Cloning of the *trkAH* gene cluster and characterization of the Trk K⁺-uptake system of *Vibrio alginolyticus*

Tatsunosuke Nakamura,¹ Naoya Yamamuro,¹ Stefan Stumpe,^{2†}
Tsutomu Unemoto¹ and Evert P. Bakker²

Author for correspondence: Tatsunosuke Nakamura. Tel: +81 43 290 2932. Fax: +81 43 290 3021.
e-mail: tnakha@p.chiba-u.ac.jp

¹ Laboratory of Membrane Biochemistry, Faculty of Pharmaceutical Science, Chiba University, 1-33 Yayoi-cho, Inage-ku, Chiba 263, Japan

² Abteilung Mikrobiologie, Universität Osnabrück, D-49069 Osnabrück, Germany

K⁺-uptake genes of *Vibrio alginolyticus* were identified by cloning chromosomal DNA fragments of this organism into plasmids, followed by electroporation and selection for growth at low K⁺ concentrations of cells of an *Escherichia coli* strain defective in K⁺ uptake. A 4.1 kb DNA fragment contained a cluster of three ORFs on the same DNA strand: the previously identified *trkA* gene, a gene similar to *E. coli trkH* (*V. alginolyticus trkH*) and a new gene, *orf1*, whose function is not clear. Products of *V. alginolyticus trkA* and *orf1* were detected in *E. coli* minicells. *trkA* and *trkH* from *V. alginolyticus* restored growth at low K⁺ concentrations of an *E. coli* $\Delta trkA$ and an *E. coli* $\Delta trkG \Delta trkH$ strain, respectively, suggesting that these *V. alginolyticus* genes can functionally replace their *E. coli* counterparts. In addition, a plasmid containing *V. alginolyticus trkAH* permitted growth of an *E. coli* $\Delta sapABCDF$ ($\Delta trkE$) strain at low K⁺ concentrations. This effect was mainly due to *V. alginolyticus trkH* and was enhanced by *trkA* from this organism. Measurements of net K⁺-uptake rates indicated that the presence of these genes in *E. coli* renders the Trk systems independent of products from the *E. coli sapABCDF* (*trkE*) operon.

Keywords: K⁺ uptake, *Vibrio*, *trkA*, *trkH*, *fmv*

INTRODUCTION

In prokaryotes, K⁺ is essential for the activation of enzymes, for turgor pressure homeostasis and for salt tolerance (Csonka & Epstein, 1996; Stumpe *et al.*, 1996). Since K⁺ is supplied by accumulation, K⁺-uptake systems are indispensable. Bacteria contain multiple K⁺-uptake systems, and in *Escherichia coli* at least four systems can be present: Kdp, Kup (TrkD), TrkG and TrkH (Bakker, 1993; Silver, 1996; Stumpe *et al.*, 1996). The inducible high-affinity Kdp system belongs to the P-type ATPases (Hesse *et al.*, 1984). Kup (formerly TrkD) is constitutive, has an intermediate affinity for K⁺, and is encoded by a single gene. TrkG and TrkH form two almost identical systems. *E. coli* K-12 strains contain

two of these systems, since the prophage *rac* region contains with *trkG* a second copy of the *trkH* gene (Schlösser *et al.*, 1995). Other bacteria are likely to possess only TrkH and not TrkG (Stumpe *et al.*, 1996). These two *E. coli* systems show one significant difference: TrkH requires all three *trkA*, *trkE* and *trkH* gene products, but TrkG still shows considerable activity in the absence of *trkE* (Dosch *et al.*, 1991). The *trkA* gene product (TrkA) is a peripheral membrane protein bound to the inner side of the cytoplasmic membrane (Bossemeyer *et al.*, 1989). Isolated TrkA binds NAD⁺ (Schlösser *et al.*, 1993). *trkA* genes have been identified in a number of bacteria, including *Vibrio alginolyticus* (Nakamura *et al.*, 1994b) and four archaea (Bult *et al.*, 1996; Klenk *et al.*, 1997; Macario *et al.*, 1993; Smith *et al.*, 1997). The *trkH* and *trkG* gene products are integral membrane proteins and probably form the K⁺-translocating subunit of the complex (Schlösser *et al.*, 1995). In *Salmonella typhimurium*, *trkE* is within an operon encoding the ABC system, SapABCDF, of unknown function (Parra-Lopez *et al.*, 1993). Recent work with *E. coli* suggests that *trkE*

† Present address: Institut fuer Molekulare Biotechnologie, Abteilung Strukturbiologie, Arbeitsgruppe Physikalische DNA-Analytik, D-07745 Jena, Germany.

The GenBank/EMBL/DBJ accession number for the sequence reported in this paper is D86411.

