

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 tran-

scriptionally 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⁺]_es 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 stand-alone 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⁺]_es.

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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 $\Delta ptsN$ 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 $\Delta ptsN$ 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 $\Delta ptsN$ mutant, implicating YcgO to be the mediator of the K^s of the $\Delta ptsN$ 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 $\Delta ptsN$ 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 $\Delta ptsN::Kan$, $\Delta ycgO::Kan$, $\Delta kdpA::Kan$, and $\Delta amtB::Kan$ 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	$\Delta(argF-lac)U169 rpsL150 relA1 spoT1 araD139 flbB5301 deoC1 ptsF25$
JD17	MC4100 $\Delta ptsN::Kan$
JD466	MC4100 $\Delta ycgO::Kan$
JD509	MC4100 $\Delta ptsN \Delta ycgO::Kan$
JD624	MC4100 <i>kup trkA</i>
JD637	JD624 $\Delta kdpA$
JD656	MC4100 $\Delta kdpA$
JD657	JD637 <i>zha-203::Tn10</i>
JD658	JD657 $\Delta ptsN::Kan$
JD660	JD624 $\Delta ptsN::Kan$
JD662	JD656 $\Delta ptsN::Kan$
JD693	JD637 $\Delta ycgO::Kan$
JD694	JD656 $\Delta ycgO::Kan$
JD696	JD624 $\Delta ycgO$
JD710	JD624 $\Delta ycgO \Delta ptsN::Kan$
JD714	JD637 $\Delta ycgO \Delta ptsN::Kan zha-203::Tn10$
JD723	JD624 <i>ycgO_{FL} ΔKan</i>
JD724	JD723 $\Delta ptsN::Kan$
JD725	JD656 $\Delta ycgO \Delta ptsN::Kan$
JD726	MC4100 <i>attλ::(P_{trc}99A bla)</i>
JD727	MC4100 <i>attλ::(P_{trc}ycgO bla)</i>
JD728	JD624 <i>attλ::(P_{trc}99A bla)</i>
JD729	JD624 <i>attλ::(P_{trc}ycgO bla)</i>
JD732	JD624 <i>attλ::(P_{trc}ycgO bla::Kan)</i>
JD734	JD637 <i>attλ::(P_{trc}99A bla)</i>
JD735	JD637 <i>attλ::(P_{trc}ycgO bla)</i>
GJ14949	JD656 <i>attλ::(P_{trc}99A bla)</i>
GJ14950	JD656 <i>attλ::(P_{trc}ycgO bla)</i>
Plasmids	
pHYD3025	Derivative of plasmid pTrc99A, described in reference 41
pHYD724	pTrc99A in which <i>kdpA'</i> , 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 <i>kup</i> expressed from P _{trc} promoter, present within EcoRI and HindIII sites
pHYD1858	pTrc99A containing <i>ycgO</i> , present within NcoI and HindIII sites, and expressed from P _{trc} promoter
pHYD5006	pHYD3025 containing <i>ptsN</i> 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 $\Delta ptsN::Kan$, $\Delta ycgO::Kan$, and $\Delta kdpA::Kan$ 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::Tn10* 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 *attλ::(P_{trc}ycgO bla)*, *attλ::(P_{trc}99A bla)*, and *attλ::(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.

$\Delta ptsN::Kan$ 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 $\Delta ptsN::Kan$ 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 $\Delta ptsN::Kan$ derivative of the triple K^+ transporter-defective strain, the $\Delta ptsN::Kan$ allele was introduced via cotransduction with the *zha-203::Tn10* insertion derived from strain CAG12072 (33), with a primary selection for Tet-resistant transductants using a P1 lysate prepared on strain JD650 (MC4100 $\Delta ptsN::Kan$ *zha-203::Tn10*) on KML agar plates. *zha-203::Tn10* 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::Tn10* marker did not affect the K^s of the $\Delta ptsN::Kan$ 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 $\Delta ptsN::Kan$ mutation on growth rates in different K^+ transporter-defective backgrounds, pairs of $ptsN^+$ and $\Delta ptsN::Kan$ 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 \pm standard errors (SE) of values obtained from three independent measurements.

