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Biochimica et Biophysica Acta 1277 (1996) 201–208

BIOCHIMICA ET BIOPHYSICA ACTA
BBA

Cloning and sequencing of novel genes from *Vibrio alginolyticus* that support the growth of K^+ uptake-deficient mutant of *Escherichia coli*

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Received 3 June 1996; revised 26 July 1996; accepted 31 July 1996

Abstract

Novel genes that functionally complement the growth of K^+ uptake-deficient mutant strain of *Escherichia coli* TK420 have been cloned from the marine bacterium *Vibrio alginolyticus*. The nucleotide sequence revealed three open reading frames. The second gene was homologous to *proC* gene and allowed the growth of *proC*-defective mutant strain of *E. coli* χ342 in the absence of proline. The first and third genes, but not *proC*, were required for the growth of TK420 in a synthetic medium containing 10 mM K^+ and 100 mM Na^+. Since K^+ uptake activity of TK420 was restored by the introduction of these genes, these two genes were considered to be directly related to K^+ transport. Homologous genes were found in *E. coli*, but their functions have not been reported.

Keywords: Potassium uptake; Potassium-translocating gene; DNA sequence; Marine bacteria; (*V. alginolyticus*)

1. Introduction

Intracellular concentrations of K^+ and Na^+ are regulated by transport systems [1,2], and K^+ is required for the activity of enzymes and the maintenance of cell turgor pressure [3,4]. K^+ and Na^+ transport systems of *Escherichia coli* have been well studied and several genes have been cloned and sequenced [1–3]. It is generally considered that Na^+ extrusion systems are required for cell growth when cells face certain environmental conditions [2,4], whereas K^+ transport systems are indispensable for cell growth and multiple K^+ transport systems exist in cells [1].

The marine bacterium *Vibrio alginolyticus* requires 0.5 M NaCl for optimal growth and has a respiration-driven Na^+ pump [5,6], Na^+/H^+ antiporter(s) [7–9], a K^+/H^+ antiporter [10–12] and K^+ uptake systems [13–15]. Using mutant strains of *E. coli*, we previously selected some genes from *V. alginolyticus* that functionally compensated a defect in Na^+ extrusion [8] or K^+ transport [14]. At present, the information on K^+ transport systems of *V. alginolyticus* is meager. This paper reports the cloning and sequencing of novel genes from *V. alginolyticus* that restore the growth and K^+ accumulation of K^+ uptake-deficient *E. coli* strain TK420.

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PII S0005-2728(96)00097-7

2. Materials and methods

2.1. Bacterial strains and culture conditions

V. alginolyticus strain 138-2 was grown in a rich medium containing 0.5% polypeptone, 0.5% yeast extract, 0.4% K_2HPO_4 and 3% NaCl (pH 7.3) as described previously [13]. *E. coli* strain TK420 [16] defective in K^+ transport genes (*kdpABC5, trkD1, trkAam*) was supplied by Wolfgang Epstein (University of Chicago). To examine the growth of TK420 in a synthetic medium containing varied concentrations of K^+ and Na^+, a basal medium containing 4.6 mM Na_2HPO_4, 2.3 mM NaH_2PO_4, 8.0 mM $(NH_4)_2SO_4$, 6 μM $FeSO_4$, 1 μg/ml of thiamine, 0.4% glucose and 0.4 mM $MgCl_2$ (pH adjusted to 7.0 with HCl) was employed. This medium contained 11.5 mM Na^+ and less than 0.02 mM K^+. Required amounts of KCl and NaCl were added to the basal medium and it was described as the medium K*X*Na*Y*, where *X* and *Y* indicated the final concentrations of K^+ and Na^+, respectively, in mM. Cells were grown aerobically in a Monod tube at 37°C and the growth was monitored by measuring the absorbance at 600 nm in a Perkin-Elmer model 35 spectrophotometer.

The *E. coli proC* mutant χ342 (*proC29*, λ^-, *relA1, spoT1, melB1*) was obtained from Barbara J. Bachmann (*E. coli* Genetic Stock Center). Strain χ342 is unable to grow on M9 minimal medium [17] lacking proline.