Table 1. *E. coli* K-12 strains and plasmids

Strain/plasmid	Characteristics*	Reference	Source†
Strains			
DK6	$\Delta uncB-C rpsL hsdR minAB purE pdxC his ile met$	Klionsky <i>et al.</i> (1984)	R. D. Simoni
LB650	TK1001 $\Delta trkH::Cam^r \Delta trkG::Kan^r$	Stumpe & Bakker (1997)	
LB670	TK1001 $\Delta sapABCDF::Kan^r$	Stumpe & Bakker (1997)	
LB680	TK1001 $\Delta trkH::Cam^r \Delta sapABCDF::Kan^r$	Stumpe & Bakker (1997)	
LB700	LB2003 $\Delta sapABCDF::Kan^r$	This study	
LB2003	TK1001 $\Delta trkA$	Stumpe & Bakker (1997)	
NovaBlue	$endA1 hsdR17 (r_k^- m_k^+) supE44 thi-1 recA1 gyrA96 relA1$ $\Delta(lac-proAB) F' [proAB^+ lacI^q lacZ\Delta M15::Tn10]$		Novagen
TG1	$supE hsdD5 thi \Delta(lac-proAB) F' [traD36 proAB^+ lacI^q lacZ\Delta M15]$	Sambrook <i>et al.</i> (1989)	Amersham
TK420	$\Delta kdpFABC5 trkD1 trkAam thi$		W. Epstein
TK1001	$F^- thi lacZ gal rha \Delta kdpFABC5 trkD1$	Rhoads & Epstein (1977)	W. Epstein
TK2450	$F^- thi rha lacZ gal \Delta kdpFABC5 trkD1 \Delta trkH::Cam^r \Delta trkG::Kan^r$	Schlösser <i>et al.</i> (1995)	W. Epstein
TK2691	$F^- rha \Delta kdpFABC5 trkD1 trkH1 trkG90 thi nadA$		W. Epstein
TK2693	$\Delta kdpFABC5 trkD1 trkE80 trkG90 nadA$		W. Epstein
Plasmids			
pHG165	Can ^R , vector plasmid from which the pKT series of plasmids is derived	Stewart <i>et al.</i> (1986)	
pKT6	Can ^R , $trkA trkH orf1$	This work	
pKT61	Can ^R , $trkH orf1$	This work	
pKT62	Can ^R , $trkH orf1$	This work	
pKT622	Can ^R , $trkH11\ddagger$	This work	
pKT623	Can ^R	This work	
pKT63	Can ^R , $orf1$	This work	
pKT64	Can ^R , $trkA orf1$	This work	
pKT65	Can ^R , $trkA trkH12§$	This work	
pKT66	Can ^R , $trkA trkH orf1a $	This work	
pKT662	Can ^R , $trkH orf1b¶$	This work	
pSS420	Can ^R , derived from pBR322, carries <i>E. coli trkA</i>	Stumpe & Bakker (1997)	
pYMK1	Cam ^R , $trkA$	Nakamura <i>et al.</i> (1994b)	
pTWE341	Can ^R , contains <i>E. coli sapABCDF</i> under the control of the T7 promoter	Bossemeyer <i>et al.</i> (1989)	

* The *V. alginolyticus trk* genes present on each plasmid are indicated. Can^R and Cam^R indicate that the plasmids confer resistance towards carbenicillin and chloramphenicol, respectively.

† R. D. Simoni, Stanford University, USA; W. Epstein, University of Chicago, USA.

‡ Due to plasmid construction, the codon for the C-terminal amino acid residue Arg₄₈₁ from *trkH* has been replaced by codons for Gly-Ile-Arg-Asn-His-Gly-His-Ser-Cys-Phe-Leu-Cys-Glu-Ile-Val-Ile-Arg-Ser-Gln-Phe-His-Thr-Thr-Tyr-Glu-Pro-Glu-Ala₅₀₈ in *trkH11*.

§ Due to plasmid construction, the codon for the C-terminal amino acid residue Arg₄₈₁ from *trkH* has been replaced by codons for Gly-Asn-Ser₄₈₃ in *trkH12*.

|| Due to plasmid construction, the codons for the C-terminal amino acid residues from Leu₁₁₃ to Arg₁₉₄ of Orf1 were deleted and replaced by Ser-Ile-Arg₁₁₅ in *orf1a*.

¶ Due to plasmid construction, the codons for the C-terminal amino acid residues from Ala₈₇ to Arg₁₉₄ of Orf1 were deleted and replaced by Gly-Asn-Ser₈₉ in *orf1b*.

corresponds to *sapD* and *sapF*, which both encode ATP-binding proteins (Parra-Lopez *et al.*, 1993; Stumpe *et al.*, 1996; C. Harms, W. Epstein, R. Tewes, E. Nölker & E. P. Bakker, unpublished observations). These proteins may confer to Trk its previously documented ATP dependence (Rhoads & Epstein, 1977; Stewart *et al.*, 1985). Finally, in all bacteria studied thus far, the *trk* genes lie dispersed on the chromosome.

In the marine bacterium *V. alginolyticus*, K⁺ uptake is tightly coupled to Na⁺ extrusion, and transmembrane

K⁺ movement participates in the regulation of internal pH (Nakamura *et al.*, 1984, 1992). To understand these phenomena, it is essential to identify the K⁺-transport genes from *V. alginolyticus* and characterize their products. This bacterium lacks the high-affinity Kdp system, but contains an inducible K⁺-uptake system of intermediate affinity (K_m for K⁺ of 0.3 mM) and a constitutive low-affinity uptake system (K_m for K⁺ of 3 mM) (Nakamura *et al.*, 1994c).

We successfully isolated ion-transport genes from *V.*

alginolyticus by using mutant strains of *E. coli* for selection (Nakamura *et al.*, 1994a, b, 1996a, b). This study was undertaken to clone *trkH* from *V. alginolyticus*. A new gene library was constructed for this purpose, and plasmid pKT6 was identified by its ability to confer growth at low K⁺ concentrations to the *trkD* Δ kdpABC *trkH trkG* strain TK2691. Unexpectedly, the cloned DNA fragment contained in a cluster *trkA*, *trkH* and a third gene of unknown function.

METHODS

Bacterial strains, gene bank, plasmids and growth conditions. The strains and plasmids used in this study are listed in Table 1. *E. coli* strain TG1 was used for the production of plasmids. Plasmid pYMK1 containing *V. alginolyticus trkA* as well as a 2 kb region 5' to this gene has been described previously (Nakamura *et al.*, 1994b).

