Growth inhibition by external potassium of *Escherichia coli* lacking PtsN ($EIIA^{Ntr}$) is caused by potassium limitation mediated by YcgO

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SUPPLEMENTAL MATERIAL

Plasmid construction. The gene encoding *kup* was amplified using the primer pair JGVTRKDF and JGVTRKDR with chromosomal DNA of the strain MC4100 serving as template and the PCR product was appropriately cloned to generate plasmid pHYD1852. Using the chromosomal DNA of the strain NCM3722 (1) as template, *amtB* was amplified using the primer pair JGVAMTBF and JGVAMTBR and plasmid pHYD1853 was generated following cloning of this PCR product. Codon alterations in *amtB* that generated the *amtB*^{K} variant encoding AmtB bearing the H168D and H318E double substitution were introduced by overlap extension PCR using the plasmid pHYD1853 as template. The primer pair JGVH168DF and JGVH168DR was used to generate the codon change in *amtB* leading to the H168D substitution and the primer pair JGBH318EF and JGBH318ER was used to generate the codon change for the H318E substitution. A pair of primers JGLPTRFP and JGLPTRRP flanking *amtB* cloned in the plasmid pHYD1853 was used in conjunction with the aforementioned two pairs of primers for performing overlap extension PCR mutagenesis. Cloning of the PCR product led to the construction of the plasmid pHYD1855. The gene encoding *ycgO* was amplified from the genomic DNA of the strain MC4100 using the primer pair JGKYCGOF and JGKYCGOR and the PCR product was suitably cloned to generate the plasmid pHYD1858. The primer pair JGPTSNF and JGVPTSNR was used to amplify *ptsN* using genomic DNA of MC4100 as template and the PCR product was cloned to construct the plasmid pHYD5006. Codon changes that led to the incorporation of alanine (A), aspartate (D) and glutamate (E) in place of histidine (H) 73 in *ptsN* were introduced by overlap extension PCR using pHYD5006 as the template. The primer pairs JGH73F/ JGH73R, JGVPTSNDF / JGVPTSNDR and JGVPTSNEF / JGVPTSNER were separately used along with a pair of primers JGLPTRFP and JGLPTRRP, flanking *ptsN*

present in the plasmid pHYD5006 in the overlap extension PCR reactions. Appropriate cloning of the PCR products led to the introduction of codon alterations responsible from the H73A, H73D and H73E substitutions in PtsN encoded by the plasmids pHYD5007, pHYD5008 and pHYD5009 respectively. pHYD586 (constructed by R. Harinarayanan) is a derivative of the plasmid pACYC184(2), bearing a deletion extending from the XbaI to the BamHI site, leading to the removal of a substantial portion of *tetA*, imparting therefore a tetracycline sensitive phenotype.

Methods. The gene encoding YcgO was transferred to the *attB* site on the chromosome of the strain MG1655 with its associated P_{trc} promoter from the plasmid pHYD1858, using the procedure of λInCh (3). The resultant strain was designated GJ14938. An MG1655 derivative, GJ14814 bearing the product of an integration via λInCh of the plasmid pTrc99A, was provided by J. Krishna Leela. The aforementioned chromosomal λInCh constructs were introduced into other strains by P1 transduction (4) employing a selection for Amp resistant (Amp^R) transductants on agar plates containing 25 μg/ml of Amp. For a strain that possessed all K^+ uptake systems or one that lacked the Kdp transporter Amp^R transductants were isolated on LB agar plates whereas for a triple transporter defective strain or for its Kdp^+ derivative Amp^R transductants were isolated on KML agar plates (5).

The *bla* gene (encoding the Amp^R determinant) of GJ14938, was replaced with the gene encoding the Kan^R determinant from the plasmid pKD4 by recombineering (6). The primer pair JGHBLAP1 and JGHBLAP2, bearing terminal homologies to 5' and the 3' portions of *bla*, was used to amplify by PCR the Kan^R determinant of the plasmid pKD4 followed by electroporation of the PCR product into GJ14938. GJ14938 contained the plasmid pHYD2384 (constructed by J. Krishna Leela) which is a derivative of the RED recombinase expressing plasmid pKD46 (6)

bearing a replacement of its *bla* gene with the gene encoding the Cm^R determinant from the plasmid pKD3. GJ14938 bearing pHYD2384 was cultivated as described (6). Kan^R recombinants were obtained at 42°C and those that were Amp sensitive (Amp^S), indicative of replacement of the *bla* gene with the Kan^R determinant, were sought by screening. One recombinant displaying an Amp^S Kan^R phenotype, GJ14946 was chosen for further study.