2.2. Plasmids

Chromosomal DNA from *V. alginolyticus* was partially digested by *Sau*3AI and then ligated into the *Bam*HI site of pHG165 [18]. Plasmid pKTV9 was selected from this gene library, which allowed the growth of TK420 in a synthetic medium containing 10 mM K^+ and 115 mM Na^+ described in Ref. [19]. The size of pKTV9 was about 6.4 kbp including pHG165 (3.4 kbp). To prepare deletion plasmids from pKTV9, the plasmid was first cut by specific restriction enzymes, purified by agarose gel electrophoresis, cleaned by a Geneclean Kit (Bio101, CA), and finally blunted and ligated using DNA Blunting and Ligation Kit (Takara, Japan). Plasmid pYK5 was selected for K^+ uptake measurements from a newly constructed DNA library prepared in the same way as for the selection of pKTV9.

Plasmid pACYC184 [20] was also used for the preparation of gene library, and pPRO4 was selected from this library, which allowed the growth of χ342 in M9 medium containing 20 μg/ml chloramphenicol and lacking proline.

2.3. DNA sequencing

The insert of pKTV9 was cut and ligated in M13mp18 and 19. DNA was sequenced in both directions by the dideoxy method using *Bca*BEST™ Dideoxy Sequencing Kit (Takara, Japan) and Sequenase Ver. 2.0 (Amersham, UK). The primers were synthesized with an Oligo 1000 DNA Synthesizer (Beckman, USA). All deleted and ligated sites of pKTV9-derived plasmids were confirmed by sequencing using the double-stranded plasmid DNA as templates. The complete sequencing of *orf3* gene was performed by primer extension using the denatured double-stranded pPRO4 DNA as a template [17]. The inserted DNA fragment in pYK5 was also determined by sequencing which confirmed the existence of complete *orf1*, *proC* and *orf3* genes. Sequences were analyzed using the Genetyx-Mac.

2.4. Intracellular amino acid content

TK420/pHG165 or TK420/pKTV9 cells were first cultured in the medium K30Na11.5 to the cell density of 0.3 to 0.4 (A_{600}) and then diluted 10 times with a synthetic medium containing definite concentrations of K^+ and Na^+. Cells were collected by centrifugation, intracellular amino acids were extracted and then determined by using an automatic amino acid analyzer (Hitachi 835-10) [21].

2.5. Measurement of K^+ uptake

K^+-depleted cells were prepared by a Tris-EDTA treatment as described in Ref. [22]. Cells were finally suspended in 200 mM NaCl and 10 mM Hepes/NaOH (pH 7.5). For the measurement of K^+ accumulation, a filtration method [10] was used. The reaction was carried out at 37°C in 200 mM NaCl, 10 mM Hepes/NaOH (pH 7.5), 10 mM glucose and different concentrations of K^+ at a cell density of

0.8–1.0 mg cell protein ml^{-1}. K^+ was determined by flame photometry using a Perkin-Elmer 403 atomic absorption spectrophotometer. Protein was determined by the Lowry method with bovine serum albumin as a standard [23].

3. Results and discussion

3.1. Cloning of genes from V. alginolyticus that functionally complement the growth of K^+ uptake-deficient E. coli TK420

E. coli TK420 is unable to grow in the synthetic medium containing 10 mM K^+ and 115 mM Na^+ (K10Na115). Using this strain, we cloned from the genomic DNA of *V. alginolyticus* and selected two plasmids, pYMK1 and pKTV9, which had the ability to restore the growth of TK420 in the medium K10Na115. One of the plasmids, pYMK1, contained the *trkA* gene from *V. alginolyticus* [14]. TK420 harboring pYMK1 could grow in a medium containing only 0.1 mM K^+. On the other hand, plasmid pKTV9, containing no *trkA* gene, could restore the growth of TK420 in the medium of K10Na115, but not in K0.1 Na115.

Fig. 1 shows the effects of K^+ and Na^+ on the growth of TK420 containing pHG165 (control) or pKTV9. TK420/pHG165 showed significant growth in the medium K10Na11.5, but the growth was severely inhibited in K10Na100. Although an increase in K^+ concentration promoted the growth (K30Na11.5), the addition of 400 mM Na^+ negated the effect of K^+ (K30Na400). These results indicated that TK420 having deficiencies in K^+ uptake is very sensitive to high external Na^+ concentrations in addition to the requirement for K^+. To support the cell growth, internal Na^+ concentrations must be maintained to a low level in *E. coli* [2]. Although Na^+ extrusion is mediated by Na^+/H^+ antiporters [2,3], the present results suggest the importance of K^+ uptake for the effective extrusion of Na^+ in a high NaCl medium.