To generate a new DNA library from *V. alginolyticus*, chromosomal DNA from this organism was partially digested with the restriction enzyme *Sau3AI*. Fragments obtained were introduced into the *Bam*HI site of plasmid pHG165 (Stewart *et al.*, 1986). Cells of strain NovaBlue (Novagen) were transformed with the plasmid mixture by electroporation. About 66% of the colonies were white. A plasmid pool was isolated from approximately 30000 colonies and brought into strain TK2450 (Δ kdpFABC5 *trkD1* Δ trkH Δ trkG) by electroporation. About 100 colonies were obtained on plates containing 3 mM KCl, 4.6 mM Na₂HPO₄, 2.3 mM NaH₂PO₄, 0.1 M NaCl, 8 mM (NH₄)₂SO₄, 6 μ M FeSO₄, 0.4 mM MgSO₄, thiamin, kanamycin, chloramphenicol, ampicillin at the concentration specified below and 22 mM glucose, final pH 7.0 (K3Na111.5 medium, in which the numbers specify the K⁺ and Na⁺ concentrations in mM) (Nakamura *et al.*, 1996b). Plasmid pKT6 was selected for its ability to support growth of strain TK420 (Δ kdpFABC5 *trkD1 trkAam*) in K3Na111.5 medium plus thiamin and ampicillin. If appropriate, supplements or antibiotics were present at the following concentrations: thiamin and nicotinic acid, 1 mg l⁻¹; kanamycin, 10 mg l⁻¹; chloramphenicol, 20 mg l⁻¹; ampicillin, 75 mg l⁻¹. Deletion plasmids from pKT6 were made as described in the legend of Fig. 1.

Nucleotide sequencing. Restriction mapping was done according to standard methods (Sambrook *et al.*, 1989). DNA nucleotide sequences of both strands of the chromosomal insert from plasmids pKT6 and pYMK1 (Nakamura *et al.*, 1994b) were determined by the dideoxy chain-termination method using DNA ligated with bacteriophage M13mp18 or M13mp19 DNA as a template, the BcaBest sequencing kit (Takara) and Sequenase version 2.0 (Amersham). DNA primers used for sequencing were made in an Oligo 1000 DNA synthesizer (Beckman). Parts of the nucleotide sequence of subcloned chromosomal inserts in plasmids were determined using a *Taq* cycle sequencing kit (Takara), PCR (PTC-150 instrument; MJ Research) and automated fluorescence sequencing (DSQ-1000 instrument; Shimadzu). Nucleotide sequences were analysed with the GENETYX-MAC program (Software Development). Protein similarity search was done with the program TFASTA (Pearson & Lipman, 1988) at the Human Genome Center, GenomeNet, Kyoto Center, Japan.

Other methods. Minicell experiments were carried out as reported previously (Reeve, 1984; Schlösser *et al.*, 1991), except that the minicell-forming strain DK6 (Klionsky *et al.*,

1984) was used. Cells used for K⁺-transport assays were cultured in minimal K30Na85 medium (Epstein & Kim, 1971). Net K⁺ uptake by K⁺-depleted cells was measured using a silicone-oil centrifugation technique for separating the cells from the medium (Bakker & Mangerich, 1981). At *t* = 0, K⁺ was added at a concentration of 1 or 2 mM to two separate cell suspensions made from the same batch of cells. Handling of the samples and K⁺ determination with an Eppendorf flame photometer were as described by Bakker & Mangerich (1981). The volume of the cytosol was assumed to equal 1.45 μ l (mg dry wt cells)⁻¹ (Bakker & Mangerich, 1981).

RESULTS

Cloning and nucleotide sequencing

E. coli strain TK2691 was used for the characterization of plasmids carrying K⁺-uptake genes from *V. alginolyticus*. This strain lacks functional TrkH and TrkG systems due to its *trkH1* and *trkG90* alleles and is also defective in K⁺ uptake via its Kdp and Kup systems (Table 1). It grows poorly at K⁺ concentrations $\leq$ 10 mM. Plasmid pKT6 was selected by its ability to support growth of strains TK2450 (Δ trkH Δ trkG) and TK420 (*trkAam*) at low K⁺ concentrations as well as strain TK2691 at 3 mM K⁺.

The nucleotide sequence of the 4.1 kb *V. alginolyticus* chromosomal DNA insert from pKT6 was determined. It contained *trkA*, which had already been cloned in plasmid pYMK1 (Nakamura *et al.*, 1994b). Two additional complete ORFs were located at the 3'-end of *trkA*, and all three frames had the same direction of transcription (Fig. 1). The middle gene started nine nucleotides behind the stop codon of *trkA*. It encoded a protein of 481 amino acids with a predicted molecular mass of 53 214 Da. This sequence was 33–25% identical to *trkH/trkG* gene products from three other prokaryotes. *E. coli trkH* has the normal G + C content for this organism of 50 mol%, whereas the prophage *trkG* gene has only 37 mol%. The G + C content of the cloned *trkH/G*-like gene was 50 mol%. This value is close to that of the *V. alginolyticus* genome (45–47 mol%; Baumann & Schubert, 1984) and *E. coli trkH*. Hence we have called this gene *V. alginolyticus trkH*. The third gene started 25 nucleotides behind the stop codon of *trkH*. It encoded a hydrophilic protein consisting of 194 amino acid residues with a molecular mass of 21 153 Da. Database searches showed no striking similarity of the 21 kDa protein with any other complete protein. We have called the third gene *orf1*.

Nucleotide sequence of flanking regions

At the 3' end of *orf1*, plasmid pKT6 contains the end of an ORF, encoded by the opposite DNA strand. This frame is followed by a sequence of nucleotides forming a putative rho-independent terminator of transcription. The nucleotide sequence 5' to *trkA*, as present in plasmids pKT6 and pYMK1, was also determined. In this region, the chromosome organization of *V. alginolyticus* is identical to that of *E. coli* and *Haemophilus*

Fig. 1. *trkAH* and *orf1* region of *V. alginolyticus*. Chromosome inserts present in plasmid pYMK1 and the series of deletion plasmids from pKT6 are indicated. Plasmids containing different segments of pKT6 were made by cutting with the indicated enzymes, blunting ends with T4 DNA polymerase where not compatible, and then ligation.

influenzae, with the genes *fmt* and *fmu* being located in this order upstream of, and on the same DNA strand as, *trkA* (Fleischmann *et al.*, 1995; Berlyn *et al.*, 1996). However, in contrast to these genome data, '*fmu*' from *V. alginolyticus* consists of two partially overlapping genes, which we have called *fmu* and *fmv* (Fig. 1 and accession no. D86411). *E. coli* *fmt* encodes Met-tRNA^{Met} formyltransferase (Guillon *et al.*, 1992). The functions of *fmu* and *fmv* are not known.