Isolation of a transposon insertion in *ycgO*. Following transposon Tn10dCm 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 $\Delta ptsN$ 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 Tn10dCm insertion in one of the suppressors wherein the Tn10dCm insertion caused the suppressor phenotype was determined by cloning the Tn10dCm element with the aid of the mini-Mu cloning procedure (38). The transposon junction site was sequenced with the primer 5'-TCCCTCTGTTCAGCTACTGA-3', which reads out from Tn10dCm, 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 $\Delta ptsN::Kan$ 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 phthalate. 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 \pm standard deviations (SD) of single measurements conducted on three independent cultures. For cultures of a strain bearing the $\Delta ptsN$ 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 \times 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 $\Delta ptsN::Kan$ 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 ($\Delta kdp \Delta ptsN::Kan$) was similar to that displayed by JD17 in K_{20} , K_{40} , and K_{115} media. In the lower $[K^+]_e$

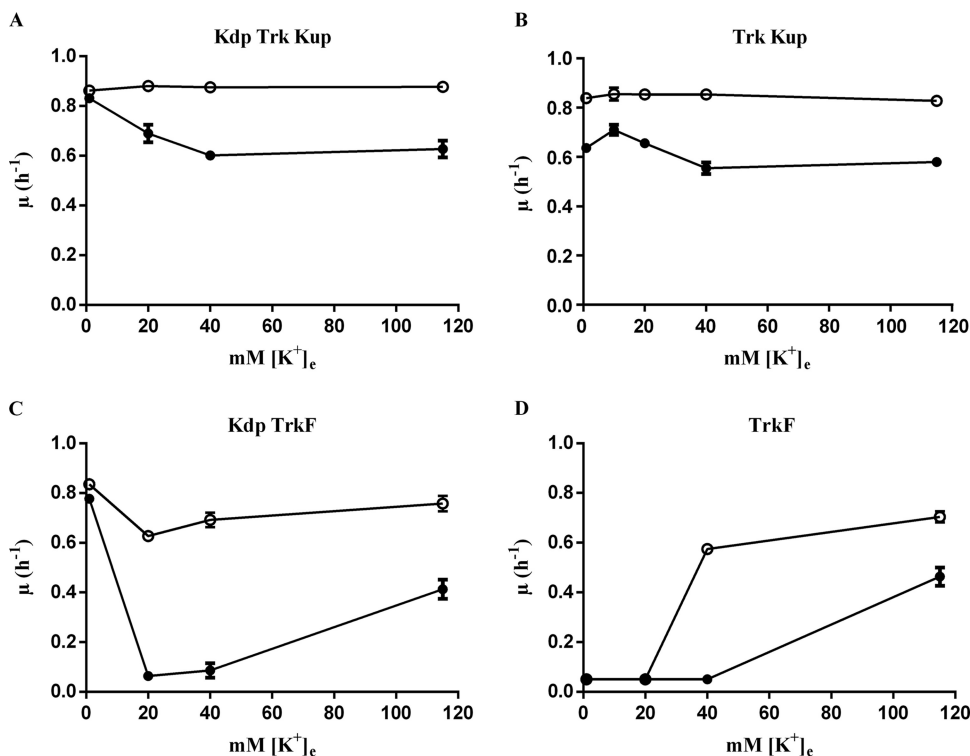


FIG 1 K^+ -sensitive growth of the ΔptsN mutant and its modulation by K^+ uptake systems. Shown are growth rates (μ) of pairs of isogenic ptsN^+ (open circles) and ΔptsN (solid circles) strains bearing combinations of K^+ uptake systems with respect to $[\text{K}^+]_e$. The predominant K^+ transport 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 ΔptsN 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 $[\text{K}^+]_e$, 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) $[\text{K}^+]_e$ s (15, 16, 40). The inhibition of the expression and/or activity of the Kdp system by $[\text{K}^+]_e$ 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 $[\text{K}^+]_e$ 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) $[\text{K}^+]_e$ 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 $\Delta\text{ptsN}::\text{Kan}$ mutation persisted in the absence of the three active K^+ uptake systems. Both JD657, which is a triple K^+ transporter-defective strain, and its $\Delta\text{ptsN}::\text{Kan}$ derivative, JD658, did not grow in K_1 or K_{20} medium. However, while the growth rate of JD657 increased as the $[\text{K}^+]_e$ 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 ΔptsN 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 ΔptsN 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 ΔptsN 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 $\Delta\text{ptsN}::\text{Kan}$ derivative, JD660, after their growth in K_1 medium followed by a shift for 2 h into 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-

