Deletion of one of two *Escherichia coli* genes encoding putative Na⁺/H⁺ exchangers (*ycgO*) perturbs cytoplasmic alkali cation balance at low osmolarity

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Two genes in the *Escherichia coli* genome, b4065 (*yjcE*) and b1191 (*ycgO*), are similar to genes encoding eukaryotic Na⁺/H⁺ exchangers. Mutants were constructed in which *yjcE* (GRN11), *ycgO* (GRF55) or both (GRD22) were inactivated. There was no change in respiration-driven Na⁺ efflux in any of the mutants when grown in media containing 50–500 mM Na⁺. The only striking finding was that growth of GRF55 was impaired at low osmolarity. In complex low-salt medium, GRF55 grew at a wild-type rate for three to four generations but then stopped; the growth was partially recovered after a pause, the length of which was dependent on salt concentration. Measurement of cytoplasmic alkali cations showed that an abrupt loss of about one-half of the intracellular K⁺ preceded the pause. When grown in low-salt medium with only 20 mM added Na⁺, GRF55 also lost the ability to maintain a sodium concentration gradient. However, this phenomenon appears to be a secondary effect of the *ycgO* deletion. The double mutant GRD22 has the same properties as GRF55; no additional effect was found. The data indicate that neither *ycgO* nor *yjcE* participates in respiration-driven Na⁺ extrusion. Instead, *ycgO* is required for growth at low osmolarity. Hence it is concluded that *ycgO* participates in cell volume regulation, and accordingly it is suggested that *ycgO* be renamed *cvrA*.

Keywords: NHE-like proteins, *Escherichia coli*, osmosensitivity

INTRODUCTION

Na⁺/H⁺ exchangers (NHEs) are found in a wide variety of eukaryotic cell types and have been studied intensively. These proteins, which catalyse coupled Na⁺ and H⁺ ion fluxes, are localized in the plasma membrane, endomembranes and the mitochondrial inner membrane. NHEs have been suggested to eliminate excess acid from actively metabolizing cells, and are also important for the regulation of cell volume and reabsorption of NaCl across epithelia (Wakabayashi *et al.*, 1997; Orłowski & Grinstein, 1997). They are found in virtually all mammalian (Wakabayashi *et al.*, 1997) and in some crustacean tissues (Towle *et al.*, 1997). We have previously made note of two genes, b4065 (*yjcE*) and b1191 (*ycgO*), in the *Escherichia coli* genome with similar sequences to the eukaryotic NHE genes (Verkhovskaya *et al.*, 1998). Neither of these genes is

related to the *E. coli* *nhaA* and *nhaB* genes that have been suggested to catalyse electrogenic Na⁺/nH⁺ exchange (see Padan & Schuldiner, 1996, for a review). We have shown that mutant cells in which *nhaA* and *nhaB* have been inactivated are still capable of pumping Na⁺ in a respiration-dependent manner (Verkhovskaya *et al.*, 1998), suggesting that other genes must be responsible for Na⁺ transport under these conditions. A putative NHE-like protein could be a good candidate for this function. The work presented here is a first attempt to discover the role of *yjcE* and *ycgO* in bacterial physiology. The data obtained allow us to conclude that neither gene product is involved in energy-dependent Na⁺ efflux. However, the *ycgO* gene product apparently participates in control of cell volume when *E. coli* cells grow in low-osmolarity conditions.

METHODS

Bacterial strains and plasmids. The *E. coli* strains and plasmids used in this study are listed in Table 1. The de-

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Abbreviation: EIPA, *N*-ethyl-*N*-isopropylamiloride.

