δ*ptsN* mutation in this background was not altered by the Δ*ycgO* mutation. In the triple K^+ transporter-defective strain, the Δ*ycgO* mutation suppressed the K^s of the Δ*ptsN* mutant in K_{40} and K_{115} media. Absence of YcgO in MC4100 or in its Δ*kdp* and Δ*kdp trkA kup* derivatives, JD656 and JD637, respectively, did not lead to any discernible alteration in growth in media of various $[K^+]_e$s (Table 3), indicating that the Δ*ycgO* mutation does not influence K^+ uptake occurring via the Kdp, TrkA, Kup, and TrkF systems.

Overproduction of YcgO yields a K^s with similarities to that displayed by the Δ*ptsN* mutation. IPTG-induced overexpression of *ycgO* placed under the expression control of the P_{trc} promoter integrated in chromosomal *attB* conferred a K^s that was similar to that conferred by the Δ*ptsN* mutation. In MC4100 and its Δ*kdp* derivative, overexpression of *ycgO* caused a K^s as the medium $[K^+]_e$ was raised (Fig. 5A and B). In a strain that contained Kdp as the sole active K^+ uptake system, overexpression of *ycgO* led to a K^s of increased severity in K_{20}, K_{40}, and K_{115} media (Fig. 5C). In the triple K^+ transporter-defective strain, *ycgO* overexpression led to K^s in K_{40} and K_{115} media (Fig. 5D). Isogenic strains bearing a vector equivalent integrated in *attB* displayed growth commensurate with their K^+ uptake capacities (Fig. 5A to D). The K^s in K_{40} and K_{115} media caused by overexpression of *ycgO* in the *kdp*$^+$ *trk kup* strain JD729 was associated with reduced cellular K^+ content in JD729 in comparison to its vector-bearing counterpart, JD728 (Table 4). Furthermore, co-overexpression of *kup* from plasmid pHYD1852 in JD732 (a strain similar to JD729) suppressed the K^s caused by P_{trc}-driven expression of *ycgO* integrated in *attB* (see Fig. S3 in the supplemental material), which in turn correlated with elevation of the K^+ content of JD732 in K_{40} and K_{115} media (Table 4). Overexpression of *ycgO* in JD732 bearing the vector pHYD3025 led to the K^s (see Fig. S3 in the supplemental material), and the K^+ content of this strain in K_{40} and K_{115} media remained at a level lower than that of its counterpart that contained the plasmid pHYD1852 (Table 4). The K^s caused by overexpression of *ycgO* was unaffected by the PtsP/PtsO status of the strain (data not shown). These observations indicate that the K^s of *ycgO* overexpression is similar to that displayed by the Δ*ptsN* mutant in more ways than one.

To test if the Δ*ptsN* mutation led to any alterations in the expression of YcgO, we constructed a strain encoding C-terminally 3× FLAG-tagged YcgO ($YcgO_{FL}$) expressed from its native chromosomal location by recombineering. Appending the 3× FLAG tag to the C terminus of YcgO did not alter YcgO function as the absence of PtsN in a strain expressing $YcgO_{FL}$ led to K^s and a strain bearing $YcgO_{FL}$ did not display K^s (data not shown). $YcgO_{FL}$ levels in the parent strain, JD723, were comparable to those in its Δ*ptsN*::Kan derivative, JD724, following growth in K_1 medium (Fig. 5E). No differences in $YcgO_{FL}$ levels were seen when the aforementioned two strains were grown for 2 h in K_{40} medium following growth to the early exponential phase in K_1 medium (data not shown). These observations indicate that the K^s caused by the Δ*ptsN* mutation does not result from overexpression of *ycgO*.

Effect of amino acid substitutions at histidine 73 of PtsN on K^s. An earlier study showed that PtsN is phosphorylated at a single site, namely histidine 73 (48), whose replacement with aspartate or alanine led to absence of phosphorylated PtsN (48). We constructed a plasmid, pHYD5006, expressing under the control of the P_{trc} promoter *ptsN*, and its derivative plasmids, pHYD5007, pHYD5008, and pHYD5009, encoding PtsN bearing the H73A, H73D, and H73E amino acid substitutions, respectively. Basal-level expression of *ptsN* from the aforementioned plasmids in strain JD17 (MC4100 Δ*ptsN*::Kan) complemented its K^s, whereas JD17 bearing the vector showed the K^s (Fig. 6). Transformants of MC4100 bearing the indicated *ptsN*-expressing plasmids did not