As shown in Fig. 1, the introduction of plasmid pKTV9 to TK420 restored the growth in the medium K10Na100. TK420/pKTV9 showed a slow growth even in K30Na400. These results strongly suggested that pKTV9 carries genetic information from *V. alginolyticus* that complements some defects of TK420 for the growth at a low K^+/high Na^+ medium.

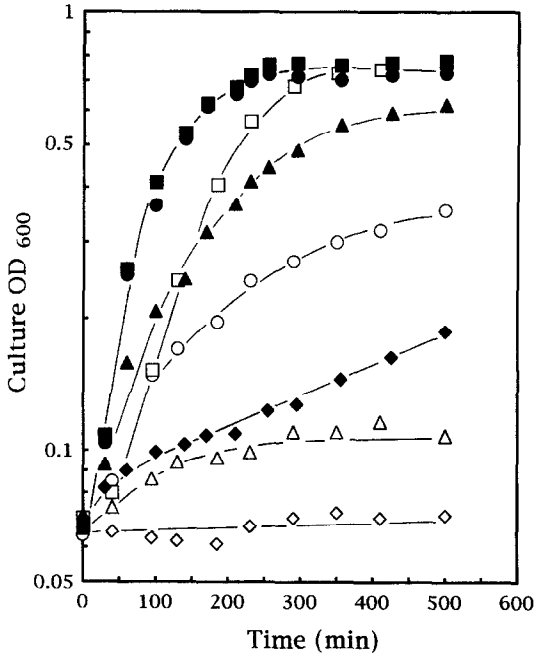

Fig. 1. Effects of K^+ and Na^+ on the growth of *E. coli* TK420 carrying pHG165 (open symbols) or pKTV9 (closed symbols). Cells were precultured in the synthetic medium containing 10 mM K^+ and 11.5 mM Na^+ (K10Na11.5) with 75 μg/ml of ampicillin, and then diluted into the synthetic medium containing different final concentrations of K^+, Na^+ and 75 μg/ml of ampicillin. K10Na11.5 (circles), K10Na100 (triangles), K30Na11.5 (squares) or K30Na400 (diamonds). A typical result among three independent experiments is presented.

The restriction mapping and subcloning of pKTV9 were performed by standard methods [17]. First, we constructed deletion plasmids pKTV91 to pKTV95 as shown in Fig. 2. Each of the constructs was transformed in TK420 and screened for the ability to restore the growth of TK420 in K10Na115 (Fig. 2). The active locus was found downstream of *Eco*RV site.

3.2. Nucleotide sequence of the active region and predicted amino acid sequence

The right side insert from the *Sal*I site of pKTV9 was sequenced (Fig. 3, accession number D50472). Four open reading frames (Orf1 to 4) were revealed. Comparison of amino acid sequences with the GenBank database showed that these sequences had ho-