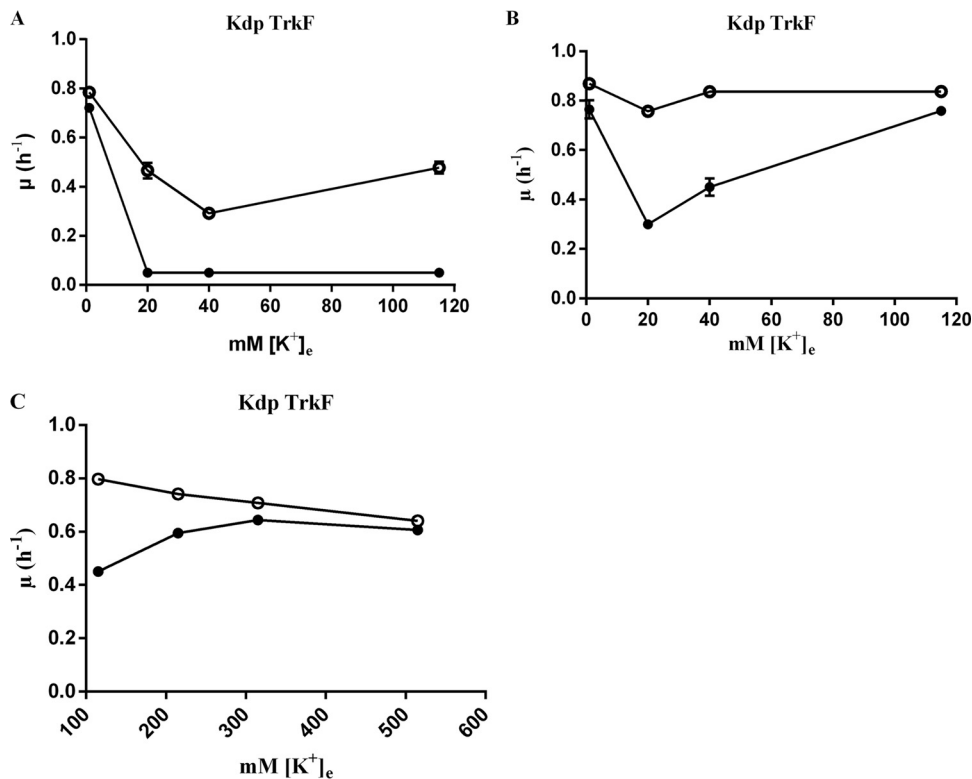


FIG 2 Modulation of the K^+ -sensitive growth of the $\Delta ptsN$ mutant by alterations in external pH and its alleviation by elevated $[K^+]_e$. Shown are the growth rates (μ) of JD624 (open circles) and its $\Delta ptsN::Kan$ derivative, JD660 (solid circles), with respect to $[K^+]_e$. The K^+ transporters 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 $\Delta ptsN::Kan$ mutation could be modulated by alterations in the pH of the medium. Growth of the $\Delta ptsN::Kan$ 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 $\Delta ptsN$ mutant and its restoration by the $\Delta ycgO$ mutation and by overexpression of *kup*

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

^a Shown is the cellular K^+ content of strains JD660 ($\Delta ptsN$), JD696 ($\Delta ycgO$), and JD710 ($\Delta ycgO \Delta ptsN$) 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 \pm 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.