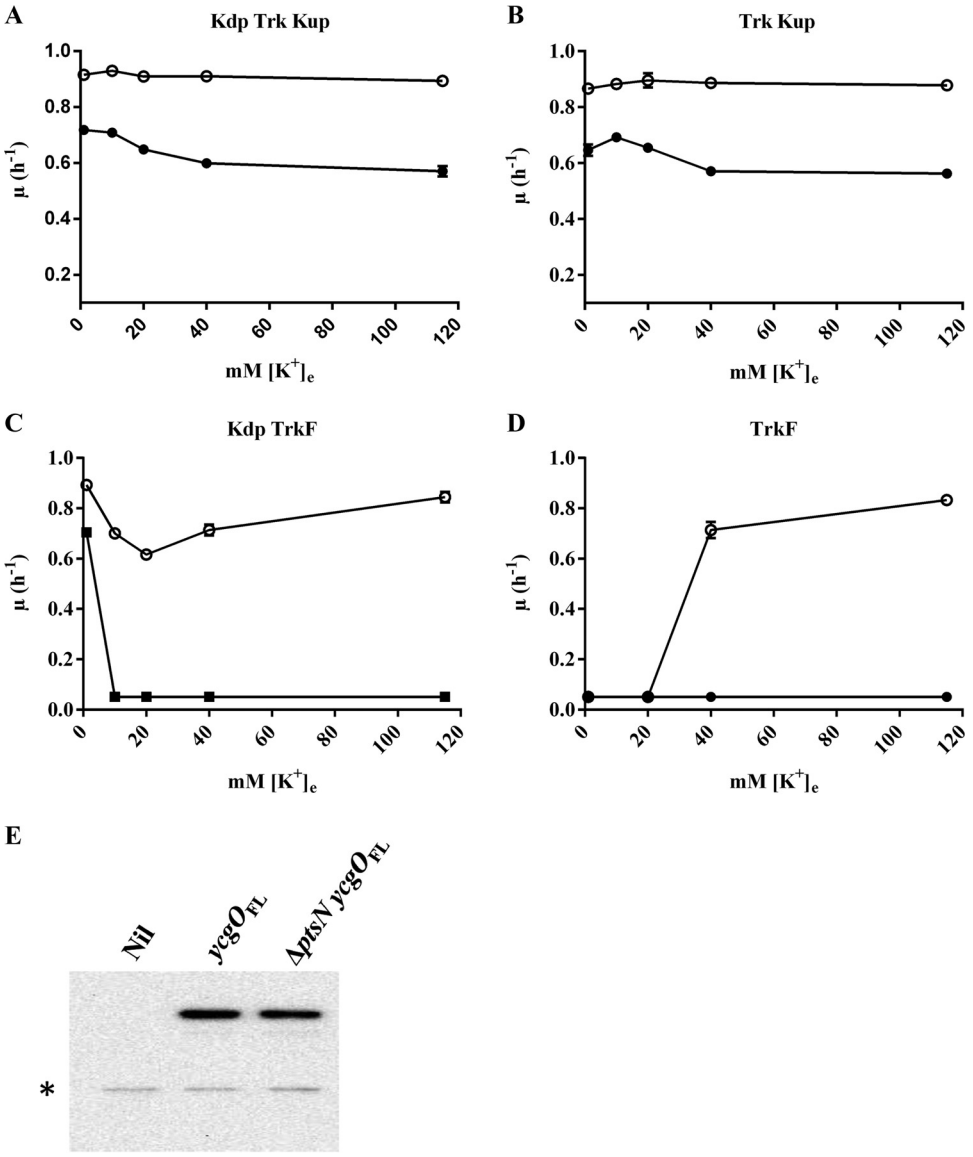

FIG 5 K^s associated with overexpression of *ycgO* and its modulation by K^+ uptake systems and levels of YcgO in the parent strain and Δ*ptsN* mutant. Shown are the growth rates (μ) of pairs of isogenic strains bearing single-copy integrations of the vector (open circles) and *ycgO* under the expression control of the P_{trc} promoter (solid circles) in *attB* with respect to $[K^+]_e$. The predominant K^+ uptake systems present in the pairs of strains are indicated (A to D). Growth rates of all strains were determined following their growth in K_1, K_{20}, K_{40}, and K_{115} media containing 0.1 mM IPTG. The strain pairs employed are represented by open and solid symbols, respectively: JD726 and JD727 (A), GJ14949 and GJ14950 (B), JD728 and JD729 (C), and JD734 and JD735 (D). (E) Expression levels of a 3× FLAG-tagged YcgO encoded by $ycgO_{FL}$ in JD723 ($ycgO_{FL}$) and JD724 (Δ*ptsN* $ycgO_{FL}$). The lane labeled "Nil" contains a whole-cell extract of strain JD624. Cultures of JD624, JD723, and JD724 obtained after growth in K_1 medium to mid-exponential phase were centrifuged and suspended in SDS sample buffer at an A_{600} of 0.002/μl. Equal volumes were loaded onto a 12% SDS-PAGE gel that was processed and immunoblotted with anti-FLAG M2 monoclonal antibody. A nonspecific anti-FLAG immunoreactive material indicative of equal loading is marked with an asterisk.

display the K^s (Fig. 6). We noted that in K_1 medium, the presence of the indicated *ptsN*-expressing plasmids but not the vector in both MC4100 and JD17 caused a modest impairment of their growth (data not shown): the reasons for this at present are ill understood.

K^s associated with the Δ*ptsN* mutation persists in a strain bearing acetohydroxy acid synthase II. Besides displaying the K^s, the Δ*ptsN* mutant has been reported to be leucine sensitive (Leu^s) in a minimal medium at a $[K^+]_e$ of 20 mM (25, 26, 49). In *E. coli* K-12, the activities of the two acetohydroxy acid synthases (AHASs) encoded by the *ilvBN* (AHASI) and *ilvHI* (AHASIII) operons constitute the primary committed step in the biosynthesis of branched-chain amino acids leucine, isoleucine, and valine (reviewed in reference 50). The Leu^s phenotype has been proposed to arise due to a combination of elevated intracellular L-leucine and K^+ levels in the Δ*ptsN* mutant that perturb cellular AHAS activity synergistically, giving rise to a state of isoleucine pseudoauxotrophy (26, 49). *E. coli* K-12 lacks AHASII due to the presence of a frameshift mutation in *ilvG*. One group has also reported that both the Leu^s phenotype and the K^s of the Δ*ptsN* mutant are absent in a strain with an intact *ilvG*, implying that in *E. coli* K-12, elevated cellular K^+ content in the Δ*ptsN* mutant perturbs AHASI

TABLE 4 Reduction in the cellular K^+ content by overexpression of *ycgO* in the parent and its restoration by overexpression of *kup*

Strain	Relevant genotype	Relative K^+ content after growth in medium[a]:		
		K_1	K_{40}	K_{115}
JD728	*kdp*$^+$ *kup trkA att*λ::(P_{trc}99A *bla*)	1 (0.18)	1 (0.12)	1 (0.01)
JD729	*kdp*$^+$ *kup trkA att*λ::(P_{trc}*ycgO bla*)	0.73 (0.14)	0.62 (0.04)	0.57 (0.7)
JD732/pHYD3025[b]	*kdp*$^+$ *kup trkA att*λ::(P_{trc}*ycgO bla*::Kan)	0.9 (0.11)	0.8 (0.16)	0.71 (0.08)
JD732/pHYD1852[c]	*kdp*$^+$ *kup trkA att*λ::(P_{trc}*ycgO bla*::Kan)	1.14 (0.02)	1.33 (0.04)	1.07 (0.04)

[a] The K^+ content obtained for strain JD728 bearing integration of the plasmid pTrc99A in *attB* [*att*λ::(P_{trc}99A *bla*)] in K_1, K_{40}, and K_{115} media was set at 100% = 1, and the relative K^+ contents for the other strains are indicated. K^+ content was determined following 2 h of growth of the indicated strains in K_1, K_{40}, and K_{115} media containing 0.1 mM IPTG. The relative standard deviations (SD) for the measurements are indicated in parentheses. The K^+ contents (mean ± SD) for JD728 following its growth in K_1, K_{40}, and K_{115} media were 162 ± 29.4, 147 ± 17.3, and 229 ± 3.4 nmol K^+/A_{600}, respectively.

[b] Vector.

[c] Plasmid bearing *kup* under the expression control of the P_{trc} promoter.

and AHASII activities, and the cellular casualty in the Δ*ptsN* mutant is the pathway of branched-chain amino acid biosynthesis (26).