An MG1655 derivative GJ14932 expressing a chromosomally encoded C-terminal 3xFLAG epitope tagged version of YcgO ($YcgO_{FL}$) was constructed by recombineering with the primer pair JGVYCGOF1 and JGVYCGOF2. A PCR product using the plasmid pSUB11 (7) as the template obtained with the primer pair JGVYCGOF1 and JGVYCGOF2 was electroporated into MG1655 bearing the plasmid pKD46 that was cultivated according to reference 6. A Kan^R recombinant GJ14932 obtained was chosen and the authenticity of the attachment of the DNA sequence encoding 3xFLAG epitope to the 3' end of *ycgO* was confirmed with DNA sequencing and the entire *ycgO* gene was also sequenced. The gene encoding the 3xFLAG tagged YcgO ($ycgO_{FL}$) was introduced into other strains by P1 transduction with a selection for the adjacent Kan^R determinant. Whenever required the Kan^R determinant was excised following transformation of the appropriate strain with the plasmid pCP20 (6).

E. coli K-12 derivatives bearing $ilvG^+$ were constructed in a two step procedure. Firstly, a P1 lysate prepared on BL21DE3 (8) which is $r_B^- m_B^-$ was transduced into JD247 which is a leu^+*araD139ilv-500*::Tn*10* derivative of the $r_K^- m_K^+$ strain FP011, obtained from M. Dreyfus, with a selection for isoleucine valine (Ilv^+) prototrophy. A valine resistant, Ilv^+ prototroph JD260, bearing the coinherited $ilvG^+$, was used as a donor in subsequent transductions to introduce $ilvG^+$ into appropriate K-12 strains using the procedure involving selection for Ilv^+ prototrophy.

For immunoblotting experiments, mid-exponential phase cultures of appropriate strains normalized to A_{600} 1 were suspended in SDS loading buffer at an A_{600} of 0.002 per µl and 20 µl of boiled samples were loaded on a 12 % SDS PAGE gel. Separated proteins were transferred to a PVDF membrane using the wet transfer method. Following blocking of the PVDF membrane in Tris-buffered saline containing 5% skimmed milk and 0.1% tween-20 (buffer A) for 30 minutes, the membrane was probed with anti-FLAG M2 monoclonal antibody (Sigma), at 4°C at a dilution of 1:10,000 in buffer A, then washed three times in buffer A. The PVDF membrane was then probed with anti-mouse horseradish peroxidase-conjugated antibody for 2 hours at a dilution of 1:10,000 in buffer A at room temperature and washed thrice with buffer A. Western blot was developed with the ECL solution Kit and visualized in Protein Simple Chemidoc.

Table S1 Oligonucleotide primers used in this study (restriction sites in the primers, for cloning are underlined).

Primer Designation	**Sequence of Primer (5'to 3')**
JGVTRKDF	TGTGGGCCAGAATTCTAAGCACACATTTCATATTTC
JGVTRKDR	GGCGTCTGGCGAAGCTTTTAGATTTCGACCTGAGTACCCA
JGVAMTBF	CCGACGAAGCCATGGTGTAATCTCTGGCACACAGCA
JGVAMTBR	TTTTTTGCAGAAGCTTTTACGCGTTATAGGCATTC
JGVH168DF	TTCGCGGGTGGCACCGTGGTGGACATTAACGCCGCAATCGCC