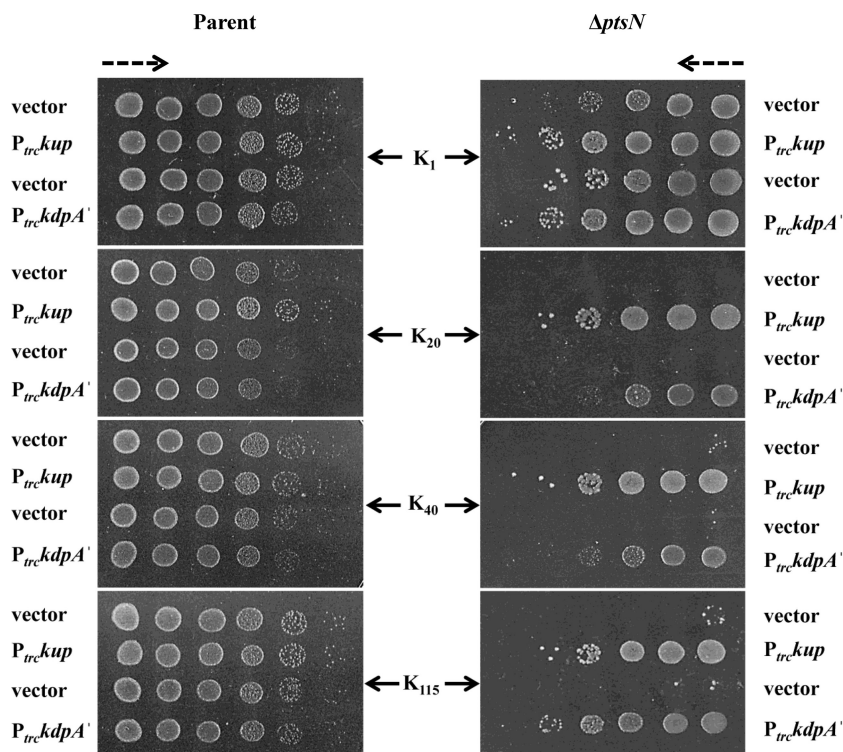


FIG 3 Alleviation of the K^s of the $\Delta ptsN$ mutant by overproduction of K⁺ uptake proteins Kup and KdpA'. Tenfold serial dilutions of cultures of the parent strain (JD624 [*kdp⁺ trkA kup*]) and its $\Delta ptsN::Kan$ derivative, JD660 ($\Delta ptsN$), bearing the plasmids pHYD3025 (vector), pHYD1852 (P_{trc}*kup*), pTrc99A (vector), and pHYD724 (P_{trc}*kdpA'*), were spotted on the surface of K₁, K₂₀, K₄₀, and K₁₁₅ 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 K₁ 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⁺]_es 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⁺]_es 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 $\Delta ptsN::Kan$ mutant JD660 in K₄₀ and K₁₁₅ media but not in K₂₀ medium (Fig. 2B). A probable explanation for this and also for the K^s in K₂₀ medium imparted by the $\Delta ptsN$ mutation in a strain that bears Kdp as the only active K⁺ transporter is provided in the section describing the effect of the $\Delta ycgO$ mutation on K^s.

Increasing the [K⁺]_e of the K₁₁₅ 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 $\Delta ptsN::Kan$ 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 K₁₁₅ 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 K₄₀ and K₁₁₅ 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 $\Delta ptsN::Kan$ $\Delta amtB$ 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 $\Delta ptsN::Kan$ derivative of MC4100, JD17, alleviated its K^s , and overexpression of *kup* in the $\Delta ptsN::Kan$ 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 $\Delta ptsN$ 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 $\Delta ptsN$ 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 TrxA 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 Tn10dCm 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* ($\Delta ycgO::Kan$) also suppressed the K^s of the *hns trxA* double mutant (data not shown). We found that the K^s of the $\Delta ptsN$ mutant was also suppressed by the $\Delta ycgO::Kan$ 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 $\Delta ptsN::Kan$ mutant JD660 displayed a K^s that was prominent in K_{10} , K_{20} , and K_{40} media, whereas its $\Delta ycgO$ 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 $\Delta ycgO$ mutation does not suppress the K^s imparted by the $\Delta ptsN$ 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 $[K^+]_e$ s, 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

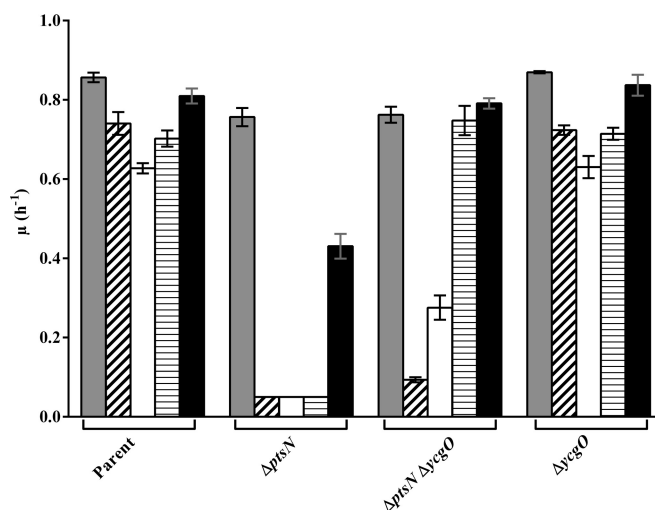


FIG 4 Involvement of YcgO in the K^s of the $\Delta ptsN$ mutant. Shown are the growth rates (μ) of the parent strain (JD624 [*kdp⁺ trkA kup*]) and its $\Delta ptsN$ (JD660), $\Delta ptsN \Delta ycgO$ (JD710), and $\Delta ycgO$ (JD696) derivatives in media of various $[K^+]_e$ s. The indicated strains 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.