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Description	Source or reference
Strains		
<i>E. coli</i>		
GR70N	F ⁻ <i>thi rpsL gal</i> Sm ^R	R. Gennis (University of Illinois, Urbana, USA)
SM10(λ pir)	<i>thi-1 thr leu tonA lacY supE recA::RP3-2-Tc::Mu Km^R λpir</i>	Miller & Mekalanos (1988)
TOP10	F ⁻ <i>mcrA</i> Δ (<i>mrr-hsdRMS-mcrBC</i>) ϕ 80 <i>lacZ</i> Δ M15 Δ <i>lacX74 recA-1 deoR araD139</i> Δ (<i>ara-leu</i>)7697 <i>galU galK rpsL</i> (Sm ^R) <i>endA-1 nupG</i>	Invitrogen
GRN11	GR70N <i>yjcE::Cm^R</i>	This study
GRF55	GR70N Δ <i>ycgO</i> , Km ^R	This study
GRD11	GR70N Δ <i>ycgO</i> , Km ^R , <i>yjcE::Cm^R</i>	This study
GRF72	GR70N Δ <i>ycgO</i> , Km ^R , pBAD- <i>ycgO</i> His-tagged N-term	This study
Plasmids		
Litmus-28	Cloning vector; Ap ^R	New England Biolabs
pBluescript SK(–)	Cloning vector; Ap ^R	Stratagene
pGP704	Knockout suicide vector; Ap ^R ; <i>ori</i> R6K, <i>mob</i> RP4, MCS of M13tg131	Miller & Mekalanos (1988)
TOPO-TA	One step cloning vector; Ap ^R	Invitrogen
pBAD/His	One step cloning vector; Ap ^R , expression is driven by <i>araBAD</i> promoter, N-terminal polyhistidine region	Invitrogen
pBAD- <i>ycgO</i>	pBAD/His containing His-tagged N-term <i>ycgO</i> under <i>araBAD</i> promoter	This study
pKO3*	Deletion plasmid; Psc101-ts <i>sacB</i> Cm ^R	Link <i>et al.</i> (1997)

* <http://arep.med.harvard.edu/labgc/pko3.html>

letion plasmid pKO3 (<http://arep.med.harvard.edu/labgc/pko3.html>) (Link *et al.*, 1997), which carries a temperature-sensitive origin of replication (Psc101-ts) and *sacB* (levansucrase gene; toxic for *E. coli* in the presence of sucrose), was generously provided by Dr G. M. Church (Harvard Medical School, Boston, USA).

Media and growth conditions. All strains were grown in LB medium supplemented with ampicillin (50 μ g ml⁻¹), kanamycin (50 μ g ml⁻¹) or chloramphenicol (20 μ g ml⁻¹), as needed. For selection against the levansucrase gene (*sacB*), the medium was supplemented with 5% (w/v) sucrose. In order to test mutants for sensitivity to low-salt content, two different media were used: TY (7.5 g tryptone l⁻¹ and 3.5 g yeast extract l⁻¹ and approx. 5 mM K⁺ and 9 mM Na⁺ as contaminants) and LSG medium (low salt glycerol medium: 40 mM MOPS/1,3-bis[tris(hydroxymethyl)methylamino] propane, pH 7.0; 2 mM NH₄Cl; 1 mM KH₂PO₄; 1 mM MgSO₄; 10 μ g thiamin ml⁻¹; 0.01%, w/v, yeast extract; 0.5%, v/v, glycerol; and 30–50 μ M Na⁺ as a contaminant).

General DNA techniques. Genomic DNA and plasmid DNA were prepared using Wizard genomic and plasmid DNA purification systems (Promega). DNA fragments were prepared for cloning using the QIAEX II gel extraction system (Qiagen) and DNA sequencing was performed using an ALF express DNA sequencer (Pharmacia). *Taq* polymerase (Promega) was used for the PCR reactions.