Table S1contd…

JGVH168DR	GGCGATTGCGGCGTTAATGTCCACCACGGTGCCACCCGCGAA
JGBH318EF	TGCGATGTCTTCGGTGTGGAGGGCGTTTGTGGCATTGTC
JGBH318ER	GACAATGCCACAAACGCCCTCCACACCGAAGACATCGCA
JGLPTRFP	CGACATCATAACGGTTCTGG
JGLPTRRP	TGGGACCACCGCGCTA
JGKYCGOF	TAACCGACCGCCATGGATGCCACAACAATAATTAG
JGKYCGOR	AGATGCTTCAAAGCTTTTAAGATTCAGCTTCCTC
JGPTSNF	AGGTGAACATATGACAAATAATGATACAAC
JGVPTSNR	TAACCCAAGCTTCTAATGGTGATGATGGTGGTGCGCTTCATCCGGA GTACCTTCGGTA
JGH73F	ATTGCCATTCCGGCTGGCAAACTCGAGGAAGATACTCTG
JGH73R	CAGAGTATCTTCCTCGAGTTTGCCAGCCGGAATGGCAAT
JGVPTSNDF	GCAATGGTATTGCCATTCCGGATGGCAAACTGGAAGAAGATAC
JGVPTSNDR	GTATCTTCTTCCAGTTTGCCATCCGGAATGGCAATACCATTGC

Table S1contd…

JGVPTSNEF	GCAATGGTATTGCCATTCCGGAGGGCAAACTGGAAGAAGATAC
JGVPTSNER	GTATCTTCTTCCAGTTTGCCCTCCGGAATGGCAATACCATTGC
JGHBLAP1	TTCCGTGTCGCCCTTATTCCCTTTTTTGCGGCATTTTGCCTTCCTGTT GTGTAGGCTGGAGCTGCTTC
JGHBLAP2	CAGTGAGGCACCTATCTCAGCGATCTGTCTATTTCGTTCATCCATA GTTCATATGAATATCCTCCTTAG
JGVYCGOF1	GTGTTGAAGATTGGCGTACGGGTGGCTGAAGAGGAAGCTGAATCT GACTACAAAGACCATGACGG
JGVYCGOF2	TTCAGCAAAGCATCGCAACCGGCGATAGATGCTTCATTAAATTTAC ATATGAATATCCTCCTTAG

Table S2 Strains and plasmids

Strain	**Genotype**
JD247	MC1061 (DE3) *malP*$_p$Δ*534*::PT7 *lacZ* RBS *lamB708 lacY' ter*T7 *leu*$^+$ *araD139 ilv-500*::Tn*10*
JD260	JD247 *ilvG*$^+$
JD271	MC4100 *ilvG*$^+$
JD274	JD271 Δ*ptsN*::Kan
JD624	MC4100 *kup trkA*
JD660	JD624 Δ*ptsN*::Kan

Table S2 contd…

JD709	JD624 Δ*amtB*
JD732	JD624 *attλ*::(P_{trc}*ycgO*, *bla*::Kan)
JD767	JD709 Δ*ptsN*::Kan
GJ14814	MG1655 *attλ*::(P_{trc}99A, *bla*)
GJ14932	MG1655 $ycgO_{FL}$::Kan

Plasmids	**Description**
pHYD586	pACYC184 bearing a deletion extending from the XbaI to the BamHI site, imparting a tetracycline sensitive phenotype.
pHYD1853	pTrc99A containing *amtB* expressed from the P_{trc} promoter, present within the NcoI and HindIII sites.
pHYD1855	Derivative of pHYD1853, in which $amtB^K$ encoding AmtB bearing the H168D and H318E amino acid substitutions is present within the NcoI and HindIII sites.
pHYD2384	A derivative of plasmid pKD46 bearing a replacement of its *bla* gene with the gene encoding the Cm^R determinant from the plasmid pKD3.

All strains listed are *E. coli* K-12 strains and MC4100 was from our laboratory collection. Deletion insertion mutations from the Keio collection (9) were introduced into appropriate strains by P1 transduction. The Δ*ptsN*::Kan deletion insertion mutation was sourced from the strain JW3171 and JW0441 (9) served as the donor of the Δ*amtB*::Kan mutation. The antibiotic cassette marking the Δ*amtB*::Kan deletion insertion was removed as described (6). The plasmids pACYC184 and pTrc99A are described in references (2) and (10) respectively.