$\Delta ptsN$ 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 $\Delta ptsN$ mutant by the $\Delta ycgO$ mutation in K_{10} and K_{20} media may be explained. The K^s caused by the $\Delta ptsN$ 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 $\Delta ycgO$ 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 $\Delta ycgO$ mutation does not suppress the K^s of a $\Delta ptsN::Kan$ 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 alkalization of K_{20} medium may also be explained (Fig. 2B), the basis being that the effect of medium alkalization 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 $\Delta ptsN::Kan$ mutation in reducing *kdp* expression may not be subject to modulation by medium alkalization. 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 $\Delta ptsN$ 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 $\Delta ptsN::Kan \Delta ycgO$ double mutant, and strain JD696, which bears the $\Delta ycgO$ 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 $\Delta ptsN::Kan$ derivative, JD660, permitted the following conclusions: (i) the absence of YcgO in a strain bearing the

TABLE 3 K^s phenotype of the $\Delta ptsN$ 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	<i>kdp⁺ kup⁺ trkA⁺</i>	0.86	0.88 ± 0.01	0.88 ± 0.01	0.88 ± 0.01
JD17 ^b	<i>kdp⁺ kup⁺ trkA⁺ ΔptsN</i>	0.83 ± 0.01	0.69 ± 0.01	0.60 ± 0.02	0.63 ± 0.03
JD466	<i>kdp⁺ kup⁺ trkA⁺ ΔycgO</i>	0.91 ± 0.01	0.89 ± 0.02	0.91 ± 0.02	0.90 ± 0.03
JD509	<i>kdp⁺ kup⁺ trkA⁺ ΔptsN ΔycgO</i>	0.83 ± 0.02	0.80 ± 0.02	0.82	0.84
JD656 ^b	<i>ΔkdpA kup⁺ trkA⁺</i>	0.84 ± 0.02	0.85 ± 0.02	0.85 ± 0.02	0.83 ± 0.02
JD662 ^b	<i>ΔkdpA kup⁺ trkA⁺ ΔptsN</i>	0.64 ± 0.02	0.66 ± 0.01	0.55 ± 0.02	0.58 ± 0.01
JD694	<i>ΔkdpA kup⁺ trkA⁺ ΔycgO</i>	0.85 ± 0.01	0.89 ± 0.02	0.89 ± 0.01	0.89
JD725	<i>ΔkdpA kup⁺ trkA⁺ ΔptsN ΔycgO</i>	0.70 ± 0.06	0.81 ± 0.02	0.82	0.82
JD657 ^{b,c}	<i>ΔkdpA kup trkA</i>	<0.05	<0.05	0.67 ± 0.01	0.83 ± 0.01
JD658 ^{b,c}	<i>ΔkdpA kup trkA ΔptsN</i>	<0.05	<0.05	<0.05	0.46 ± 0.02
JD693	<i>ΔkdpA kup trkA ΔycgO</i>	<0.05	<0.05	0.72 ± 0.03	0.86 ± 0.01
JD714 ^c	<i>ΔkdpA kup trkA ΔptsN ΔycgO</i>	<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 K₁, K₂₀, K₄₀, and K₁₁₅ media. The first quartet, MC4100 to JD509, 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::Tn10* marker.

$\Delta ptsN::Kan$ mutation led to restoration of cellular K⁺ content to a level approaching that of the parent following exposure to K₄₀ and K₁₁₅ 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 $\Delta ycgO$ mutation suppressed the K^s of the $\Delta ptsN$ mutant in backgrounds bearing mutations in K⁺ uptake systems (Table 3). In the background bearing all K⁺ uptake systems, the $\Delta ycgO$ mutation suppressed the K^s of the $\Delta ptsN$ mutant with the outcome that the $\Delta ptsN \Delta ycgO$ derivative of MC4100, JD509, grew at rates comparable to those of MC4100 in K₁, K₂₀, K₄₀, and K₁₁₅ media. In the background devoid of the Kdp transporter, the $\Delta ycgO$ mutation suppressed the K^s of the $\Delta ptsN$ mutant in K₂₀, K₄₀, and K₁₁₅ media. In K₁ medium, the slow growth associated with the presence of the $\Delta ptsN$ mutation in this background was not altered by the $\Delta ycgO$ mutation. In the triple K⁺ transporter-defective strain, the $\Delta ycgO$ mutation suppressed the K^s of the $\Delta ptsN$ mutant in K₄₀ and K₁₁₅ media. Absence of YcgO in MC4100 or in its Δkdp and $\Delta kdp trkA kup$ derivatives, JD656 and JD637, respectively, did not lead to any discernible alteration in growth in media of various [K⁺]_e (Table 3), indicating that the $\Delta ycgO$ 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 $\Delta ptsN$ 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 $\Delta ptsN$ mutation. In MC4100 and its Δkdp 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 K₂₀, K₄₀, and K₁₁₅ media (Fig. 5C). In the triple K⁺ transporter-defective strain, *ycgO* overexpression led to K^s in K₄₀ and K₁₁₅ 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 K₄₀ and K₁₁₅ 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 K₄₀ and K₁₁₅ 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 K₄₀ and K₁₁₅ 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 $\Delta ptsN$ mutant in more ways than one.

