Identification of the ABC protein SapD as the subunit that confers ATP dependence to the K⁺-uptake systems Trk^H and Trk^G from *Escherichia coli* K-12

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The activity of the two almost identical K⁺-uptake systems, Trk^H and Trk^G, from *Escherichia coli* K-12 depends completely and partially on the presence of the *trkE* gene, respectively. *trkE* maps inside the *sapABCDF* operon, which encodes an ATP-binding cassette (ABC) transporter of unknown function from the subgroup of peptide-uptake systems. This study was carried out to clarify the role of *sapABCDF* gene products in the ATP dependence of the *E. coli* Trk systems. For this purpose $\Delta sapABCDF \Delta trkG$ and $\Delta sapABCDF \Delta trkH$ strains of *E. coli* containing plasmids with *sap* genes from either *E. coli* or *Vibrio alginolyticus* were used. All five plasmid-encoded *E. coli* Sap proteins were made in *E. coli* mini-cells. The presence of the ATP-binding SapD protein from either *E. coli* or *V. alginolyticus* alone was sufficient for stimulating the K⁺ transport activity of the Trk^H and Trk^G systems. K⁺-uptake experiments with *Escherichia coli* cells containing SapD variants with changes in the Walker A box Lys-46 residue, the Walker B box Asp-183 residue and the signature motif residues Gly-162 or Gln-165 suggested that adenine nucleotide binding to SapD rather than ATP hydrolysis by this subunit is required for the activity of the *E. coli* Trk^H system. K⁺ transport via two plasmid-encoded Trk systems in a $\Delta sapABCDF$ *E. coli* strain remained dependent on both a high membrane potential and a high cytoplasmic ATP concentration, indicating that in *E. coli* ATP dependence of Trk activity can be independent of Sap proteins. These data are interpreted to mean that Trk systems can interact with an ABC protein other than SapD.

Keywords: ABC system, peptide transporter family, K⁺ transport, Kir-SUR system, regulation by adenine nucleotides

INTRODUCTION

Under most conditions of growth *Escherichia coli* K-12 cells accumulate K⁺ via two almost identical high-rate, low-affinity systems, Trk^H and Trk^G. The products of four non-linked genes, *trkA*, *trkE*, *trkG* and *trkH*

contribute to the activity of these systems (Walderhaug *et al.*, 1987; Dosch *et al.*, 1991; Schlösser *et al.*, 1995). The *trkH* and *trkG* gene products (Trk^H and Trk^G, respectively) are the K⁺-translocating subunits of each system. They are similar proteins, sharing 41 % identical amino acid residues (Schlösser *et al.*, 1995). The reason that *E. coli* K-12 possesses two Trk systems appears to be that *trkG*, a second copy of *trkH*, has been inserted with the prophage *rac* into the chromosome (Schlösser *et al.*, 1992, 1995). However, genome data show that it is an exception for prokaryotes to contain two *trkH*-like genes (Durell *et al.*, 1999). Trk^H and Trk^G belong to a superfamily of K⁺-transporters with members in plants,

Abbreviations: ABC, ATP-binding cassette; EcTrk, VaTrk, Trk from *E. coli* and *V. alginolyticus*, respectively; EcSap and EcSap, Sap proteins and *sap* genes, respectively, from *E. coli*; pmf, proton-motive force; VaSap and Vasap, Sap proteins and *sap* genes, respectively, from *V. alginolyticus*.

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fungi, bacteria and archaea. These proteins may have evolved from the simple KcsA type of K⁺ channel (Doyle *et al.*, 1998; Schrempf *et al.*, 1995) by at least two gene duplication and gene fusion events, forming covalently linked tetramers of different functions (Nakamura *et al.*, 1998b; Durell *et al.*, 1999; 2000; Durell & Guy, 1999; Tholema *et al.*, 1999).

The *trkA* gene product (TrkA) is required for the activity of the Trk systems from *E. coli* and *Salmonella typhimurium* (Dosch *et al.*, 1991; Stumpe *et al.*, 1996; Parra-Lopez *et al.*, 1994). It occurs in many prokaryotes, including *Vibrio alginolyticus* (Nakamura *et al.*, 1994; 1998a), and several archaea (Stumpe *et al.*, 1996; Durell *et al.*, 1999). TrkA is a peripheral membrane protein, attached via TrkH or TrkG to the inner side of the cytoplasmic membrane (Bossemeyer *et al.*, 1989; Parra-Lopez *et al.*, 1994; Nakamura *et al.*, 1998a). TrkA from *E. coli* (EcTrkA) contains two putative NAD⁺-binding sites, but although both NAD⁺ and NADH have been shown to bind to the isolated protein (Schlösser *et al.*, 1993), it is not known whether these dinucleotides play a role in K⁺ transport via the Trk system *in vivo* (Stumpe *et al.*, 1996).