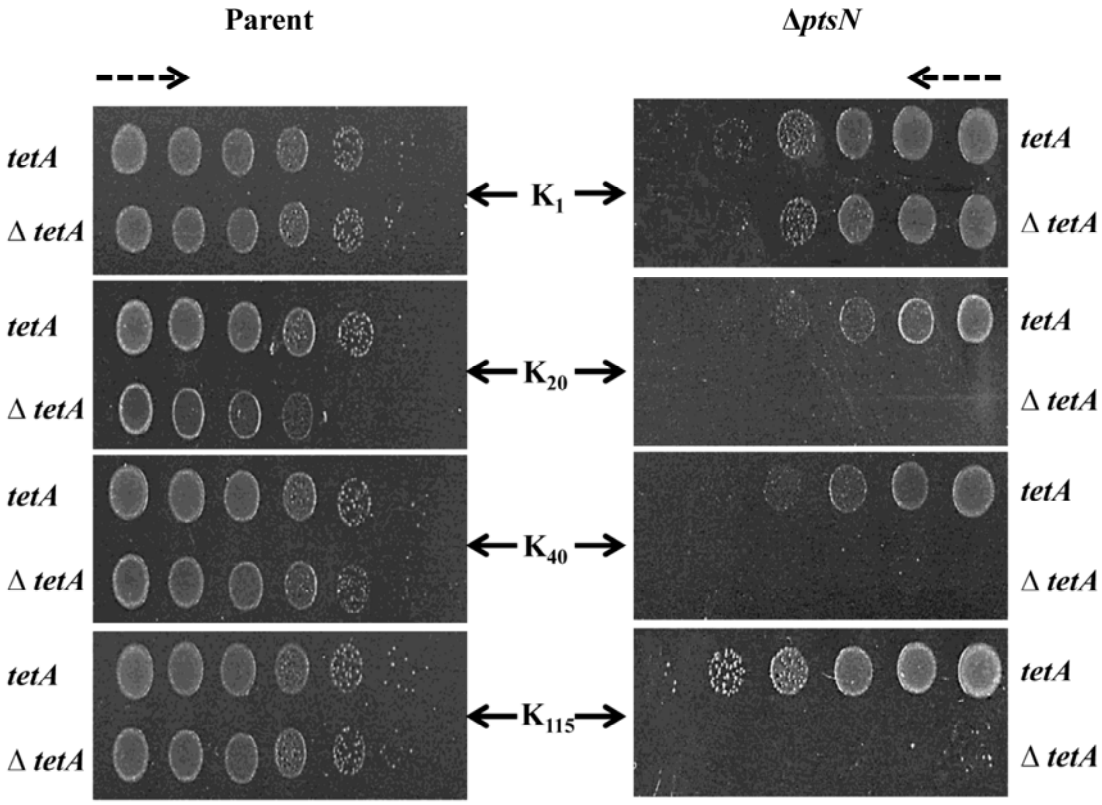

Figure S1 TetA mediated suppression of the K^S of the Δ*ptsN* mutant. 10-fold serial dilutions of cultures of the parent (JD624, *kdp*$^+$ *trkA kup*) and its Δ*ptsN*::Kan derivative JD660 (Δ*ptsN*), bearing the plasmids pACYC184 (*tetA*) and pHYD586 (Δ*tetA*), were spotted on the surface of K_1, K_{20}, K_{40} and K_{115} glucose agar plates. The interrupted arrow indicates the direction of increasing order of cell dilution.