To test if the $\Delta ptsN$ 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 $\Delta ptsN::Kan$ derivative, JD724, following growth in K₁ medium (Fig. 5E). No differences in YcgO_{FL} levels were seen when the aforementioned two strains were grown for 2 h in K₄₀ medium following growth to the early exponential phase in K₁ medium (data not shown). These observations indicate that the K^s caused by the $\Delta ptsN$ 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 $\Delta ptsN::Kan$) 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

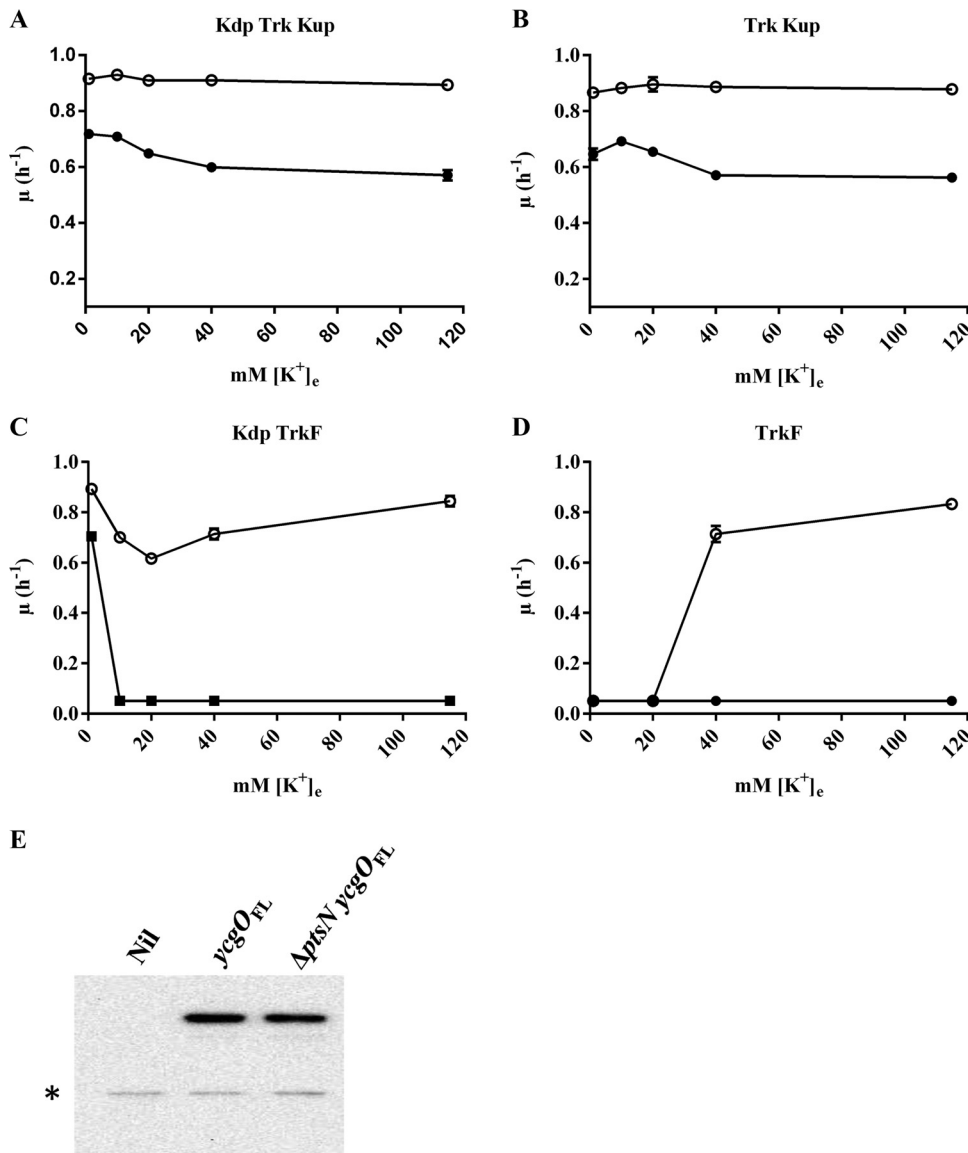


FIG 5 K^s associated with overexpression of *ycgO* and its modulation by K⁺ uptake systems and levels of YcgO in the parent strain and Δ *ptsN* mutant. Shown are the growth rates (μ) of pairs of isogenic strains bearing single-copy integrations of the vector (open circles) and *ycgO* under the expression 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 K₁, K₂₀, K₄₀, and K₁₁₅ 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 K₁ medium to mid-exponential phase were centrifuged and suspended in SDS sample buffer at an A₆₀₀ 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 K₁ 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 pseudo-auxotrophy (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	<i>kdp⁺ kup trkA attλ::(P_{trc}99A bla)</i>	1 (0.18)	1 (0.12)	1 (0.01)
JD729	<i>kdp⁺ kup trkA attλ::(P_{trc}ycgO bla)</i>	0.73 (0.14)	0.62 (0.04)	0.57 (0.7)
JD732/pHYD3025 ^b	<i>kdp⁺ kup trkA attλ::(P_{trc}ycgO bla::Kan)</i>	0.9 (0.11)	0.8 (0.16)	0.71 (0.08)
JD732/pHYD1852 ^c	<i>kdp⁺ kup trkA attλ::(P_{trc}ycgO bla::Kan)</i>	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* [*attλ::(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 \pm 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 $\Delta ptsN$ mutant is the pathway of branched-chain amino acid biosynthesis (26).