We found that the K^s of the Δ*ptsN* mutant persisted in the presence of the *ilvGM*$^+$ (*ilvG*$^+$) allele, obtained from *E. coli* strain BL21(DE3) (51). While the *ilvG*$^+$ derivative of MC4100, JD271, grew at comparable rates in K_1, K_{20}, K_{40}, and K_{115} media, its Δ*ptsN*::Kan derivative, JD274, displayed a K^s similar to that displayed by its *ilvG* ancestor, JD17 (see Fig. S4 and Table S2 in the supplemental material). In addition, the Δ*ptsN*::Kan derivative of MC4100 bearing *ilvG*$^+$ obtained from strain NCM3722 (52) also displayed K^s (data not shown). Finally, Δ*ptsN*::Kan derivatives of MG1655 bearing *ilvG*$^+$ obtained from both BL21(DE3) and NCM3722 exhibited K^s in comparison to their isogenic parents, and 5 mM concentrations each of leucine, isoleucine, and valine did not suppress the K^s of the Δ*ptsN*::Kan derivative of MC4100, JD17 (data not shown).

DISCUSSION

In this study, we have interrogated the physiological defect that renders a strain lacking PtsN to display K^s. Our proposal on the physiological basis for K^s contrasts with an earlier study (25) in two ways, which are that (i) the K^s correlates with K^+ limitation rather than a K^+ overload, and (ii) the K^+ limitation mediated by YcgO causes the K^s rather than hyperactivated Trk-mediated K^+ uptake. Observations of the present study are discussed below, a

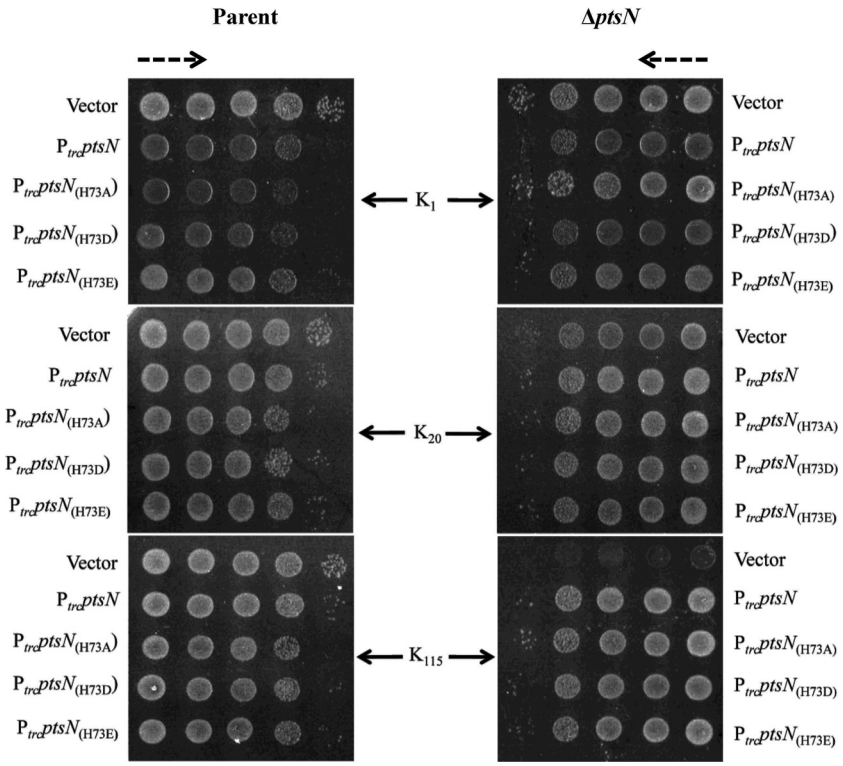

FIG 6 Complementation of the K^s of the Δ*ptsN* mutant by plasmids encoding PtsN and those encoding PtsN bearing amino acid substitutions of its phosphorylation site histidine. Tenfold serial dilutions of MC4100 (parent) and its Δ*ptsN*::Kan derivative, JD17 (Δ*ptsN*), bearing the plasmids pHYD3025 (vector), pHYD5006 (P_{trc}*ptsN*), pHYD5007 (P_{trc}*ptsN*$_{H73A}$), pHYD5008 (P_{trc}*ptsN*$_{H73D}$), and pHYD5009 (P_{trc}*ptsN*$_{H73E}$), were spotted on the surface of K_1, K_{20}, and K_{115} glucose agar plates. pHYD5006 encodes PtsN, whereas pHYD5007, pHYD5008, and pHYD5009 encode PtsN bearing the H73A, H73D, and H73E amino acid substitutions, respectively. The dashed arrows indicate the direction of increasing order of cell dilution.

model for K^+ limitation in the Δ*ptsN* mutant is presented, and the probable physiological role of YcgO is discussed.

K^s of the Δ*ptsN* mutant correlates with K^+ limitation. The present work provides credible evidence that the Δ*ptsN* mutant experiences a K^+ limitation that causes its K^s. Although for the most part we have performed comparative studies with a pair of *ptsN*$^+$ and Δ*ptsN* mutant strains bearing the repressible Kdp and the low-affinity TrkF K^+ uptake systems, the notion that the K^s correlates with K^+ limitation would remain unaffected despite this choice of strains. Exacerbation of the K^s by removal of the constitutive K^+ uptake systems (Fig. 1) is consistent with the notion that a K^+ limitation causes the K^s. If a K^+ overload was causal, then one would expect its absence to alleviate the K^s, which was not the case. Persistence of the K^s in the absence of TrkA indicates that TrkA-promoted hyperactivity of the Trk K^+ uptake system is not the cause of the K^s, which is contrary to the suggestion of Lee et al. (25). In addition, Lee et al. have also not tested whether *trkA* deficiency suppresses K^s in their mutant.

Furthermore, K^s was correlated with reduced K^+ content in high-$[K^+]_e$ media (Table 2) and overexpression of multiple K^+ uptake proteins suppressed the K^s (Fig. 3; see Fig. S1 and S2 in the supplemental material), with suppression by overexpression of *kup* leading to elevation of K^+ content in the Δ*ptsN* mutant (Table 2). Alleviation of K^s by overexpression of K^+ uptake proteins is analogous to the relief from K^+-limited growth provided by a K^+ uptake system to a triple K^+ transporter-defective strain (13). Among the K^+ uptake proteins, the effect of *amtB*^{K} expression on K^s is perhaps noteworthy. Expression of *amtB*^{K} is associated with K^s (see Fig. S2A) due to increased cytoplasmic K^+ pools (44). The presence of the two K^s-causing perturbations, namely, absence of PtsN and expression of *amtB*^{K}, in one strain led to their annihilation (see Fig. S2A and B), indicating that their origins must be opposing.