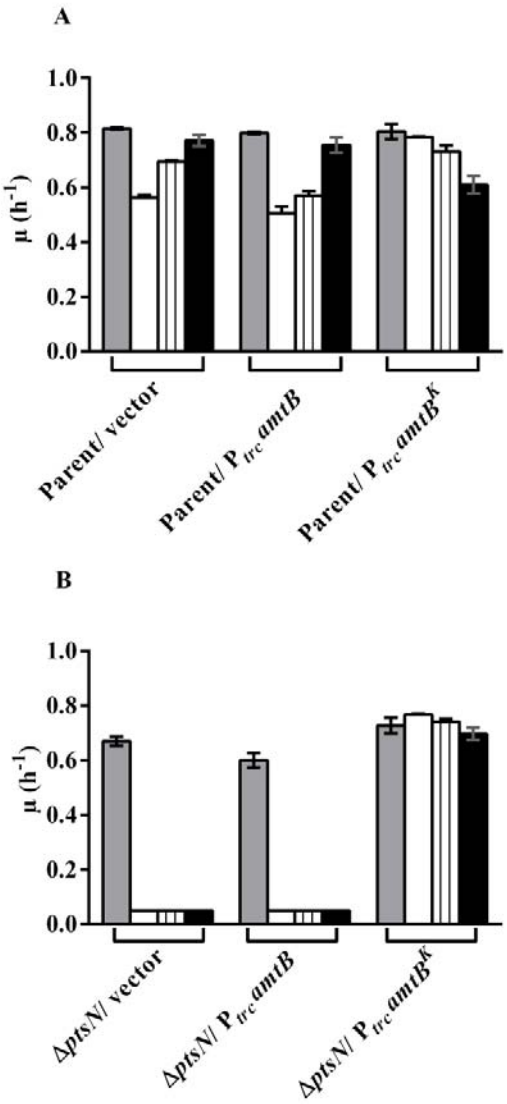

Figure S2 Suppression of the K^S of the Δ*ptsN* mutant by expression of $amtB^K$. (A) Growth rates of the parent (JD709, kdp^+ *trkA kup* Δ*amtB*) and (B) its Δ*ptsN*::Kan derivative JD767 (Δ*ptsN*) bearing the plasmids pTrc99A (vector), pHYD1853 ($P_{trc}amtB$) and pHYD1855 ($P_{trc}amtB^K$) in media of varying $[K^+]_e$. The indicated strains were grown in K_1 (gray bars), K_{20} (white bars), K_{40} (striped bars) and K_{115} (black bars) media and the growth rates were determined. The growth medium also contained 5 μM IPTG and 5 mM L-glutamine instead of ammonium sulfate as the nitrogen source.

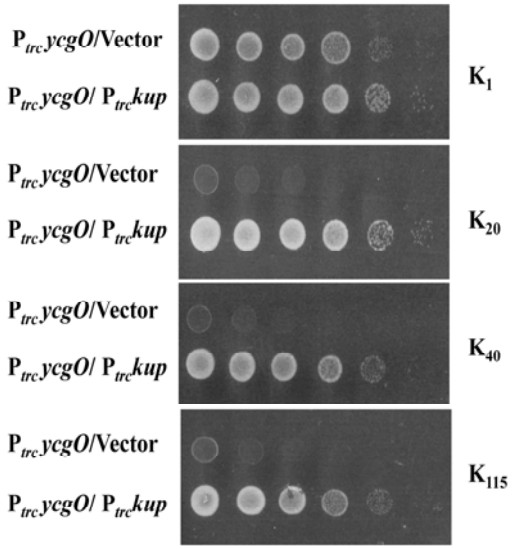

Figure S3 Alleviation of the K^S of *ycgO* overexpression by co-overexpression of *kup*. Ten-fold serial dilutions of cultures of JD732 (P_{trc}*ycgO*) bearing the plasmid pHYD3025 (vector) and pHYD1852 (P_{trc}*kup*) were spotted on the surface of K_1, K_{20}, K_{40} and K_{115} glucose agar plates containing 0.1 mM IPTG.

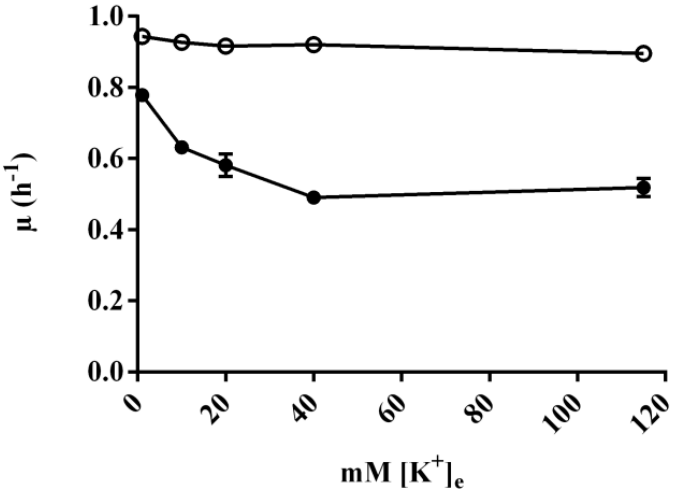

Figure S4 Persistence of the K^S of the Δ*ptsN* mutation in a strain bearing a functional IlvGM acetohydroxyacid synthase. Growth rates of an *ilvG*$^+$ derivative of MC4100, JD271 (open circles) and its Δ*ptsN*::Kan derivative JD274 (closed circles) with respect to $[K^+]_e$. Growth rates of the indicated strains were determined following their growth in K_1, K_{20}, K_{40} and K_{115} media.

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