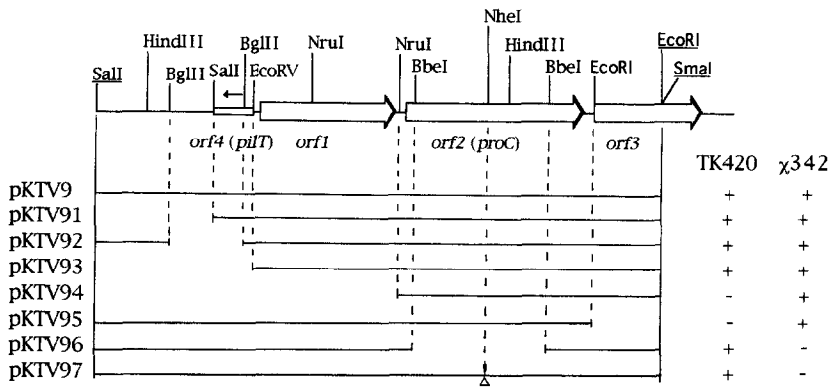

Fig. 2. Subcloning of the insert in pKTV9. Underlined *Sal*I, *Eco*RI and *Sma*I sites exist in pKTV9 on multi-cloning sites of the vector but not in the inserted DNA fragment from *V. alginolyticus*. Deletion plasmids, pKTV91 (*Sal*I cut and ligation), pKTV92 (*Bgl*II cut and ligation), pKTV93 (*Sal*I and *Eco*RV cut and blunt ligation), pKTV94 (*Sal*I and *Nru*I cut and blunt ligation), pKTV95 (*Eco*RI cut and ligation) and pKTV96 (*Bbe*I cut and ligation) were constructed. In the case of pKTV97, the *Nhe*I site (CTAG) indicated by the arrow was deleted using mung bean nuclease and ligated which produced a frame shift in *orf2* gene (indicated by Δ). TK420 or χ342 cells with plasmids were precultured in LB medium containing 100 mM K^+ and 75 μg/ml ampicillin. Each of the constructs was screened for the ability to restore the growth of two strains in synthetic agar media supplemented with ampicillin at 37°C: TK420 in K10Na115 (low K^+/high Na^+ medium) or χ342 (medium lacking proline). A + sign in the figure denotes growth.

mology with the sequences of the *pilT* and *proC* region of *Pseudomonas aeruginosa* [24,25].

Orf4 was a fragment in the opposite transcriptional orientation to the other 3 genes. As compared with the twitching motility gene product (PilT) from *P. aeruginosa*, 44 amino acids were identical among the 70 amino acid sequence of Orf4. Thus, the part of *orf4* sequenced was highly homologous to the *pilT* gene from *P. aeruginosa*.

Orf1 was comprised of 233 amino acid residues with a molecular mass of 26109 Da. The deduced amino acid sequence of Orf1 had 54% identity with an open-reading frame located between *pilT* and *proC* genes from *P. aeruginosa* [25]. The function of this gene, however, has not been reported.

Orf2 was comprised of 278 amino acid residues with a molecular mass of 29813 Da. Orf2 was 34% identical with the previously reported sequence of Δ^1-pyrroline 5-carboxylate reductase, the *proC* gene product from *E. coli* [26]. The *proC*-defective *E. coli* mutant, χ342, unable to grow on M9 medium lacking proline, was used for the complementation experiment. Although χ342/pHG165 required proline for growth, χ342 harboring pKTV94 or pKTV95 could grow in the absence of added proline (Fig. 2). These results indicated that *orf2* corresponds to the *proC* gene from *V. alginolyticus*.

The deduced amino acid sequence of Orf3 had 47% identity with a protein fragment (78 amino acid residues were reported) encoded by the flanking 3′ side gene of *proC* from *P. aeruginosa*, though its function has not been reported [24]. The *orf3* gene was incomplete in pKTV9 and the fusion point was double-underlined in Fig. 3. Four additional amino acid residues, PGNS (Pro-Gly-Asn-Ser), derived from pHG165, were added to the C-terminal part of Orf3 in pKTV9 (117 amino acid residues).

To obtain the complete *orf3* gene from *V. alginolyticus*, a new gene bank was prepared using pACYC184, and the recombinant plasmids were transformed into χ342 and screened for the ability to grow in M9 medium lacking proline. Seven plasmids were selected. After digestion with *Eco*RI, pPRO4, having the longest insert, was chosen for further experiments. The size of pPRO4 was about 7.7 kbp including pACYC184 (4.2 kbp). The complete *orf3* gene sequence was determined using pPRO4 as described in Section 2.3 and presented in Fig. 3. Orf3 was comprised of 185 amino acid residues with a molecular mass of 20580 Da. The hydropathy profile of the deduced amino acid sequence was characteristic of a membrane protein with 4 possible membrane-spanning regions (Fig. 4). Although the DNA fragment in pKTV9 included only 3 of these