The Δ*ptsN* derivative of the *kdp*$^+$ *kup trk* strain displayed an exacerbated K^s in media containing 20 to 40 mM K^+ (Fig. 1C). However, the growth rate of this strain could be significantly improved by elevating the $[K^+]_e$ (Fig. 1C and 2C). This growth pattern can be explained simply on the basis that as the $[K^+]_e$ is increased, enhanced TrkF-mediated K^+ uptake (2, 9, 16) to some extent counteracts the K^+ limitation and additionally accounts for the increased growth rate of the Δ*ptsN* derivative of the triple K^+ transporter-defective strain as the $[K^+]_e$ is increased from 20 to 115 mM (Fig. 1D). Enhanced TrkF activity also serves to explain the alleviation of the K^s of the Δ*ptsN* derivative of the *kdp*$^+$ *kup trk* strain by medium alkalinization, a positive modulator of TrkF activity (9) (Fig. 2B). Overall, these findings additionally conform to the proposal that K^s of the Δ*ptsN* mutant correlates with K^+ limitation.

YcgO is the mediator of K^s. Suppression of the K^s of the Δ*ptsN* mutant by the Δ*ycgO* mutation (Fig. 4) and the K^s of *ycgO* overexpression (Fig. 5) are phenotypes that strongly implicate YcgO to be the mediator of the K^s of the Δ*ptsN* mutant. Moreover, the Δ*ycgO* mutation yielded a K^+-related growth phenotype and affected cellular K^+ content only in the absence of PtsN (Fig. 4; Tables 2 and 3), indicating that ordinarily YcgO activity is rendered cryptic in *E. coli*. The K^s of *ycgO* overexpression in *ptsN*$^+$ strains bears striking similarities to the K^s of the Δ*ptsN* mutant. Besides correlating with K^+ limitation (Table 4), its severity could be modulated by constitutive K^+ transporters (Fig. 5A to D) and suppressed by overexpression of *kup* (Table 4; see Fig. S3 in the supplemental material), adding credence to the notion that YcgO is the mediator of K^s. Finally, the absence of any overt alteration in the level of YcgO in the Δ*ptsN* mutant (Fig. 5E) indicates that its K^s may result from enhanced YcgO activity. Overexpression of *ycgO* perhaps provokes activation of YcgO to yield the K^s.

PtsN *in vivo* can exist in the phosphorylated (phospho-PtsN) and the dephosphorylated (dephospho-PtsN) states, and two observations indicate that the K^s of the Δ*ptsN* mutant may result from to the absence of dephospho-PtsN. First, the Δ*ptsP* mutant, wherein PtsN is present in the dephosphorylated state (24, 53), did not display the K^s (data not shown), indicating that absence of phospho-PtsN does not cause K^s. Furthermore, expression of PtsN variants incapable of being phosphorylated (47, 48), and hence present *in vivo* in the dephosphorylated state, complemented the K^s (Fig. 6). Additional experimentation, though, will be required to clearly rule out the possibility that the K^s is not absolutely related to the absence of a particular phosphorylation state of PtsN but results from the absence of PtsN *per se*. We tentatively conclude that the K^S results from the absence of dephospho-PtsN.

K^+ limitation in the Δ*ptsN* mutant—a model. The K^s of the Δ*ptsN* mutant and *ycgO* overexpression correlated with reduced cellular K^+ content (Tables 2 and 4), which could occur if YcgO activity either inhibited overall K^+ uptake or led to K^+ efflux. The fact that the K^s could be modulated by the Trk and Kup status of the strain (Fig. 1 and 5A to D) and alleviated by expression of K^+ uptake proteins (Fig. 3; see Fig. S1 to S3 in the supplemental material) indicates that the activity of YcgO may constitute a pathway for K^+ efflux that is fettered, probably by dephospho-PtsN. If true, then a model for K^+ limitation in the Δ*ptsN* mutant can be outlined (Fig. 7). It is assumed that the magnitude of K^+ efflux mediated by YcgO is lower than the flux of K^+ uptake occurring separately through the Trk, Kup, and fully activated TrkF systems and is stimulated by $[K^+]_e$. A comparison of the growth rate curves for a pair of MC4100 *ptsN*$^+$ and Δ*ptsN* strains (Fig. 1A) with respect to $[K^+]_e$ shows that the growth rate of the Δ*ptsN* mutant decreases as the $[K^+]_e$ is raised from 1 to 20 mM and remains at lower but a constant value with further increases in $[K^+]_e$. It is postulated that this decrease in growth rate corresponds to the onset of YcgO-mediated K^+ efflux that attains a maximal value above a $[K^+]_e$ of 20 mM. Growth rate decreases as described above are also apparent for the Δ*kdp* Δ*ptsN* double mutant JD662 (Fig. 1B) and for MC4100 and its Δ*kdp* derivative overexpressing *ycgO* (Fig. 5A and B). The onset of YcgO-mediated K^+ efflux is suggestive of a regulatory role for $[K^+]_e$ in stimulation of YcgO activity. $[K^+]_e$-mediated regulation of the gating of the MscK (KefA) channel in *E. coli* (54) is a precedent for ionic regulation of transporter/channel activity. The $[K^+]_e$ range above which YcgO-mediated K^+ efflux reaches its maximum leads to K^+ limitation and hence the K^s. Furthermore, the repression of expression of the Kdp system by $[K^+]_e$ (15, 16) would serve to maintain a cytoplasmic K^+ deficit. The exacerbation of the K^s of the Δ*ptsN* mutant in the absence of TrkA and Kup (Fig. 1C) can be accounted for by this model.