Deletion plasmids

A series of deletion plasmids was constructed to study the expression and function of the *V. alginolyticus* *trkAH* and *orf1* genes (Fig. 1). Due to the method of construction used, plasmids pKT622 and pKT65 encode TrkH variant proteins with an extended C-terminus of 27 (TrkH11) and 2 residues (TrkH12), respectively (see Table 1).

Expression in *E. coli*

We examined which *V. alginolyticus* *trk* gene products are made in *E. coli* minicells (Fig. 2). Compared with minicells containing the vector plasmid pHG165 (lane 1) or the $\Delta trkAH$ and $\Delta orf1$ plasmid pKT623 (lane 7), cells containing plasmid pKT6 (lane 2) showed three additional polypeptides with apparent molecular masses

of approximately 54, 26 and 24 kDa. The 54 kDa band is TrkA, since it is absent in minicells containing the $\Delta trkA$ plasmids pKT62, pKT63, pKT622 or pKT623 (lanes 3, 4, 5 and 7, respectively). The 24 and 26 kDa proteins probably represent two forms of Orf1, since they were absent from minicells containing the $\Delta orf1$ plasmids pKT622, pKT65 or pKT623 (lanes 5–7, respectively). The TrkH protein was not detected (cf. minicells with plasmids pKT6, pKT622 and pKT65, encoding TrkH, TrkH11 and TrkH12, respectively, with the $\Delta trkH$ plasmid pKT623; Fig. 2, lanes 2 and 5–7). *E. coli* TrkH and TrkG migrate in the type of gels shown in Fig. 2 with a rate closely similar to that of the β -lactamase precursor (Schlösser *et al.*, 1991, 1995). It is, however, unlikely that *V. alginolyticus* TrkH was not observed in Fig. 2 due to co-migration with one of the β -lactamase forms, since neither TrkH nor its two longer variants were detected. Below we will show that *V. alginolyticus* TrkH must be made in *E. coli*, since it is active in this bacterium.

Further information on the two forms of Orf1 was obtained from a pulse-chase experiment. The results showed that the 24 kDa protein did not originate from the 26 kDa form or vice versa, since during the chase period both forms exhibited the same intensity with respect to each other. By contrast, the pre-form of β -lactamase vanished during the chase (results not shown). Neither boiling of the protein samples in sample buffer

Fig. 2. Synthesis of *V. alginolyticus trkA orf1* gene products by *E. coli* minicells. Plasmid-containing strain DK6 cells (1.0 ml) were labelled for 1 h with 10 μ Ci (370 kBq) [³⁵S]methionine as described by Schlösser *et al.* (1991). Subsequently, cell proteins were separated by SDS-PAGE. Labelled proteins were visualized by autoradiography. Lanes 1–7 show radioactive proteins produced by minicells containing plasmid pHG165 (lane 1), pKT6 (lane 2), pKT62 (lane 3), pKT63 (lane 4), pKT622 (lane 5), pKT65 (lane 6) and pKT623 (lane 7). The position of the proteins TrkA, pre- β -lactamase, β -lactamase and the two forms of Orf1 (Orf1 and Orf1*) on the autoradiogram are indicated on the left. The positions of molecular mass markers (expressed in kDa) are indicated on the right.

before loading them on the gel nor increasing the β -mercaptoethanol concentration to 15% in the sample buffer influenced the position and relative intensity of the two Orf1 bands (results not shown).

Next, the subcellular locations of TrkA and Orf1 were investigated. To this end, minicells were labelled and subsequently disrupted by sonication and the fractions of soluble proteins and membrane proteins were isolated by differential centrifugation (Schlösser *et al.*, 1991; Fig. 3). Pre- β -lactamase and the mature form of this protein occurred exclusively in the membrane and soluble protein fractions, respectively, showing that the separation of these fractions was satisfactory (Fig. 3, lanes 5–8). In agreement with previous results with *E. coli* (Bossemeyer *et al.*, 1989) and *S. typhimurium* (Parra-Lopez *et al.*, 1994), *V. alginolyticus* TrkA was present in both fractions (Fig. 3, lanes 5 and 7). This result supports the notion that TrkA is a peripheral membrane protein (Bossemeyer *et al.*, 1989). The two forms of Orf1 were mainly located in the membrane fraction (Fig. 3, lane 5). This result was not anticipated, since the nucleotide sequence of *orf1* predicted its product to be mainly hydrophilic. Closer inspection of the amino acid se-

Fig. 3. Subcellular location of the TrkA and Orf1 proteins in minicells. Two millilitres of minicells of strain DK6/pKT6 (odd-numbered lanes) or strain DK6/pHG165 (even-numbered lanes) was labelled with 20 μ Ci (740 kBq) [³⁵S]methionine for 1 h. Minicells were collected by centrifugation. The pellet was suspended in 2 ml K115 medium without sulfur compounds. Cells were broken by sonication with the small tip of a Branson Sonifier for 2 min. The suspension was then centrifuged for 10 min in an Eppendorf centrifuge at 10000 r.p.m. Subsequently, the supernatant was centrifuged for 20 min at 100000 *g* in a Beckman Mini-ultracentrifuge. The pellet (membrane fraction) was suspended in 100 μ l sample buffer; the supernatant (soluble protein fraction) was precipitated with one-tenth of a volume of 50% TCA. The precipitate was collected by centrifugation and also dissolved in sample buffer (Schlösser *et al.*, 1991). Lanes: 1 and 2, total cells; 3 and 4, low-speed pellet after sonication ('cell debris'); 5 and 6, membrane protein fraction; 7 and 8, soluble protein fraction. The positions of the TrkA, Orf1 and Orf1* proteins in the different fractions are indicated; the arrows 1 and 2 indicate the position of plasmid-encoded pre- and mature β -lactamase, respectively. The positions of standard proteins on the gel with their relative molecular mass in kDa are indicated on the left.

quence encoded by *orf1* indicates that residues 3–25 and 54–73 might just be hydrophobic enough for their localization within a membrane.