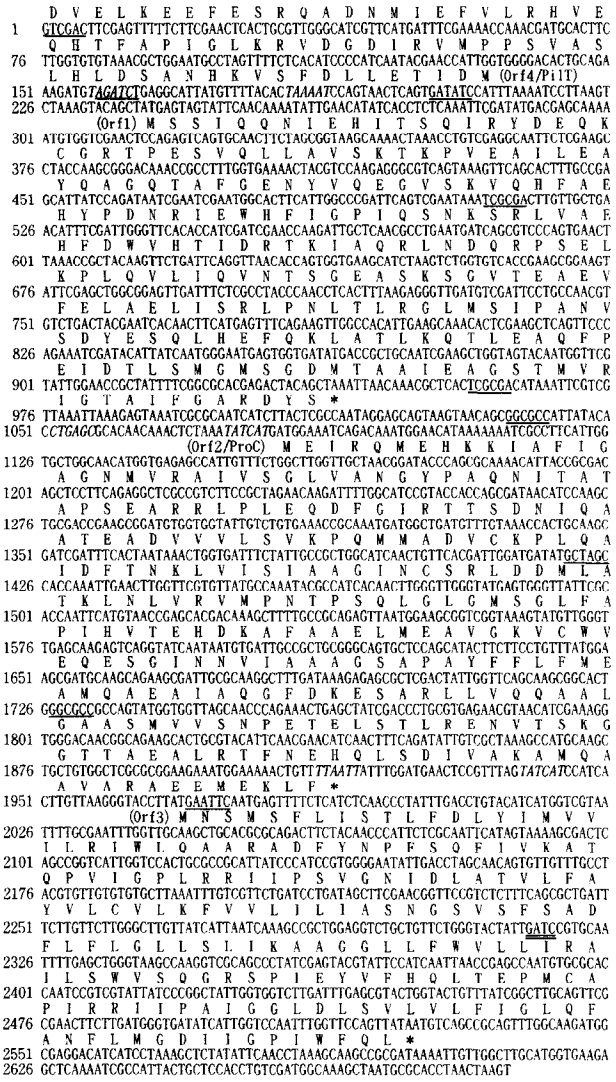

Fig. 3. Nucleotide sequence and deduced amino acid sequence of the genes from *Vibrio alginolyticus* that functionally compensate the growth of TK420 in low K^+/high Na^+ medium. Orf4 (PilT) is a fragment of opposite orientation. Predicted promoters of the *orf1*, *orf2* (*proC*) and *orf3* are shown with italic letters. The amino acid sequences are written below the nucleotide sequences for *orf1*, *orf2* and *orf3* but above them for *orf4*. Double-underlined *GATC* site was where the pKTV9 insert was ligated with the lost *Bam*HI site of pHG165. The restriction sites used for the preparation of deletion plasmids in Fig. 2 are also underlined. The nucleotide sequence reported here was submitted to the GSDB, DDBJ, EMBL and NCBI with the accession number, D50472.

membrane-spanning regions, it was effective in supporting the growth of TK420 in the medium K10Na115.

3.3. Identification of the genes restoring the growth of TK420

When K^+ accumulation is insufficient at high osmotic conditions, *E. coli* cells need to take up or synthesize compatible solutes [3,4]. Thus, K^+ uptake deficiency may influence not only K^+ accumulation but also Na^+ extrusion or an increase in compatible solutes. Proline is a compatible solute, and a high affinity proline uptake system (ProU) is induced by osmotic upshock in *E. coli* [4]. In the case of a higher plant, *Arabidopsis thaliana*, translation of one of the proline biosynthetic genes, *proC*, increases on salt treatment [27].

Since pKTV9 was found to contain *proC* gene (*orf2*) from *V. alginolyticus*, the effects of pKTV9 on the intracellular amino acid contents of *E. coli* TK420 were examined. As shown in Table 1, the glutamate content increased about 50 times in the TK420/pKTV9 cells grown in high Na^+ medium as compared with TK420/pHG165 cells grown in K10Na11.5, but the proline content was unaffected. The contents of other amino acids were also unaffected (data not shown). These results indicated that pKTV9 does not induce an increase in the proline content. Since glutamate usually acts as a counter anion to K^+ [28], a large increase in glutamate

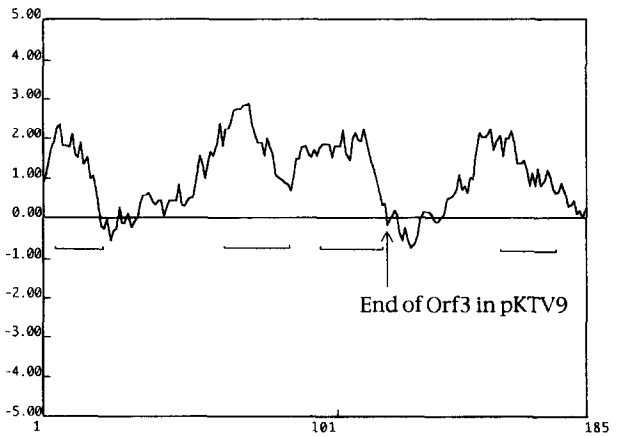

Fig. 4. Hydropathic profile of the deduced amino acid sequence of Orf3. Hydropathic values were calculated according to the method of Kyte and Doolittle [38] by using a window of 15 amino acids. Four potential transmembrane helices are indicated by horizontal bars in the figure. In pKTV9, Orf3 was interrupted at the end of predicted third membrane-spanning region.