We found that the K^s of the $\Delta ptsN$ 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 $\Delta ptsN::Kan$ 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 $\Delta ptsN::Kan$ derivative of MC4100 bearing *ilvG⁺* obtained from strain NCM3722 (52) also displayed K^s (data not shown). Finally, $\Delta ptsN::Kan$ 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 $\Delta ptsN::Kan$ 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

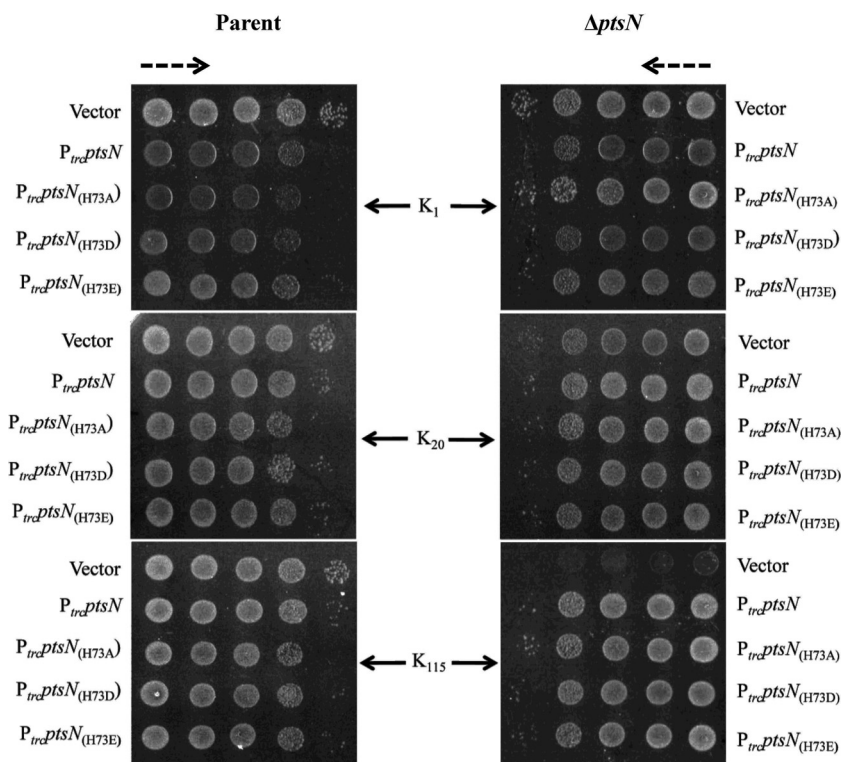


FIG 6 Complementation of the K^s of the $\Delta ptsN$ mutant by plasmids encoding PtsN and those encoding PtsN bearing amino acid substitutions of its phosphorylation site histidine. Tenfold serial dilutions of MC4100 (parent) and its $\Delta ptsN::Kan$ derivative, JD17 ($\Delta ptsN$), bearing the plasmids 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 $\Delta ptsN$ mutant is presented, and the probable physiological role of YcgO is discussed.

K^s of the $\Delta ptsN$ mutant correlates with K^+ limitation. The present work provides credible evidence that the $\Delta ptsN$ 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 $\Delta ptsN$ 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 $\Delta ptsN$ 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 $\Delta ptsN$ 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 $\Delta ptsN$ 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 $\Delta ptsN$ derivative of the *kdp⁺ kup trk* strain by medium alkalization, a positive modulator of TrkF activity (9) (Fig. 2B). Overall, these findings additionally conform to the proposal that K^s of the $\Delta ptsN$ mutant correlates with K^+ limitation.