Function of the *trkAH* and *orf1* genes

The effects of the deletion plasmids (Fig. 1) on growth of *E. coli* chromosomal K⁺-uptake mutant strains were examined. In this analysis, strains with both point mutations and deletions in different *trk* genes were included (Table 2). Only plasmids containing *V. alginolyticus trkA* (i.e. plasmids pYMK1, pKT6, pKT64 or pKT65) allowed the *E. coli trkA* strain TK420 or the

Table 2. Effects of plasmids on the growth of K⁺-uptake-defective mutants of *E. coli*

Values are the minimum K⁺ concentrations required for growth. Mutants with each plasmid were grown in K0.1, K1, K3, K10, K30 and K100 synthetic agar medium at 37 °C. Colonies were seen after 18 h.

Plasmid*	<i>E. coli</i> mutants							
	TK420 (<i>trkAam</i>)	LB2003 (Δ <i>trkA</i>)	LB700 (Δ <i>trkA</i> Δ <i>trkE</i>)	TK2691 (<i>trkH1</i> <i>trkG90</i>)	LB650 (Δ <i>trkH</i> Δ <i>trkG</i>)	TK2693 (<i>trkG90</i> <i>trkE80</i>)	LB680 (Δ <i>trkH</i> Δ <i>trkE</i>)	LB670 (Δ <i>trkE</i>)
pKT6 (<i>trkAH orf1</i>)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
pYMK1 (<i>trkA</i>)	0.1	ND	0.1	ND	ND	1.0	ND	1.0
pKT61 (<i>trkH orf1</i>)	30.0	30.0	30.0	0.1	0.1	0.1	0.1	0.1
pKT62 (<i>trkH orf1</i>)	30.0	30.0	30.0	0.1	0.1	0.1	0.1	0.1
pKT622 (<i>trkH11</i>)	30.0	30.0	30.0	10.0	30.0	30.0	3.0	3.0
pKT623 (—)	30.0	30.0	30.0	ND	30.0	30.0	3.0	3.0
pKT63 (<i>orf1</i>)	30.0	30.0	30.0	ND	30.0	30.0	3.0	3.0
pKT64 (<i>trkA orf1</i>)	0.1	1.0	1.0	ND	30.0	3.0	3.0	1.0
pKT65 (<i>trkAH12</i>)	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
pKT66 (<i>trkAH orf1a</i>)	0.1	0.1	0.1	ND	0.1	ND	0.1	0.1
pKT662 (<i>trkH orf1b</i>)	ND	ND	ND	ND	ND	ND	0.1	0.1
pHG165 (—)	30.0	30.0	30.0	30.0	30.0	30.0	3.0	3.0

* Genes from *V. alginolyticus* contained in each plasmid are indicated.

Δ *trkA* strains LB2003 and LB700 to grow at low K⁺ concentrations, suggesting that the *V. alginolyticus trkA* gene product is active in *E. coli*.

The data in Table 2 with strains TK2691 (*trkH1 trkG92*) or LB650 (Δ *trkH* Δ *trkG*) showed that *V. alginolyticus trkH* is functionally equivalent to the *E. coli trkH* and *trkG* genes, since all *V. alginolyticus trkH*-containing plasmids supported rapid growth of these strains at 1 mM K⁺. Plasmid pKT622 supported growth of these strains only at 10 mM K⁺ and above. The relatively poor complementation by this plasmid is probably due to inactivity of the TrkH11 variant due to its long C-terminal extension. By contrast, the TrkH12 variant must be active, since plasmid pKT65 allowed strains TK2691 or LB650 to grow at 0.1 mM K⁺ (Table 2).

Plasmid-pKT6-directed expression of *V. alginolyticus trkAH* was constitutive in *E. coli*, since cells of LB700 (Δ *trkA*) containing this plasmid showed identical kinetic parameters for net K⁺ uptake [K_m of about 250 μ M and a V_{max} of about 220 nmol min⁻¹ (mg dry wt)⁻¹], regardless of whether the cells were grown at 0.3 or at 30 mM K⁺ (results not shown).

Table 2 shows an additional effect in that plasmid pKT6 and some of its derivatives allowed the *E. coli* Δ *sapABCDF* strains LB670, LB680 and LB700 to grow at low K⁺ concentrations. This effect was examined further by measuring K⁺ uptake in Na⁺-loaded cells of plasmid-containing LB670 (Δ *sapABCDF*) and LB700 (Δ *sapABCDF* Δ *trkA*), grown at 30 mM K⁺ (Fig. 4a, b, respectively). The absence of *trkA* gave strain LB700 a K⁺ uptake null phenotype (e.g. strain LB700 containing the non-complementing plasmid pKT63; Fig. 4b). By

contrast, strain LB670 (Δ *sapABCDF*) showed some residual K⁺ uptake [LB670 containing the non-complementing plasmids pSS420 (carries *E. coli trkA*), pKT63 (*orf1*⁺) or pKT623 (Δ *trkAH* Δ *orf1*)]. The residual uptake by this strain is due to partial independence of the *E. coli* TrkG system of *sapDF*, formerly '*trkE*', (Dosch *et al.*, 1991; C. Harms, W. Epstein, R. Tewes, E. Noelker & E. P. Bakker, unpublished observations; see Discussion). Cells of strain LB670 grew at 3 mM K⁺ and above (Table 2).