Interruption of *yjcE* (GRN11 strain). The *yjcE* gene was obtained by PCR using the following primers: GAAGATC-TTATGGAAATCTTCTTC (forward) and CCCAAGCTT-TTACTGATTTTCCTC (reverse). The 1.6 kb PCR product was cloned into Litmus-28 as a *Bgl*II–*Hind*III fragment and sequenced in its entirety. A cassette containing a gene encoding chloramphenicol acetyltransferase (*cat*) was inserted in a

unique *Pst*I site located approximately in the middle of the *yjcE* gene. This interrupted *yjcE* gene was then cloned as a *Bgl*II–*Kpn*I fragment (approx. 3 kb) into the pGP704 suicide vector by transformation into SM10 λ *pir* competent cells (Miller & Mekalanos, 1988). Transformed SM10 λ *pir* cells were used for conjugation as a donor, and GR70N as a recipient. The ex-conjugants were selected for double-crossover events on the basis of chloramphenicol resistance and ampicillin sensitivity, after which the desired inactivation genotype was verified by PCR and designated GRN11.

Deletion and cloning of the *ycgO* gene. We first attempted to inactivate this gene by an approach similar to that used above. When this met with a great deal of difficulty, it appeared that *ycgO* might be an essential gene. For this reason, we set up a two-plasmid approach involving knockout and rescue plasmids (Link *et al.*, 1997). The knockout plasmid (pKO3) included the *ycgO* deletion construct (see below) under control of a temperature-sensitive origin of replication (Psc101-ts). The rescue plasmid (pBAD-*ycgO*) carried a viable copy of *ycgO* under control of an arabinose promoter as described by Brown *et al.* (1995).

The rescue plasmid was constructed as follows: the *ycgO* gene was obtained by PCR using the following primers: ATGCT-GGCAGGAGTCGATGGCG (forward) and AGATTCAGC-TTCCTCTTCAGC (reverse). The 1.6 kb product was cloned into pBAD-TOPO by transformation into TOP-10 cells. The sequence and orientation of the product in the vector were verified by restriction analysis and DNA sequencing.

The knockout plasmid was constructed as follows. Two 500 bp fragments that flank the ends of *ycgO* were obtained by PCR and cloned into a TA-TOPO vector. The 5' fragment (L) contained a *Sma*I site at the 5' end and a *Pst*I site at the 3' end. The 3' fragment (R) contained *Pst*I and *Sal*I sites. These

fragments were cloned, first L and then R, into pBluescript SK(–) using the restriction sites described above. A kanamycin-resistance cassette was then introduced into the *Pst*I site to give a construct containing the kanamycin-resistance gene and the *ycgO* flanking sequences (L-*kan*-R). A 2.2 kb *Not*I–*Sal*I fragment containing this L-*kan*-R sequence was then cloned into the pKO3 vector.

The actual transformation of GR70N cells with the L-*kan*-R-pKO3 construct in order to delete the *ycgO* gene was done both with and without the rescue plasmid (pBAD-*ycgO*). When the rescue plasmid was used, colonies were selected on LB agar containing kanamycin, chloramphenicol, ampicillin and 0.2% (w/v) arabinose at 42 °C and then grown at 30 °C. The shift in temperature turned off Psc101-ts and thus selected for the integration of the plasmid into the genome (frequency of integration approx. 2×10^{-5}). Return to permissive temperature selected for excisants; in the presence of sucrose and kanamycin, mutant allele in the chromosome was selected using a positive selection (sucrose and kanamycin resistance). The strain produced was designated GRF72. In the case where no rescue plasmid was used, cells were plated on LB agar containing kanamycin and chloramphenicol at 42 °C, and the resulting strain was designated GR55. In both cases, the replacement of the genomic *ycgO* by the kanamycin-resistance gene was confirmed by PCR. The same number of colonies bearing the *ycgO* deletion were obtained with or without the rescue plasmid, which indicates that the *ycgO* gene is not essential.

Expression of *ycgO* in the rescue plasmid. The pBAD expression vector includes a sequence encoding a 6 × His tag at its N-terminus. To test for arabinose-induced expression of the recombinant *ycgO* gene product, the cell membrane fraction was subjected to SDS-PAGE and Western blotting using antibodies that recognize histidine tags (tetra-His antibody; Qiagen).