YcgO is the mediator of K^s . Suppression of the K^s of the $\Delta ptsN$ mutant by the $\Delta ycgO$ 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 $\Delta ptsN$ mutant. Moreover, the $\Delta ycgO$ 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 $\Delta ptsN$ 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 $\Delta ptsN$ 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 $\Delta ptsN$ mutant may result from the absence of dephospho-PtsN. First, the $\Delta ptsP$ 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 $\Delta ptsN$ mutant—a model. The K^s of the $\Delta ptsN$ 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 $\Delta ptsN$ 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 $\Delta ptsN$ strains (Fig. 1A) with respect to $[K^+]_e$ shows that the growth rate of the $\Delta ptsN$ 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 $\Delta kdp \Delta ptsN$ double mutant JD662 (Fig. 1B) and for MC4100 and its Δkdp 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 $\Delta ptsN$ mutant in the absence of TrkA and Kup (Fig. 1C) can be accounted for by this model.

In the Δkdp background, the $\Delta ptsN$ mutation additionally retarded the growth rate in the $[K^+]_e$ range below 10 mM (Fig. 1B), which was not suppressed by the $\Delta ycgO$ 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

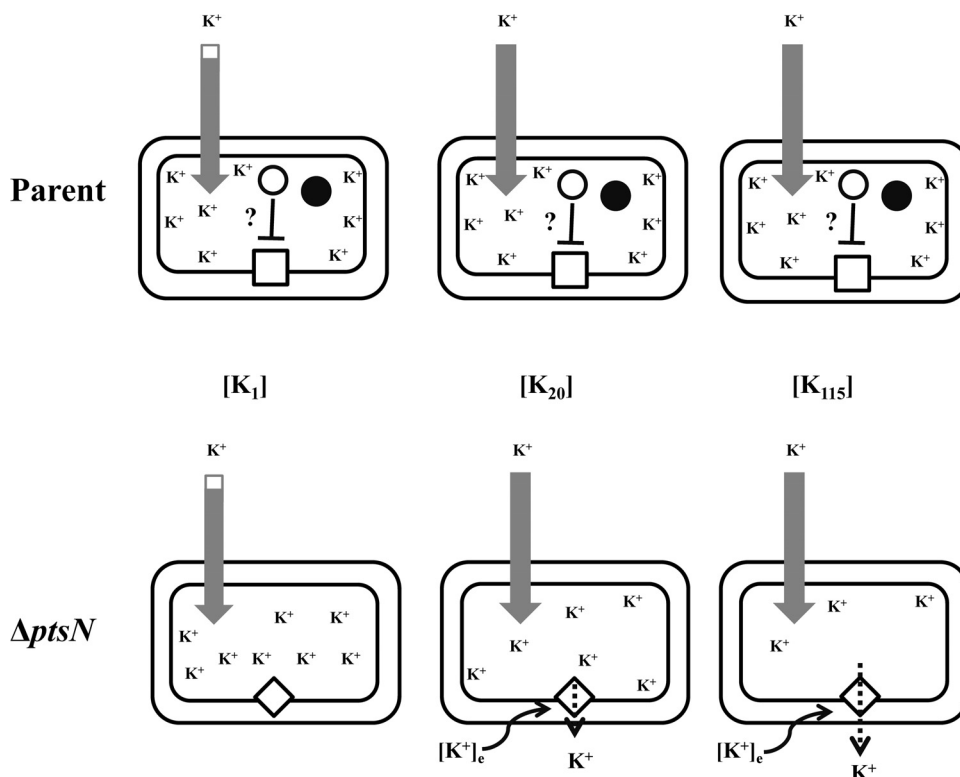


FIG 7 A model for K^+ limitation mediated by YcgO in the $\Delta ptsN$ mutant. Scheme depicting cellular K^+ content in the parent strain and its reduction in the $\Delta ptsN$ mutant. K^+ content in the two strains is shown in media with $[K^+]_e$ s of 1 ($[K_1]$), 20 ($[K_{20}]$), and 115 ($[K_{115}]$) 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 $\Delta ycgO$ 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 $\Delta ycgO$ 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 $\Delta ycgO$ 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 re-

ferred 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 YcgO 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 $\Delta ptsN$ mutant. However, an earlier study (26) implicates impaired AHAS I and AHAS III activities in the *E. coli* K-12 $\Delta ptsN$ 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 AHAS II 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 $\Delta ptsN$ mutant that perturbs AHAS I and AHAS III activities and (ii) that AHAS II is insensitive to elevated cellular K^+ content (26). We found that the K^s persisted in the $\Delta ptsN$ mutant bearing a functional AHAS II (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 $\Delta ptsN$ 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 AHAS II-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 dephosphorylation of 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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