In the Δ*kdp* background, the Δ*ptsN* mutation additionally retarded the growth rate in the $[K^+]_e$ range below 10 mM (Fig. 1B), which was not suppressed by the Δ*ycgO* mutation (Table 3). The reason for this growth retardation is not known, but it is indicative of a K^+ limitation because it was absent in a Kdp$^+$ strain (Fig. 1A) and may represent another aspect of K^+ ion metabolism that is

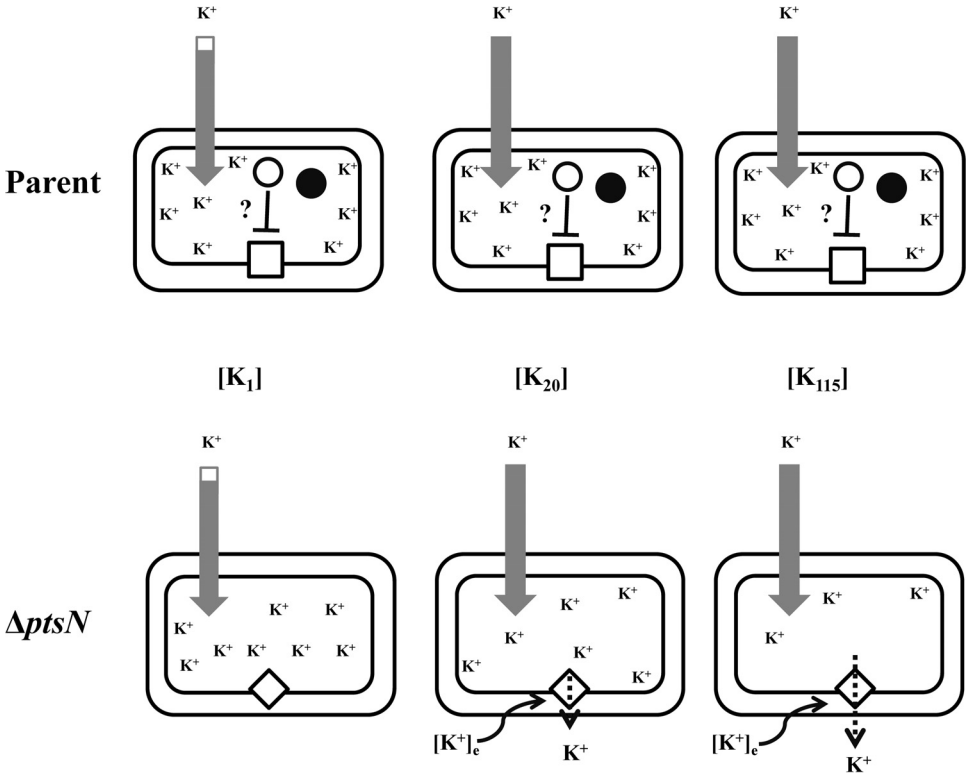

FIG 7 A model for K^+ limitation mediated by YcgO in the Δ*ptsN* mutant. Scheme depicting cellular K^+ content in the parent strain and its reduction in the Δ*ptsN* mutant. K^+ content in the two strains is shown in media with $[K^+]_e$s of 1 ($[K_1]$), 20 ($[K_{20}]$), and 115 ($[K_{115}]$) mM. A double-colored arrow represents the contribution to cellular K^+ content due to K^+ uptake mediated by the Kdp (white) and the Trk plus Kup (gray) transporters, whereas a single-colored gray arrow represents K^+ uptake occurring via the Trk and Kup transporters and reflects the repression of the Kdp system by $[K^+]_e$. The contribution of the TrkF activity to the cellular K^+ content in strains bearing all K^+ uptake systems is considered to be negligible. K^+ efflux mediated by YcgO is represented as a dashed arrow, and its stimulation by $[K^+]_e$ is represented by a wavy arrow. The heights of the arrows representing K^+ uptake and efflux are proportional to their K^+ transport fluxes. Dephospho-PtsN, presumed to fetter YcgO, and the phosphorylated form of PtsN are represented as open and solid circles, respectively. The open square and diamond, respectively, represent the fettered and unfettered states of YcgO.

regulated by PtsN, one that occurs independent of YcgO and is apparent in the absence of Kdp.

A probable physiological role for YcgO. The present work implies that in *E. coli*, YcgO is rendered cryptic, probably by dephospho-PtsN, whose absence leads to K^+ limitation via YcgO-mediated K^+ efflux. That the phenotype of the Δ*ycgO* mutant was seen only in the absence of PtsN (Table 3) is consistent with this implication and is indicative of a specialized physiological role for YcgO. What is the likely physiological role of YcgO? An earlier study implicated a role for YcgO in growth adaptation to low medium osmolarity, because the Δ*ycgO* mutant displayed highly impaired growth in certain low-osmolarity media (27). However, we did not observe any significant differences in the growth rates between MC4100 and its Δ*ycgO* derivative in the low-osmolarity media employed in the aforementioned study (data not shown). YcgO belongs to the CPA1 family of proteins that mediate monovalent cation/proton antiport (55), and its orthologs in *Vibrio parahaemolyticus* (56) and *Vibrio cholerae* (57) have been reported to mediate K^+ efflux by functioning as K^+/H^+ antiporters. It is thus plausible that YcgO-mediated K^+ efflux may occur via K^+/H^+ antiport.

A role for K^+ efflux as an adaptive, stress-relieving mechanism has previously been documented for the KefG/B and KefF/C K^+/H^+ (Kef) antiporters. K^+ efflux via Kef proteins has been referred to as protective K^+ efflux because it is thought to provide adaptation to cytoplasmic electrophile stress (8). Allowing for the notion that dephospho-PtsN fetters YcgO, by analogy to the Kef system it is possible that certain stresses alter the balance of the phosphorylation status of PtsN toward phospho-PtsN and lead to a decline in growth via YcgO-mediated K^+ limitation. Impaired *kdp* expression resulting from the absence or lowered levels of dephospho-PtsN reported earlier (47) may then be rationalized as a factor in maintenance of K^+ limitation. *E. coli* thus can reduce its growth rate via K^+ limitation, serving as an adaptive response for stress tolerance. Our results therefore suggest that perhaps one of the functions of the PtsP-PtsO-PtsN phosphorelay is in the control of a stress-responsive K^+ efflux pathway.

The PtsP-PtsO-PtsN phosphorelay appears to possess a sensing attribute, which is the presence of a GAF domain thought to function as a sensor of small molecules (58), located in the N terminus of PtsP (22). Recently glutamine and α-ketoglutarate were shown to reciprocally affect the phosphorylation status of PtsN, with the former elevating and the latter decreasing the level of dephospho-PtsN (24). Glutamine and α-ketoglutarate were shown to exert their effects by inhibiting and stimulating, respectively, a GAF domain-dependent autophosphorylation of PtsP (24). The ratio of glutamine and α-ketoglutarate is thought to be a measure of the state of the cell with regard to its nitrogen suffi-

ciency or deficiency, with the ratios being high and low in nitrogen-rich and nitrogen-limiting environments, respectively (59). Given this, it has been proposed that the PtsP-PtsO-PtsN phosphorelay serves as a sensor of nitrogen availability (24). However, the reason or reasons for a linkage between nitrogen metabolism, the PtsP-PtsO-PtsN phosphorelay, and K^+ metabolism remain somewhat obscure. Perhaps such a linkage may be realized in the context of a microbial community setting such as a biofilm, wherein a metabolic stress signal(s) conveyed via YcgO-mediated K^+ efflux could be a means of propagating the signal within the community. Under this scenario, the postulated stimulation of YcgO K^+ efflux by $[K^+]_e$ could serve to amplify the signal. Recently, K^+ efflux via the YugO K^+ channel and its dispersal from the interior to the periphery of the *Bacillus subtilis* biofilm have been proposed to coordinate glutamate utilization by cells in the two spatially separated zones of the biofilm (60).