GRD22 double mutant. A mutant strain that features both interruption of the *yjcE* gene (from the GRN11 strain) and deletion of the *ycgO* gene (from the GRF55 strain) was generated by P1 transduction. Preparation of lysates and transduction were carried out according to Silhavy *et al.* (1982). Transductants were grown on LB agar with chloramphenicol and kanamycin. The acquisition of the silenced gene from the lysate was checked by PCR using primers located 50 bp above and below the coding region.

Measurements of Na⁺ and K⁺ content in cells. Sodium and potassium content of cells and membrane vesicles was determined by flame photometry as described by Verkhovskaya *et al.* (1996). Na⁺-loaded cells were produced by diethanolamine treatment as described by Nakamura *et al.* (1982). All transport experiments were reproduced three to four times. The individual curves are presented.

RESULTS

Na⁺ transport

To check whether the *yjcE* and *ycgO* gene products are involved in respiration-driven Na⁺ efflux, the intracellular content of Na⁺ in growing cells, and Na⁺ transport out of Na⁺-loaded cells, were measured in the GRN11 (*yjcE*[–]), GRF55 (*ycgO*[–]) and GRD22 (*yjcE*[–] *ycgO*[–]) mutant strains. When grown on rich medium, TY, or defined succinate media (Verkhovskaya *et al.*,

1996) containing 100–500 mM Na⁺, all of the mutant strains maintained Na⁺ gradients as high as in wild-type cells. Sodium extrusion from Na⁺-loaded cells or membrane vesicles from these strains, grown under the same conditions, did not differ significantly from that in the parent, GR70N, strain (not shown). Nevertheless, there was a possibility that Na⁺ transport was carried out by the *yjcE* and *ycgO* gene products in wild-type cells, but that other systems took on this function when *yjcE* and *ycgO* were inactivated. Data obtained with the use of EIPA, an amiloride analogue which inhibits eukaryotic Na⁺/H⁺ exchangers (Chambrey *et al.*, 1997), did not support this suggestion.

We found that EIPA is a specific inhibitor of Na⁺ transport in *E. coli*. As shown in Fig. 1(a), Na⁺ efflux from wild-type *E. coli* was blocked by 100 µM EIPA. This inhibition takes place without affecting the rate of respiration or ΔΨ across the cell membrane (not shown). Since the enzyme generally believed to be the main Na⁺ transport system in *E. coli*, NhaA, is not sensitive to EIPA (Padan & Schuldiner, 1996), these findings could be an indication that NHE-like proteins are involved in Na⁺ transport in *E. coli*. However, Na⁺ transport in the GRD22 double mutant showed the same sensitivity to EIPA as wild-type cells (Fig. 1b). This shows that there must be a Na⁺ transporter in *E. coli*, which (1) is different from NhaA, since it is EIPA-sensitive, and (2) is not an NHE-like protein, since the activity was observed even after the genes for the only two such proteins in *E. coli* (*yjcE* and *ycgO*) were inactivated.

Impairment of growth

The growth of the GRN11, GRF55 and GRD22 mutant strains was independent of the Na⁺ concentration in the medium. These strains were capable of growing as fast as the wild-type in rich (TY) or defined (LSG) media containing as little as 30–50 µM Na⁺ (the background contamination level) or as much as 500 mM NaCl. In the absence of added NaCl, the media were supplemented with 0.4 M mannitol to keep osmolarity close to that in standard LB medium. At high pH (8.8), no inhibition of growth was found relative to the parent strain GR70N. At low pH (4.5), the growth of the double mutant strain, GRD22, was approximately 25% slower than that of the other strains.