Table 1
Amino acid contents of TK420/pKTV9 and TK420/pHG165 grown in the synthetic medium containing varied concentrations of K^+ and Na^+

Growth medium	TK420/pKTV9		TK420/pHG165	
	Glutamate	Proline	Glutamate	Proline
	(nmol/mg cell protein)			
K30Na11.5	6.5	0.7	1.4	0.8
K10Na11.5	n.d.	n.d.	1.2	0.8
K10Na100	36.4	1.8	–	–
K10Na200	60.7	1.1	–	–
K10Na300	58.8	1.3	–	–

The average of two or more determinations is presented.
n.d., not determined; –, no growth within 24 h.

strongly suggested an increase in the accumulation of K^+ in TK420/pKTV9.

We constructed new deletion plasmids, pKTV96 and 97, from unveiled restriction sites (see Figs. 2 and 3). These plasmids were transformed into TK420 or χ342, and examined for the ability to restore the growth. It was confirmed that the genetic information for the growth of χ342 in the medium lacking proline resided in *proC* (*orf2*). On the other hand, both *orf1* and *orf3*, but not *proC*, were required for the growth of TK420. Since pKTV94 restored the growth of χ342, *orf1* and *proC* genes seemed to have their own promoters. The direction of *orf1*, *proC* and *orf3* transcription were the opposite of the β-galactosidase gene in pHG165. The predicted promoter regions of *orf1*, *proC* and *orf3* genes were indicated in Fig. 3 with italics.

An *Eco*RV restriction site was located inside the predicted promoter region of *orf1*. pKTV92 had this promoter region but pKTV93 partially lost this region. We examined the time course of the growth of TK420 bearing these deletion plasmids. pKTV91 and 92 had almost the same growth activity as pKTV9, but pKTV93 showed a slow growth (data not shown). This result supported the idea that the promoter region of *orf1* was necessary for the growth of TK420 in low K^+/high Na^+ medium.

3.4. K^+ transport activity of TK420 bearing pYK5

Since pKTV9 contained an incomplete *orf3* gene, a new plasmid, pYK5, that functionally complements the growth of TK420 and χ342 was selected. The size of pYK5 was about 7.6 kbp including pHG165 (3.3 kbp). We confirmed by sequencing that pYK5 has the whole DNA fragment shown in Fig. 2. From restriction mapping, pYK5 was found to have about 1.2 and 0.4 kbp of extra DNA on the 5′ and 3′ sides, respectively, of the DNA shown in Fig. 2.

TK420 cells bearing pYK5 were cultured with the medium K10Na100 at the cell density of 0.8 (A_{600}). K^+-depleted cells were prepared and K^+ accumulation was measured as in 2.5. TK420 cells with pYK5 quickly took up K^+ in 10 mM K^+ (Fig. 5), which was in contrast to the K^+ uptake activity of TK420 with the vector pHG165. These results strongly suggested that *orf1* and *orf3* genes in pYK5 are directly related to K^+ transport genes from *V. alginolyticus*.

3.5. Homologous genes to orf1 and orf3 from different organisms

As described in the above section, the *orf1* and *orf3* genes are likely to be the same genes as the