PtsN and AHAS activity. In this study, performed with *E. coli* K-12 strains, K^+ limitation mediated by YcgO is implicated to be the cause of the K^s of the Δ*ptsN* mutant. However, an earlier study (26) implicates impaired AHASI and AHASIII activities in the *E. coli* K-12 Δ*ptsN* mutant to be the cellular casualty of its K^s, due to the observation that the K^s could be alleviated by the presence of a functional AHASII that otherwise is absent in *E. coli* K-12 (50). That study made two presumptions, namely (i) that cellular K^+ content is elevated in the Δ*ptsN* mutant that perturbs AHASI and AHASIII activities and (ii) that AHASII is insensitive to elevated cellular K^+ content (26). We found that the K^s persisted in the Δ*ptsN* mutant bearing a functional AHASII (see Fig. S4 in the supplemental material) and was not alleviated by leucine, isoleucine, and valine supplementation, indicating that impaired AHAS activity may not be the sole correlate of the physiological defect in the Δ*ptsN* mutant. The reason or reasons for this discrepancy are at present unclear but may be the result of a genetic variation or variations between the AHASII-bearing strains used in this study and the aforementioned study, which remain to be characterized.

In conclusion, this study implicates a role for PtsN in preventing K^+ limitation in *E. coli* by a mechanism that may involve fettering of YcgO-mediated K^+ efflux, probably by dephospho-PtsN. It is speculated that K^+ limitation may be conditional such that under favorable growth conditions, futile efflux of K^+ is prevented. YcgO-mediated K^+ limitation may occur under certain physiological stresses that may exert its effects on the PtsP-PtsO-PtsN phosphorelay by shifting the balance between the two phosphorylation states of PtsN toward phospho-PtsN. The ensuing K^+ limitation and reduced growth may serve as means to tolerate the stress. Future studies in this regard will need to establish unambiguously the phosphorylation state of PtsN that negatively regulates YcgO activity, in particular, whether YcgO displays selective interaction with a phosphorylated state of PtsN, and obtain direct evidence that YcgO functions as a K^+ efflux protein.

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REFERENCES

1. **Csonka LN, Epstein W.** 1996. Osmoregulation, p 1210–1223. *In* Neidhardt FC, Curtiss R, III, Ingraham JL, Lin ECC, Low KB, Magasanik B, Reznikoff WS, Riley M, Schaechter M, Umbarger HE (ed), *Escherichia coli* and *Salmonella*: cellular and molecular biology 2nd ed, vol 1. American Society for Microbiology, Washington, DC.
2. **Epstein W.** 2003. The roles and regulation of potassium in bacteria. Prog Nucleic Acid Res Mol Biol **75:**293–320. http://dx.doi.org/10.1016/S0079-6603(03)75008-9.
3. **Epstein W, Schultz SG.** 1965. Cation transport in *Escherichia coli*. V. Regulation of cation content. J Gen Physiol **49:**221–234.
4. **Richey B, Cayley DS, Mossing MC, Kolka C, Anderson CF, Farrar TC, Record MT, Jr.** 1987. Variability in the intracellular ionic environment of *Escherichia coli*: differences between in vitro and in vivo effects of ion concentrations on protein-DNA interactions and gene expression. J Biol Chem **262:**7157–7164.
5. **Dinnbier U, Limpinsel E, Schmid R, Bakker EP.** 1988. Transient accumulation of potassium glutamate and its replacement by trehalose during adaptation of growing cells of *Escherichia coli* K-12 to elevated sodium chloride concentrations. Arch Microbiol **150:**348–357. http://dx.doi.org/10.1007/BF00408306.
6. **Cayley S, Lewis BA, Guttman HJ, Record MT, Jr.** 1991. Characterization of the cytoplasm of *Escherichia coli* K-12 as a function of external osmolarity: implications for protein-DNA interactions *in vivo*. J Mol Biol. **222:**281–300. http://dx.doi.org/10.1016/0022-2836(91)90212-O.
7. **Ninfa AJ.** 2007. Regulation of carbon and nitrogen metabolism: adding regulation of ion channels and another second messenger to the mix. Proc Natl Acad Sci U S A **104:**4243–4244. http://dx.doi.org/10.1073/pnas.0700325104.
8. **Booth IR.** 29 March 2005. Glycerol and methylglyoxal metabolism. EcoSal Plus 2005 http://dx.doi.org/10.1128/ecosalplus.3.4.3.
9. **Buurman ET, McLaggan D, Naprstek J, Epstein W.** 2004. Multiple paths for nonphysiological transport of K^+ in *Escherichia coli*. J Bacteriol **186:**4238–4245. http://dx.doi.org/10.1128/JB.186.13.4238-4245.2004.
10. **Bakker EP, Booth IR, Dinnbier U, Epstein W, Gajewska A.** 1987. Evidence for multiple K^+ export systems in *Escherichia coli*. J Bacteriol **169:**3743–3749.
11. **Epstein W, Whitelaw V, Hesse J.** 1978. A K^+ transport ATPase in *Escherichia coli*. J Biol Chem **253:**6666–6668.
12. **Hesse JE, Wieczorek L, Altendorf K, Reicin AS, Dorus E, Epstein W.** 1984. Sequence homology between two membrane transport ATPases, the Kdp-ATPase of *Escherichia coli* and the Ca^{2+}-ATPase of sarcoplasmic reticulum. Proc Natl Acad Sci U S A **81:**4746–4750. http://dx.doi.org/10.1073/pnas.81.15.4746.
13. **Laimins LA, Rhoads DB, Epstein W.** 1981. Osmotic control of *kdp* operon expression in *Escherichia coli*. Proc Natl Acad Sci U S A **78:**464–468. http://dx.doi.org/10.1073/pnas.78.1.464.
14. **Asha H, Gowrishankar J.** 1993. Regulation of *kdp* operon expression in