However, GRF55 was sensitive to low osmolarity. (GRN11 may also be sensitive but to a much smaller extent.) When GRF55 was inoculated into rich medium without added salt (TY), or into a synthetic low-salt glycerol medium (LSG), growth was observed for 2–3 h, but it then stopped completely, and no further growth was observed in the next 8 h (Fig. 2a, b). When LSG medium was initially supplemented with 50 mM KCl, 50 mM LiCl, 30 mM MgSO₄ or 200 mM mannitol (but not sucrose), GRF55 grew at the wild-type rate (Fig. 2c). Similar data were obtained with TY medium. However, when grown in TY supplemented with only 20 mM KCl

Fig. 1. Inhibition by EIPA of alkali cation transport in wild-type *E. coli* (a) and the double mutant (GRD22) deficient in two NHE-like proteins (b). The cells were grown in LB medium, loaded with Na⁺ and suspended in a medium containing 150 mM NaCl, 50 mM HEPES/NaOH, pH 7.5. Alkali cation transport was initiated by the addition of 10 mM KCl; 100 μ M EIPA was added at time zero. ■, Intracellular Na⁺; ●, intracellular K⁺. Left-hand panels, without EIPA; right-hand panels, 100 μ M EIPA added.

or NaCl, growth stopped after three to four generations. Growth resumed after a delay, but only at a lower rate. Both the length of this pause and the growth rate thereafter were strictly dependent on salt concentration (shown for NaCl in Fig. 2d). The double mutant (*ycgO*⁻*yjcE*⁻) showed the same phenotype as the *ycgO*⁻ mutant; no additional effects of the *yjcE* interruption on growth were found.

To confirm that this phenotype was due to the lack of the *ycgO* gene, the 'rescue strain' (GRF72) was studied. This strain consists of GRF55 with the addition of a plasmid containing a viable copy of *ycgO* under the control of the arabinose promoter and labelled with a 6 × His tag (Methods). Arabinose-induced expression of the *ycgO* gene was confirmed by Western blotting with anti-His-tag antibodies, which revealed a band corresponding to a molecular mass of approximately 60 kDa in agreement with the prediction from the gene sequence. Unlike GRF55, the 'rescue strain' was able to grow on TY medium supplemented with 0.002% (w/v) arabinose; there was no longer a pause in growth, but the rate of growth was approximately half that of the wild-type (not shown), perhaps indicating that an excess of the *ycgO* gene product is toxic.

Intracellular alkali cation levels and cation transport activity in GRF55 grown at low osmolarity

To analyse the capability of the GRF55 strain for alkali cation transport, a relatively low osmolarity (20 mM NaCl added to rich medium) was chosen for cell growth. The cells were collected when growth had recovered after the delay (see Fig. 2d), loaded with Na⁺ and suspended in medium containing NaCl and buffer. Cation transport was initiated by the addition of 10 mM KCl. Under these conditions, transport of both K⁺ and Na⁺ was significantly lower in GRF55 than in the parent, GR70N, strain (Fig. 3a). To exclude the possibility that the altered alkali cation transport in GRF55 was due to the mutant being more susceptible to cell damage during the Na⁺ loading procedure, 100 mM NaCl was added directly to the growth medium, and the intracellular alkali cation levels were followed. Fig. 3(b) shows that exposing GRF55 cells to a high Na⁺ concentration caused a significant rise in intracellular Na⁺ content, in contrast to the case with the parent strain. Potassium accumulation due to osmotic up-shock was not altered. The observed increase in the intracellular Na⁺ level in GRF55 could be due to either lower Na⁺ efflux or higher Na⁺ influx in the mutant

Fig. 2. Growth of the single deletion mutant GRF55 (*ycgO*⁻) is impaired at low osmolarity. (a, b) Growth of wild-type, GR70N (●), and GRF55 (■) strains in TY medium in the presence of 100 mM NaCl (a) or without added salt (b). (c) Growth of GRF55 in LSG medium in the absence of additions (□) or in the presence of 200 mM sucrose (●), 200 mM mannitol (■), 50 mM LiCl (▼), 30 mM MgSO₄ (▲) and 50 mM KCl (◆). (d) Growth of GRF55 in TY medium with different amounts of added NaCl: none (○), 10 mM (▲), 20 mM (■), 40 mM (●). The TY medium itself contained 9 mM Na⁺.

cells. To resolve this question, the intracellular Na⁺ content was measured in mutant cells in the absence of K⁺, under conditions where active Na⁺ efflux was impossible due to lack of a counterion. Fig. 3(c) shows the change in intracellular alkali cation content in GRF55 and wild-type (GR70N) cells grown at low osmolarity and transferred to a medium containing 100 mM NaCl and 34 mM HEPES/NaOH, pH 7.5. In GRF55, the Na⁺ content increased rapidly, and became as high as K⁺, whereas in the parent strain the change was insignificant. This confirms that GRF55 has a higher Na⁺ permeability.