Plasmid pKT6 (*trkAH orf1*⁺) stimulated K⁺ uptake by both Δ *sapABCDF* strains to a level similar to that of plasmid pTWE341 (*sapABCDF*⁺) in strain LB670. This might suggest that *orf1* from *V. alginolyticus* replaces *sapDF* from *E. coli*. However, the following experiment shows that this is not the case: plasmid pKT65 (*trkAH*⁺ Δ *orf1*) and plasmid pKT6 (*trkAH orf1*⁺) enabled both strains to take up K⁺ with approximately the same rapid rate (Fig. 4). We then examined whether *V. alginolyticus trkA*, *trkH* or both genes are required for this stimulatory effect. The major effect was caused by *V. alginolyticus trkH*, since plasmid pKT662 (*trkH*⁺) stimulated K⁺ uptake by strain LB670 to an extent closely similar to that of strain LB670/pKT65 (*trkAH*⁺) (Fig. 4a). Also, *V. alginolyticus trkA* exerted a small but significant effect, since in strain LB670 plasmid pKT64 (*trkA*⁺) stimulated K⁺ uptake twofold compared to background activity (Fig. 4). This effect was specific for *trkA* from *V. alginolyticus*, since the *E. coli trkA*-containing plasmid pSS420 did not stimulate strain LB670, and only enhanced K⁺ uptake by strain LB700 to a level similar to that of strain LB670 without a complementing plasmid (Fig. 4).

Fig. 4. K⁺ uptake by plasmid-containing cells of strain LB670 (a) or LB700 (b). K⁺-depleted cells were preincubated aerobically for 10 min in a medium containing 0.2 M HEPES (sodium salt) (pH 7.5) and 10 mM glucose at 1 mg dry wt ml⁻¹. At zero time, 1 mM KCl was added. The initial rate of uptake by K⁺-depleted cells was measured. ●, Cells containing plasmid pTWE341 (*trkE*⁺ from *E. coli*); ■, cells containing pSS420 (*trkA*⁺ from *E. coli*); □, cells containing pYMK1 (*trkA*⁺); ○, cells containing pKT6 (*trkAH*⁺); △, cells containing pKT623; ▽, cells containing pKT63; ◇, cells containing pKT64 (*trkA*⁺); ▲, cells containing pKT65 (*trkAH*⁺); ▼, cells containing pKT662 (*trkH*⁺).

DISCUSSION

Archaeoglobus fulgidus (Klenk *et al.*, 1997) and *V. alginolyticus* (this work) are the first two prokaryotes in which it has been found that the *trkA* and *trkH* genes cluster together on the chromosome. For *V. alginolyticus*, it is not yet known whether these *trkA* and *trkH*

genes form an operon. The small intergenic distances and the related function of the *trkA* and *trkH* gene products are in favour of this notion. Coordinated expression of *trkA* and *trkH* may be advantageous to the cells. However, at present there is no evidence that this expression is inducible, since K⁺ uptake via plasmid-pKT6-encoded *V. alginolyticus* TrkAH was, in *E. coli*, independent of the K⁺ concentration at which the cells were grown (see Results). The Trk systems from *E. coli* are constitutive (Rhoads *et al.*, 1976). Hence we assume that cloned *trkAH* genes from *V. alginolyticus* encode the previously described constitutive K⁺-uptake system of this bacterium (Nakamura *et al.*, 1994c). An argument against this notion might be that at 0.25 mM, the observed *K_m* for K⁺ uptake via plasmid-pKT6-encoded *V. alginolyticus* TrkAH was similar to that of the inducible K⁺-uptake system (*K_m* 0.3 mM), rather than to that of the constitutive system (*K_m* 3 mM) of that bacterium (Nakamura *et al.*, 1994c). However, we attribute this phenomenon to a multiple-copy effect, since for *E. coli* it has been shown that plasmid-encoded TrkG lowers the *K_m* for K⁺ uptake via the Trk systems by a factor of four (Schlösser *et al.*, 1991, 1995). Moreover, we have cloned a second set of *V. alginolyticus* genes, which encode high-affinity K⁺-uptake activity in *E. coli* (*K_m* for K⁺ uptake below 50 μM). We assume that this system represents the inducible, high-affinity system from *V. alginolyticus* (Nakamura *et al.*, 1998).

Both TrkA and TrkH from *V. alginolyticus* restored K⁺ uptake by *E. coli* strains from which the corresponding genes had been deleted (Table 2; Fig. 4b). Since the *E. coli* Trk systems consist of several components (Stumpe *et al.*, 1996), these data suggest that hybrids are formed between *E. coli* and *V. alginolyticus* *trk* gene products. A special case of such an interaction concerns that between *V. alginolyticus* TrkAH and *E. coli* SapDF ('TrkE'). It was already known that the *E. coli* TrkH system requires 'trkE', but that the *E. coli* TrkG system depends only partially on it (Dosch *et al.*, 1991). Here we show that the activity of *V. alginolyticus* TrkAH is completely independent of *E. coli* SapDF ('TrkE') (Fig. 4). The interpretation of this result is not straightforward, but we will provide two alternative hypotheses. The first rests on the unproven assumption that the ATP-binding proteins SapDF confer ATP dependence to the Trk systems. Independence of K⁺ uptake of *sapABCDF* genes would then suggest that this process has become independent of ATP. It is, however, difficult to understand why Trk systems from the two relatively closely related organisms *V. alginolyticus* and *E. coli* would exhibit a major difference with respect to their ATP requirement. We therefore favour the second hypothesis, which is based on the observation that both *E. coli* and *S. typhimurium* contain several peptide transporters closely related to SapABCDF (Parra-Lopez *et al.*, 1993). We propose that in *E. coli*, *V. alginolyticus* Trk 'borrows' ATP-binding subunits from ABC transporters other than Sap, and that the same is to some extent true for *E. coli* TrkG. Recent work on sugar

uptake by *Streptomyces lividans* suggests that the ATP-binding subunit MsiK from the cellobiose-uptake system can also be used by the maltose-uptake system of this organism (Schlösser *et al.*, 1997), suggesting that ATP-binding components of ABC transporters are exchangeable among different systems.