Escherichia coli: evidence against turgor as signal for transcriptional control. J Bacteriol **175:**4528–4537.
15. **Roe AJ, McLaggan D, O'Byrne CP, Booth IR.** 2000. Rapid inactivation of the *Escherichia coli* Kdp K^+ uptake system by high potassium concentrations. Mol Microbiol **35:**1235–1243. http://dx.doi.org/10.1046/j.1365-2958.2000.01793.x.
16. **Laermann V, Cudic E, Kipschull K, Zimmann P, Altendorf K.** 2013. The sensor kinase KdpD of *Escherichia coli* senses external K^+. Mol Microbiol **88:**1194–1204. http://dx.doi.org/10.1111/mmi.12251.
17. **Postma PW, Lengeler JW, Jacobson GR.** 1993. Phosphoenolpyruvate: carbohydrate phosphotransferase systems of bacteria. Microbiol Rev **57:**543–594.
18. **Saier MH, Jr.** 2001. The bacterial phosphotransferase system: structure, function, regulation and evolution. J Mol Microbiol Biotechnol **3:**325–327.
19. **Deutscher J, Francke C, Postma PW.** 2006. How phosphotransferase system-related protein phosphorylation regulates carbohydrate metabolism in bacteria. Microbiol Mol Biol Rev **70:**939–1031. http://dx.doi.org/10.1128/MMBR.00024-06.
20. **Deutscher J, Ake FM, Derkaoui M, Zebre AC, Cao TN, Bouraoui H, Kentache T, Mokhtari A, Milohanic E, Joyet P.** 2014. The bacterial phosphoenolpyruvate:carbohydrate phosphotransferase system: regulation by protein phosphorylation and phosphorylation-dependent protein-protein interactions. Microbiol Mol Biol Rev **78:**231–256. http://dx.doi.org/10.1128/MMBR.00001-14.
21. **Powell BS, Court DL, Inada T, Nakamura Y, Michotey V, Cui X, Reizer A, Saier MH, Jr, Reizer J.** 1995. Novel proteins of the phosphotransferase system encoded within the *rpoN* operon of *Escherichia coli*. Enzyme IIA^{Ntr} affects growth on organic nitrogen and the conditional lethality of an era^{ts} mutant. J Biol Chem **270:**4822–4839.
22. **Reizer J, Reizer A, Merrick MJ, Plunkett G, III, Rose DJ, Saier MH, Jr.** 1996. Novel phosphotransferase-encoding genes revealed by analysis of the *Escherichia coli* genome: a chimeric gene encoding an enzyme I homologue that possesses a putative sensory transduction domain. Gene **181:**103–108. http://dx.doi.org/10.1016/S0378-1119(96)00481-7.
23. **Rabus R, Reizer J, Paulsen I, Saier MH, Jr.** 1999. Enzyme I^{Ntr} from *Escherichia coli*. A novel enzyme of the phosphoenolpyruvate-dependent phosphotransferase system exhibiting strict specificity for its phosphoryl acceptor, NPr. J Biol Chem **274:**26185–26191.
24. **Lee CR, Park YH, Kim M, Kim YR, Park S, Peterkofsky A, Seok YJ.** 2013. Reciprocal regulation of the autophosphorylation of enzyme I^{Ntr} by glutamine and alpha-ketoglutarate in *Escherichia coli*. Mol Microbiol **88:**473–485. http://dx.doi.org/10.1111/mmi.12196.
25. **Lee CR, Cho SH, Yoon MJ, Peterkofsky A, Seok YJ.** 2007. *Escherichia coli* enzyme IIA^{Ntr} regulates the K^+ transporter TrkA. Proc Natl Acad Sci U S A **104:**4124–4129. http://dx.doi.org/10.1073/pnas.0609897104.
26. **Reaves ML, Rabinowitz JD.** 2011. Characteristic phenotypes associated with *ptsN*-null mutants in *Escherichia coli* K-12 are absent in strains with functional *ilvG*. J Bacteriol **193:**4576–4581. http://dx.doi.org/10.1128/JB.00325-11.
27. **Verkhovskaya ML, Barquera B, Wikstrom M.** 2001. Deletion of one of two *Escherichia coli* genes encoding putative Na^+/H^+ exchangers (*ycgO*) perturbs cytoplasmic alkali cation balance at low osmolarity. Microbiology **147:**3005–3013. http://dx.doi.org/10.1099/00221287-147-11-3005.
28. **Baba T, Ara T, Hasegawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H.** 2006. Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol Syst Biol **2:**2006.0008.
29. **Miller JH.** 1992. A short course in bacterial genetics: a laboratory manual and handbook for *Escherichia coli* and related bacteria. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
30. **Datsenko KA, Wanner BL.** 2000. One-step inactivation of chromosomal genes in *Escherichia coli* K-12 using PCR products. Proc Natl Acad Sci U S A **97:**6640–6645. http://dx.doi.org/10.1073/pnas.120163297.
31. **Epstein W, Davies M.** 1970. Potassium-dependent mutants of *Escherichia coli* K-12. J Bacteriol **101:**836–843.
32. **Epstein W, Kim BS.** 1971. Potassium transport loci in *Escherichia coli* K-12. J Bacteriol **108:**639–644.
33. **Singer M, Baker TA, Schnitzler G, Deischel SM, Goel M, Dove W, Jaacks KJ, Grossman AD, Erickson JW, Gross CA.** 1989. A collection of strains containing genetically linked alternating antibiotic resistance elements for genetic mapping of *Escherichia coli*. Microbiol Rev **53:**1–24.
34. **Amann E, Ochs B, Abel KJ.** 1988. Tightly regulated *tac* promoter vectors useful for the expression of unfused and fused proteins in *Escherichia coli*. Gene **69:**301–315. http://dx.doi.org/10.1016/0378-1119(88)90440-4.
35. **Chang AC, Cohen SN.** 1978. Construction and characterization of amplifiable multicopy DNA cloning vehicles derived from the P15A cryptic miniplasmid. J Bacteriol **134:**1141–1156.
36. **Sambrook J, Russell DW.** 2001. Molecular cloning: a laboratory manual, 3rd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
37. **Kleckner N, Bender J, Gottesman S.** 1991. Uses of transposons with emphasis on Tn*10*. Methods Enzymol **204:**139–180. http://dx.doi.org/10.1016/0076-6879(91)04009-D.
38. **Groisman EA, Casadaban MJ.** 1986. Mini-mu bacteriophage with plasmid replicons for in vivo cloning and *lac* gene fusing. J Bacteriol **168:**357–364.
39. **Papp-Wallace KM, Maguire ME.** 2008. Regulation of CorA Mg^{2+} channel function affects the virulence of *Salmonella enterica* serovar Typhimurium. J Bacteriol **190:**6509–6516. http://dx.doi.org/10.1128/JB.00144-08.
40. **Frymier JS, Reed TD, Fletcher SA, Csonka LN.** 1997. Characterization of transcriptional regulation of the *kdp* operon of *Salmonella typhimurium*. J Bacteriol **179:**3061–3063.
41. **Pathania A, Sardesai AA.** 2015. Distinct paths for basic amino acid export in *Escherichia coli*: YbjE (LysO) mediates export of L-lysine. J Bacteriol **197:**2036–2047. http://dx.doi.org/10.1128/JB.02505-14.
42. **Dosch DC, Salvacion FF, Epstein W.** 1984. Tetracycline resistance element of pBR322 mediates potassium transport. J Bacteriol **160:**1188–1190.
43. **Sardesai AA, Gowrishankar J.** 2001. Improvement in K^+-limited growth rate associated with expression of the N-terminal fragment of one subunit (KdpA) of the multisubunit Kdp transporter in *Escherichia coli*. J Bacteriol **183:**3515–3520. http://dx.doi.org/10.1128/JB.183.11.3515-3520.2001.
44. **Hall JA, Yan D.** 2013. The molecular basis of K^+ exclusion by the *Escherichia coli* ammonium channel AmtB. J Biol Chem **288:**14080–14086. http://dx.doi.org/10.1074/jbc.M113.457952.
45. **Dillon SC, Dorman CJ.** 2010. Bacterial nucleoid-associated proteins, nucleoid structure and gene expression. Nat Rev Microbiol **8:**185–195. http://dx.doi.org/10.1038/nrmicro2261.
46. **Ortenberg R, Beckwith J.** 2003. Functions of thiol-disulfide oxidoreductases in *E. coli*: redox myths, realities, and practicalities. Antioxid Redox Signal **5:**403–411. http://dx.doi.org/10.1089/152308603768295140.
47. **Luttmann D, Heermann R, Zimmer B, Hillmann A, Rampp IS, Jung K, Gorke B.** 2009. Stimulation of the potassium sensor KdpD kinase activity by interaction with the phosphotransferase protein IIA^{Ntr} in *Escherichia coli*. Mol Microbiol **72:**978–994. http://dx.doi.org/10.1111/j.1365-2958.2009.06704.x.
48. **Zimmer B, Hillmann A, Görke B.** 2008. Requirements for the phosphorylation of the *Escherichia coli* $EIIA^{Ntr}$ protein in vivo. FEMS Microbiol Lett **286:**96–102. http://dx.doi.org/10.1111/j.1574-6968.2008.01262.x.
49. **Lee CR, Koo BM, Cho SH, Kim YJ, Yoon MJ, Peterkofsky A, Seok YJ.** 2005. Requirement of the dephospho-form of enzyme IIA^{Ntr} for derepression of *Escherichia coli* K-12 *ilvBN* expression. Mol Microbiol **58:**334–344. http://dx.doi.org/10.1111/j.1365-2958.2005.04834.x.
50. **Salmon KA, Yang CR, Hatfield GW.** 28 February 2006. Biosynthesis and regulation of the branched-chain amino acids. EcoSal Plus 2006 http://dx.doi.org/10.1128/ecosalplus.3.6.1.5.
51. **Studier FW, Moffatt BA.** 1986. Use of bacteriophage T7 RNA polymerase to direct selective high-level expression of cloned genes. J Mol Biol **189:**113–130. http://dx.doi.org/10.1016/0022-2836(86)90385-2.
52. **Soupene E, van Heeswijk WC, Plumbridge J, Stewart V, Bertenthal D, Lee H, Prasad D, Paliy O, Charernnoppakul P, Kustu S.** 2003. Physiological studies of *Escherichia coli* strain MG1655: growth defects and apparent cross-regulation of gene expression. J Bacteriol **185:**5611–5626. http://dx.doi.org/10.1128/JB.185.18.5611-5626.2003.
53. **Bahr T, Lüttmann D, März W, Rak B, Görke B.** 2011. Insight into bacterial phosphotransferase system-mediated signaling by interspecies transplantation of a transcriptional regulator. J Bacteriol **193:**2013–2026. http://dx.doi.org/10.1128/JB.01459-10.
54. **Li Y, Moe PC, Chandrasekaran S, Booth IR, Blount P.** 2002. Ionic regulation of MscK, a mechanosensitive channel from *Escherichia coli*. EMBO J **21:**5323–5330. http://dx.doi.org/10.1093/emboj/cdf537.
55. **Saier MH, Jr, Reddy VS, Tamang DG, Västermark A.** 2014. The transporter classification database. Nucleic Acids Res **42**(Database issue): D251–D258. http://dx.doi.org/10.1093/nar/gkt1097.