Although it is clear that the deletion of *ycgO* results in altered Na⁺ permeability under certain circumstances, there is no indication of direct involvement of the *ycgO*

gene product in Na⁺ movement across the cell membrane. In fact, GRF55 grown in TY in the presence of 20 mM KCl, instead of NaCl, did not show altered Na⁺ influx, but nevertheless retained the osmosensitive phenotype. This suggests that the *ycgO* product does not participate in Na⁺ transport directly, and that the observed imbalance of intracellular Na⁺ may rather be a result of events that took place in the cells during the growth delay. We therefore took a closer look at this phase.

Lack of *ycgO* results in loss of intracellular K⁺ upon growth at low osmolarity

Cell protein and intracellular alkali cation content were measured during growth of GRF55 and wild-type

Fig. 3. Alkali cation transport is impaired in GRF55 (*ycgO*⁻) cells grown at low osmolarity. Cells of wild-type (GR70N) and mutant (GRF55) strains of *E. coli* were grown in TY medium with 20 mM added NaCl. Levels of intracellular K⁺ (●) and Na⁺ (■) were measured under the following conditions. (a) Cells were loaded with Na⁺ and suspended in 150 mM NaCl, 50 mM HEPES/NaOH, pH 7.5, with 4 mM DTT and 50 μ M ubiquinone 1; alkali cation transport was initiated by the addition of 10 mM KCl. (b) NaCl (200 mM) was added to growing cultures at the time indicated as zero. (c) Cells were removed from growing cultures and transferred to a medium containing 100 mM NaCl and 34 mM HEPES/NaOH, pH 7.5.

(GR70N) cells in TY + 20 mM NaCl (Fig. 4). During the first 2.5–3.5 h, when growth of the two strains was identical, Na⁺ and K⁺ levels in the GRF55 cells paralleled those in GR70N. However, at some point the

K⁺ content in GRF55 dropped and simultaneously Na⁺ began to accumulate until the content of the two cations was equal (Fig. 4). Then growth of GRF55 halted abruptly, while GR70N continued to grow. No Na⁺

Fig. 4. GRF55 (*ycgO*⁻) cells are unable to maintain normal levels of intracellular K⁺ during growth at low osmolarity. Wild-type (GR70N) and mutant (GRF55) strains were grown in TY medium with 20 mM added NaCl. Cell protein (▲), intracellular K⁺ (●) and intracellular Na⁺ (■) were followed during growth.

could be detected in GR70N cells during growth, while the intracellular K⁺ content initially increased and then gradually decreased. After some hours, cell growth in GRF55 recovered partially; K⁺ gradually accumulated and Na⁺ was expelled, but the ion gradients did not return to wild-type levels (Fig. 4).

DISCUSSION

Two *E. coli* genes, *yjcE* and *ycgO*, which are similar to the eukaryotic genes for NHEs and belong to the CPA1 family of cation-proton antiporters, according to Saier's classification of transport proteins (Saier *et al.*, 1999), are not unique in the bacterial world. Members of this family are widely spread among bacteria, and alignment of amino acid sequences of a number of these putative proteins (MULTALIN; Corpet, 1988) reveals an evident

pattern of conservation, all the residues of which are also conserved in the eukaryotic NHEs (Fig. 5) (Towle *et al.*, 1997; Nass *et al.*, 1997). NHE-like proteins in bacteria might play an important role in Na⁺ transport and pH regulation of the cytoplasm, as they do in eukaryotic cells (for a review, see Wakabayashi *et al.*, 1997; Orłowski & Grinstein, 1997). However, the data on *E. coli* presented in this paper indicate that the *yjcE* gene product participates in regulation of cation transport under some circumstances rather than operating as a transport system itself.