The function of the *orf1* gene product is not clear. Deletion of the 3' 70% of the gene did not affect K⁺ uptake via the Trk systems in *E. coli* (Table 2 and Fig. 4). Moreover, the *E. coli* genome does not contain an *orf1* gene (Blattner *et al.*, 1997), suggesting that the gene is not essential for the activity of the *E. coli* Trk systems. In several bacteria, the gene 3' to *trkA* is *mscL*, which encodes a stretch-activated channel (Parra-Lopez *et al.*, 1994; Sukharev *et al.*, 1994; Fleischmann *et al.*, 1995). In *V. alginolyticus*, *orf1* has a location and direction of transcription similar to that of *mscL* in these other bacteria but Orf1 does not show significant sequence similarity with MscL. However, *E. coli* contains other mechanosensitive channels without homology with MscL (Sukharev *et al.*, 1994; Blattner *et al.*, 1997). Membranes derived from *E. coli* cells expressing *orf1* did not show stretch-activated channel activity attributable to Orf1 (Paul Blount, University of Wisconsin, personal communication), suggesting that *orf1* does not encode a mechanosensitive channel.

ACKNOWLEDGEMENTS

We thank Eva Limpinsel and Tomomi Kurita for excellent technical assistance, Wolfgang Epstein (University of Chicago) for his kind gift of TK strains, and Dale A. Webster (Illinois Institute of Technology) for critical reading of a previous version of this manuscript. This work was supported by a grant-in-aid for scientific research from the Japanese Ministry of Education, Science and Culture, and by grants from the Deutsche Forschungsgemeinschaft, SFB171, project C1, and by the Fonds der Chemischen Industrie.

REFERENCES

- Bakker, E. P. (1993). Cell K⁺ and K⁺ transport systems in prokaryotes. In *Alkali Cation Transport Systems in Prokaryotes*, pp. 205–224. Edited by E. P. Bakker. Boca Raton, FL: CRC Press.
- Bakker, E. P. & Mangerich, W. E. (1981). Interconversion of components of the bacterial proton motive force by electrogenic potassium transport. *J Bacteriol* **147**, 820–826.
- Baumann, P. & Schubert, R. H. W. (1984). Family II. Vibrionaceae Veron 1965, 5245^{AL}. In *Bergey's Manual of Systematic Bacteriology*, vol. 1, pp. 516–550. Edited by N. R. Krieg & J. G. Holt. Baltimore: Williams & Wilkins.
- Berlyn, M. K. B., Low, K. B. & Rudd, K. E. (1996). Linkage map of *Escherichia coli* K-12. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, pp. 1715–1902. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- Blattner, F. R., Plunkett, G., Bloch, C. A. & 14 other authors (1997). The complete genome sequence of *Escherichia coli* K-12. *Science* **277**, 1453–1474.
- Bossemeyer, D., Borchard, A., Dosch, D. C., Helmer, G. C., Epstein, W., Booth, I. R. & Bakker, E. P. (1989). K⁺ transport protein TrkA of *Escherichia coli* is a peripheral membrane protein that requires other *trk* gene products for attachment to the cytoplasmic membrane. *J Biol Chem* **264**, 16403–16410.
- Bult, C. J., White, O., Olsen, G. J. & 37 other authors (1996). Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. *Science* **273**, 1058–1073.
- Csonka, L. N. & Epstein, W. (1996). Osmoregulation. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, pp. 1210–1223. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- Dosch, D. C., Helmer, G. L., Sutton, S. H., Salvacion, F. F. & Epstein, W. (1991). Genetic analysis of potassium transport loci in *Escherichia coli*: evidence for three constitutive systems mediating uptake of potassium. *J Bacteriol* **173**, 687–696.
- Epstein, W. & Kim, B. S. (1971). Potassium transport loci in *Escherichia coli* K-12. *J Bacteriol* **108**, 639–644.
- Fleischmann, R. D., Adams, M. D., White, O. & 37 other authors (1995). Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269**, 496–512.
- Guillon, J.-M., Mechulam, Y., Schmitter, J.-M., Blanquet, S. & Fayat, G. (1992). Disruption of the gene for Met-tRNA^{Met} formyltransferase severely impairs growth of *Escherichia coli*. *J Bacteriol* **174**, 4294–4301.
- Hesse, J. E., Wiczorek, L., Altendorf, K., Reicin, A. S., Dorus, E. & Epstein, W. (1984). Sequence homology between two membrane transport ATPases, the Kdp-ATPase of *Escherichia coli* and Ca²⁺-ATPase of sarcoplasmic reticulum. *Proc Natl Acad Sci USA* **81**, 4746–4750.
- Klenk, H.-P., Clayton, R. A., Tomb, J.-F. & 48 other authors (1997). The complete genome sequence of the hyperthermophilic, sulphate-reducing archaeon *Archaeoglobus fulgidus*. *Nature* **390**, 364–370.
- Klionsky, D. J., Brusilow, W. S. A. & Simoni, R. D. (1984). *In vivo* evidence for the role of the ϵ subunit as an inhibitor of the proton-translocating ATPase of *Escherichia coli*. *J Bacteriol* **160**, 1055–1060.
- Macario, A. J., Dugan, C. B. & de Macario, E. C. (1993). An archaeal *trkA* homologue near *dnaK* and *dnaJ*. *Biochim Biophys Acta* **1216**, 495–498.
- Nakamura, T., Tokuda, H. & Unemoto, T. (1984). K⁺/H⁺ antiporter functions as a regulator of cytoplasmic pH in a marine bacterium, *Vibrio alginolyticus*. *Biochim Biophys Acta* **776**, 330–336.
- Nakamura, T., Kawasaki, S. & Unemoto, T. (1992). Roles of K⁺ and Na⁺ in pH homeostasis and growth of the marine bacterium *Vibrio alginolyticus*. *J Gen Microbiol* **138**, 1271–1276.
- Nakamura, T., Komano, Y., Itaya, E., Tsukamoto, K., Tsuchiya, T. & Unemoto, T. (1994a). Cloning and sequencing of an Na⁺/H⁺ antiporter gene from the marine bacterium *Vibrio alginolyticus*. *Biochim Biophys Acta* **1190**, 465–468.
- Nakamura, T., Matsuba, Y., Yamamuro, N., Booth, I. R. & Unemoto, T. (1994b). Cloning and sequencing of a K⁺ transport gene (*trkA*) from the marine bacterium *Vibrio alginolyticus*. *Biochim Biophys Acta* **1219**, 701–705.
- Nakamura, T., Suzuki, F., Abe, M., Matsuba, Y. & Unemoto, T. (1994c). K⁺ transport in *Vibrio alginolyticus*: isolation of a mutant defective in an inducible K⁺ transport system. *Microbiology* **140**, 1781–1785.
- Nakamura, T., Enomoto, H. & Unemoto, T. (1996a). Cloning and sequencing of the *nhaB* gene encoding an Na⁺/H⁺ antiporter from *Vibrio alginolyticus*. *Biochim Biophys Acta* **1275**, 157–160.
- Nakamura, T., Katoh, Y., Shimizu, Y., Matsuba, Y. & Unemoto, T. (1996b). Cloning and sequencing of novel genes from *Vibrio*