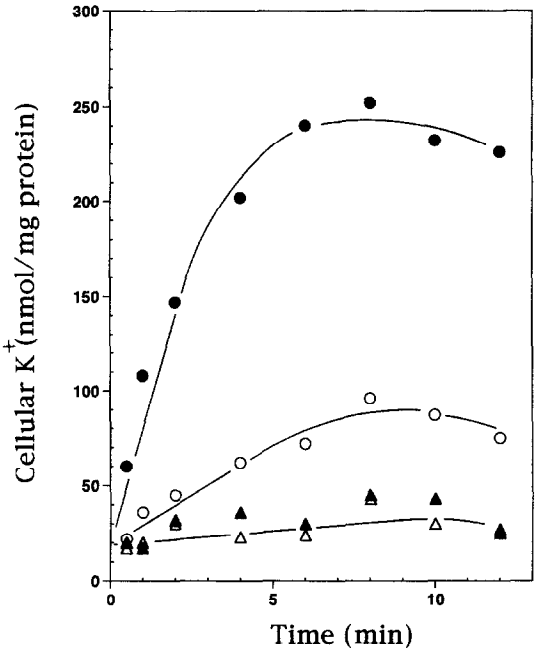

Fig. 5. Time course of K^+ uptake. TK420/pHG165 (open symbols) cells were cultured in the medium K30Na11.5 and TK420/pYK5 (closed symbols) cells were cultured in K10Na100. Cellular K^+ was depleted by Tris-EDTA treatment. The experiment was started by suspending the K^+-depleted cells in 200 mM NaCl, 10 mM Hepes/NaOH (pH 7.5), 10 mM glucose without addition of KCl (triangles) or in the presence of 10 mM KCl (circles) at 37°C. K^+ uptake was measured by the filtration method. The results shown are the average of three separate experiments.

flanking genes upstream and downstream of the *proC* gene from *P. aeruginosa*. These 2 genes, however, had no homology with the flanking genes of *proC* from *E. coli* and other reported organisms.

The deduced Orf3 sequence with 185 amino acids (a.a.) had some similarities to parts of other K^+ transport proteins, TrkH (18% identical in 131 a.a.) [29], Kup (TrkD) (16% identical in 166 a.a.) [30] and KefC (22% identical in 141 a.a.) [31], and with Na^+/H^+ antiporter NhaB (22% identical in 101 a.a.) [32] from *E. coli*. Orf3 was also similar to TetA(C) of pACYC184 (21% identical in 92 a.a.), which has been reported to have K^+ uptake activity [33,34]. These results suggested that Orf3 is possible to constitute a membrane segment of an ion transporter.

From the homology search program, tfasta, at GenomeNet (Kyoto center), DNA sequences homologous to *orf3* were found in *E. coli* (accession number U28377) and *Haemophilus influenzae* (accession number U32784) [35], but the functions of these genes have not been reported. Orf3 from *V. alginolyticus* was 51% and 40% identical with Orf3 from *E. coli* (188 a.a.) and *H. influenzae* (184 a.a.), respectively.

Homologous DNA sequences to *orf1* were also found in *E. coli* (accession number U28377), *H. influenzae* [35], *Synechocystis sp* (accession number D64006) [36] and *Saccharomyces cerevisiae* (accession number X78214) [37], but the functions of these genes have not been reported. Orf1 from *V. alginolyticus* (233 a.a.) was 61%, 58%, 42% and 41% identical with Orf1 from *E. coli* (234 a.a.), *H. influenzae* (237 a.a.), *Synechocystis* (233 a.a.) and *S. cerevisiae* (257 a.a.), respectively. In spite of the evolutionary differences, 44 amino acids residues (about 19%) were conserved in Orf1 from the five organisms. This is the first report that *orf1* and *orf3* are K^+ transport-related genes.

It is interesting to note that *orf1* and *orf3* genes line up in that order in the chromosome of *E. coli* (67 min), though the *proC* gene from *E. coli* exists at 9 min. In contrast, in *H. influenzae*, *orf1* (starts #96557 bp of the complete genome, accession number U32694) and *orf3* (starts #1100270 bp of the complete genome) are widely separated in the chromosomal DNA. It should be noted that *trkA* (72 min), *trkH* (87 min) and *trkE* (29 min) gene products constitute one K^+ transporter, the TrkH system, but each gene is widely separated from others in the *E. coli* chromosome [3].

Since *E. coli* chromosome has genes very similar to *orf1* and *orf3*, it is reasonable to consider that these genes are functional in TK420. However, TK420 required the introduction of *orf1* and *orf3* from *V. alginolyticus* to grow in the medium K10Na100. This might be due to the insufficiency of K^+ transport activity of Orf1/Orf3 from *E. coli* under that growth condition. Another possibility is that TK420 is defective in *orf1* and/or *orf3*. Further studies are required to identify the precise functions of both gene products.

Acknowledgements

We greatly appreciate Wolfgang Epstein (University of Chicago) for his kind gift of strain TK420. We are grateful to Fumio Ikegami (Chiba University) for technical advice. We are also grateful to Dale A. Webster (Illinois Institute of Technology) for critical reading of this manuscript. This work was supported in part by a grant-in-aid for scientific research from the Japanese Ministry of Education, Science and Culture.

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