The *ycgO* gene product was found to be important for growth of *E. coli* at low osmolarity. Under such conditions, the *ycgO* deletion caused growth to halt completely, shortly after a partial loss of intracellular K⁺ and accumulation of Na⁺ (Fig. 4). The latter cannot be the reason for the growth delay. Rather, it has a mitigating effect on the deletion phenotype: without the addition of 20 mM NaCl to TY there was no Na⁺ accumulation in GRF55, but the growth delay was much longer (Fig. 2d). Likewise, no Na⁺ accumulation by GRF55 cells was observed during growth in TY + 20 mM KCl, but there was also loss of K⁺ and then a pause in growth. Thus it could be that it is the loss of K⁺ that causes growth to halt. Since K⁺ is the main cytoplasmic osmolyte at low medium osmolarity (Record *et al.*, 1998a, b), the observed loss of K⁺ should also cause a significant decrease of the cytoplasmic free water volume, which is normally maintained at a constant level in *E. coli* (Record *et al.*, 1988a, b). Indeed, the fraction of K⁺ retained by a Donnan effect was determined to be 56% at 0.17 osM and 38% at 1.25 osM in *E. coli* (McLaggan *et al.*, 1994); this means that the cytoplasmic free water volume depends on the 44–62% fraction of K⁺ that is osmotically regulated. In GRF55 cells, approximately 50% of the internal K⁺ was lost. This should result in the loss of a great deal of the cytoplasmic free water, causing a significant increase in macromolecular crowding and leading to a crucial drop in the diffusion coefficient for cytoplasmic components. The loss of K⁺ could also result in decrease of turgor pressure, which is believed to be an obligatory requirement for growth of the cell wall (Norris & Manners, 1993; Koch, 1990, 2000). It is worth noting that when the growth of GRF55 recovered after the delay, the level of intracellular K⁺ was also partially restored.

The loss of K was not due to non-specific inhibition of the K⁺ uptake systems, or the loss of the cell membrane integrity. During the growth delay, there was no visible indication of cell lysis or cell damage since the GRF55 cells retained the capability for K⁺ accumulation in response to osmotic up-shock (not shown). It seems reasonable that the *ycgO* gene product participates in a system that determines the amount of intracellular K⁺ that is required to keep the necessary free water volume normal at low osmolarity.

One may speculate that there are two systems for regulation of intracellular K⁺ in *E. coli* operating at low

<i>E. coli</i> 2	70	GWLF....N	LDLIE	GLLIG	AIVGSTDA	VFFLLGGKGL	NERVG	STLEI	ESGSNDPMA	V F
<i>V. cholerae</i>	112	MWLF....N	LSLLQ	GVLVG	AIVGSTDA	VFSLLKGRSL	NERVG	ATLEI	ESGTNDPMA	V F
<i>S. coelicolor</i>	111	HWLL....G	IDWRL	ALLIG	AIVSSTDA	VFAVLRALPL	PRKVS	VLVEA	ESGFNDAP	. .
<i>E. coli</i> 1	105	YWVVP....G	IPLIP	AFALA	AVLSPTDA	LSGIVGEGRI	PKKIM	GILQG	EALMNDASG	L V
<i>B. subtilis</i>	104	HWMIP....A	IPLAA	AFGLA	AILSPTDV	VSALSGRVKM	PKGIL	RLLEG	EGLMNDASG	L V
<i>M. tuberculosis</i>	115	RAL....G	MESHA	AAVLG	AVLSPTDA	VAGLAK..RL	PRRAL	TVLRG	ESLINDGTA	L V
<i>A. fulgidus</i>	105	NWIL....G	IPLAL	ALIFG	AMVSATDP	VLATFKSISA	PKKLS	ILIEG	ESVLNDGAA	I V
<i>P. aeruginosa</i>	118	YYTFPLFGWQ	VDFIY	CLLFG	ALISPTDPI	VLGILKSAGA	PKPLA	TTIVG	ESLFDNDGTA	V V
<i>M. jannaschii</i>	108	NFVFHL...P	ILSLI	GLLFG	AIVSATDPAT	LIPIFSSMDI	DPEVAI	TLEA	ESVFNDPLG	I V
Consensus		.w.....	i....	all.g	Ai.s.TD.aa	v.....	p....	. leg	Es..ND...	v