- alginolyticus* that support the growth of K⁺ uptake-deficient mutant of *Escherichia coli*. *Biochim Biophys Acta* 1277, 201–208.
- Nakamura, T., Yuda, R., Unemoto, T. & Bakker, E. P. (1998).** KtrAB, a new type of bacterial K⁺-uptake system from *Vibrio alginolyticus*. *J Bacteriol* 180 (in press).
- Parra-Lopez, C., Baer, M. T. & Groisman, E. A. (1993).** Molecular genetic analysis of a locus required for resistance to antimicrobial peptides in *Salmonella typhimurium*. *EMBO J* 12, 4053–4062.
- Parra-Lopez, C., Lin, R., Aspedon, A. & Groisman, E. A. (1994).** A *Salmonella* protein that is required for resistance to antimicrobial peptides and transport of potassium. *EMBO J* 13, 3964–3972.
- Pearson, W. R. & Lipman, D. J. (1988).** Improved tools for biological sequence comparison. *Proc Natl Acad Sci USA* 85, 2444–2448.
- Reeve, J. (1984).** Synthesis of bacteriophage and plasmid-encoded polypeptides in minicells. In *Advanced Molecular Genetics*, pp. 212–223. Edited by A. Pühler & K. N. Timmis. Berlin: Springer.
- Rhoads, D. B. & Epstein, W. (1977).** Energy coupling to net K⁺ transport in *Escherichia coli* K-12. *J Biol Chem* 253, 1394–1401.
- Rhoads, D. B., Waters, F. B. & Epstein, W. (1976).** Cation transport in *Escherichia coli*. VIII. Potassium transport mutants. *J Gen Physiol* 67, 325–341.
- Sambrook, J., Fritsch, F. & Maniatis, T. (1989).** *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Schlösser, A., Klüttig, S., Hamann, A. & Bakker, E. P. (1991).** Subcloning, nucleotide sequence, and expression of *trkG*, a gene that encodes an integral membrane protein involved in potassium uptake via the Trk system of *Escherichia coli*. *J Bacteriol* 173, 3170–3176.
- Schlösser, A., Hamann, A., Bossemeyer, D., Schneider, E. & Bakker, E. P. (1993).** NAD⁺ binding to the *Escherichia coli* K⁺-uptake protein TrkA and sequence similarity between TrkA and domains of a family of dehydrogenases suggest a role of NAD⁺ in bacterial transport. *Mol Microbiol* 9, 533–543.
- Schlösser, A., Meldorf, M., Stumpe, S., Bakker, E. P. & Epstein, W. (1995).** TrkH and its homologue, TrkG, determine the specificity and kinetics of cation transport by the Trk system of *Escherichia coli*. *J Bacteriol* 177, 1908–1910.
- Schlösser, A., Kampers, T. & Schrempf, H. (1997).** The *Streptomyces* ATP-binding component MsiK assists in cellobiose and maltose transport. *J Bacteriol* 179, 2092–2095.
- Silver, S. (1996).** Transport of inorganic cations. In *Escherichia coli and Salmonella: Cellular and Molecular Biology*, 2nd edn, pp. 1091–1102. Edited by F. C. Neidhardt and others. Washington, DC: American Society for Microbiology.
- Smith, D. R., Doucette-Stamm, L. A., Deloughery, C. & 34 other authors (1977).** Complete genome sequence of *Methanobacterium thermoautotrophicum* ΔH: functional analysis and comparative genomics. *J Bacteriol* 179, 7135–7155.
- Stewart, G. S. A. B., Lubinsky-Mink, S., Jackson, C. G. & Kuhn, J. (1986).** pHG165: a pBR322 copy number derivative of pUC8 for cloning and expression. *Plasmid* 15, 172–181.
- Stewart, L. M. D., Bakker, E. P. & Booth, I. R. (1985).** Energy coupling to K⁺ uptake via the Trk system in *Escherichia coli*: the role of ATP. *J Gen Microbiol* 131, 77–85.
- Stumpe, S. & Bakker, E. P. (1997).** Requirement of a large K⁺-uptake capacity and of extracytoplasmic protease activity for protamine resistance of *Escherichia coli*. *Arch Microbiol* 167, 126–136.
- Stumpe, S., Schlösser, A., Schleyer, M. & Bakker, E. P. (1996).** K⁺ circulation across the prokaryotic cell membrane: K⁺-uptake systems. In *Handbook of Biological Physics*, vol. 2, pp. 474–499. Edited by W. N. Konings, H. R. Kaback & J. S. Lolkema. Amsterdam: Elsevier.
- Sukharev, S. I., Blount, P., Martinac, B., Blattner, F. R. & Kung, C. (1994).** A large-conductance mechanosensitive channel in *E. coli* encoded by *mscL* alone. *Nature* 368, 265–268.

Received 5 January 1998; revised 13 March 1998; accepted 6 April 1998.