56. **Radchenko MV, Waditee R, Oshimi S, Fukuhara M, Takabe T, Nakamura T.** 2006. Cloning, functional expression and primary characterization of *Vibrio parahaemolyticus* K^+/H^+ antiporter genes in *Escherichia coli*. Mol Microbiol **59:**651–663. http://dx.doi.org/10.1111/j.1365-2958.2005.04966.x.
57. **Resch CT, Winogrodzki JL, Patterson CT, Lind EJ, Quinn MJ, Dibrov P, Häse CC.** 2010. Putative Na^+/H^+ antiporter of *Vibrio cholerae*, VcNhaP2, mediates the specific K^+/H^+ exchange in vivo. Biochemistry **49:**2520–2528. http://dx.doi.org/10.1021/bi902173y.
58. **Martinez SE, Beavo JA, Hol WG.** 2002. GAF domains: two-billion-year old molecular switches that bind cyclic nucleotides. Mol Interv **2:**317–323. http://dx.doi.org/10.1124/mi.2.5.317.
59. **Senior PJ.** 1975. Regulation of nitrogen metabolism in *Escherichia coli* and *Klebsiella aerogenes*: studies with the continuous-culture technique. J Bacteriol **123:**407–418.
60. **Prindle A, Liu J, Asally M, Ly S, Garcia-Ojalvo J, Suel GM.** 2015. Ion channels enable electrical communication in bacterial communities. Nature **527:**59–63. http://dx.doi.org/10.1038/nature15709.