<i>E. coli</i> 2	260	ALILSAWMIF	FARPLSVFAG	L.....	..LPFRGFNL	RERVF	ISWVG	LRGAVPIILA
<i>V. cholerae</i>	303	GLALAVGMIL	FARPIAVWIG	L.....	..APFKSFTA	REKWF	VSWVG	LRGAVPIILA
<i>S. coelicolor</i>	299	ALVVGLVLLL	LARPVSLLC	L.....	..LPFR.VPW	REQVF	ISWAG	LRGAVPIVLT
<i>E. coli</i> 1	319	NIILIIYAALM	LVRFGWLWTM	KKFSNRFLKK	KPMEFGSWTT	REILI	ASFAG	VRGAITLAGV
<i>B. subtilis</i>	314	YILVITFTLM	LLRF..LWL	FFWNGKWFEN	KDQNIYKPL	RSTLL	ISISG	VRGAVTLGAS
<i>M. tuberculosis</i>	316	..LAVTGVVI	ATRIAWQAT	TVLGHTVDRV	LKKPTRHVGF	RQRCV	TSWAG	FRGAVSLAAA
<i>A. fulgidus</i>	297	EILVAIAAVI	LARAFVVYPV		..MTYYRFPN	LWKHV	VFWGG	LRGVIPVALA
<i>P. aeruginosa</i>	328	AAFALGGAVL	VSRLLTVGPA	IL...VLRR	FRGANRQVPA	GTIRI	LWVGG	LRGVSVVALA
<i>M. jannaschii</i>	309	AFLCALGSIL	LARPVGLVIA	T.....	..AIPPIRPL	TERIY	LALEG	PRGVVPATLA
Consensus		..l...g...	laR...v...			re...	.sw.G	.RGa!p.ala

Fig. 5. Bacterial proteins that belong to the CPA1 family of transport proteins (Saier *et al.*, 1999). Alignment of the predicted amino acid sequences for NHE-like genes from eight bacterial genomes (*Escherichia coli* 1, *yjcE*, AAC77035; *Escherichia coli* 2, *ycgO*, AAC74275; *Vibrio cholerae*, VC2703, AE004336; *Streptomyces coelicolor*, SC4A10.04c, AL109663; *Bacillus subtilis*, *yvgP*, Z99121; *Mycobacterium tuberculosis*, YM87, Q50678; *Archeoglobus fulgidus*, *nhe2*, AE001046; *Pseudomonas aeruginosa*, *nhaP*, AB010827; and *Methanococcus jannaschii* 1, MJ1521, U67593). The MULTALIN computer program, version 5.3.3, was used (Corpet, 1988). Residues which are fully conserved across eukaryotic Na⁺/H⁺ exchangers and these bacterial proteins are shown in bold; regions of homology are shown in frames.

and high osmolarity, respectively. A decrease in osmolarity would cause a switch between these 'high' and 'low' systems, i.e. inhibition of the former and activation of the latter. If, as we suggest, the *ycgO* gene product is an essential part of the 'low' system, this switch would result in total loss of cell volume control in GRF55 cells, leading to the pause in growth. This model would also explain why there is no difference between GRF55 and GR70N in growth at low osmolarity for the first few generations, since the 'high' system would be functional in both strains. Partial recovery of growth and alkali cation gradients after the pause might be due to some reactivation of the 'high' system; this effect would be more pronounced with increasing salt concentration in the medium.

Hence, we conclude that the *ycgO* gene product participates in cell volume regulation in *E. coli* growing at low osmolarity, and we therefore suggest renaming *ycgO* as *cvrA*.

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