Little is known about the function of *trkE*. Trk^H requires *trkE*, but Trk^G shows residual activity in the absence of a functional *trkE* gene (Dosch *et al.*, 1991). Moreover, *V. alginolyticus* Trk (VaTrk) is fully active in an *E. coli* Δ *trkE* strain (Nakamura *et al.*, 1998a). In both *S. typhimurium* and *E. coli* *trkE* maps inside the *sapABCD* operon [Parra-Lopez *et al.* (1993) and W. Epstein, personal communication, respectively]. This operon encodes an ATP-binding cassette (ABC) transporter of unknown function from the subgroup of peptide-uptake systems (Parra-Lopez *et al.*, 1993; Linton & Higgins, 1998). Since the *sapABCD* operon is required for the resistance of *S. typhimurium* towards the small, strongly cationic protein protamine (Groisman *et al.*, 1992), Groisman and his colleagues have speculated that SapABCD detoxifies protamine by transporting it into the cytoplasm, where it is supposed to be degraded by proteases (Parra-Lopez *et al.*, 1993; Groisman, 1994). However, our studies with *E. coli* K-12 have shown that the role of *sapABCD* with respect to protamine resistance of *E. coli* lies in its 'trkE' function, i.e. in its role in K⁺ transport via the Trk systems (Stumpe & Bakker, 1997; Stumpe *et al.*, 1998).

The fact that *trkE* maps inside the *sapABCD* operon (Parra-Lopez *et al.*, 1993) raises new interest in early observations that K⁺ transport via the Trk system depends on ATP (Rhoads & Epstein, 1977). ATP is believed to activate the system (Stewart *et al.*, 1985), whereas the transmembrane proton-motive force (pmf) may drive the K⁺ transport process (Stumpe *et al.*, 1996). Here we address the question of the relationship between *trkE* and the *sapABCD* operon with respect to their role in K⁺ transport in *E. coli*. We show that *sapD*, one of the two operon genes encoding an ATP-binding subunit, is required for K⁺ uptake. Changing amino acid residues in the Walker A or B box of SapD led in some

situations to an almost complete loss of transport activity, suggesting that the ATP-dependence of the Trk systems is conferred via SapD. Finally, the *trkE*-independent activities of Trk^G and VaTrk activities remained ATP-dependent.

METHODS

Strains, plasmids and growth conditions. The strains and plasmids used in this study are listed in Table 1. Cells were grown in the presence of 115 mM K⁺ ions in the minimal mineral medium described by Epstein & Kim (1971) in the presence 10 mM glucose as carbon source. Strains carrying the Δ *atpB-atpC* deletion were grown in the same medium in the presence of 22 mM glucose. Mini-cells of the plasmid-containing *E. coli* DK6 strain (Klionsky *et al.*, 1984) were formed during growth for 18 h in medium KML, containing 1% tryptone, 0.5% yeast extract, 1% KCl, 100 mg carbenicillin l⁻¹ and 20 mM glucose.

Plasmids pCH100–pCH107 were generated from plasmid pTWE341 by deleting parts of the *E. coli* *sapABCD* operon with the restriction enzymes indicated in Fig. 1(a). Plasmids pT7 and pT70 contain *Sau*3AI restriction fragments with chromosomal *V. alginolyticus* *sap* genes, cloned into the *Bam*HI site of plasmid pHG165 (Stewart *et al.*, 1986). These plasmids were identified by complementation of the K⁺ transport defect of *E. coli* Δ *sapABCD* strains. Plasmids of the pKT7 series were generated from plasmids pKT7 and pKT70 by deleting parts of the *V. alginolyticus* *sap* operon with the restriction enzymes indicated in Fig. 1(b). Plasmids of the pCC series were generated with the aid of specific primers and the QuickChange site-directed mutagenesis kit from Stratagene. The correctness of the changes introduced was checked by nucleotide sequencing (Sanger *et al.*, 1977).

The *sapABCD* deletion plasmid, pCH410, was derived from plasmid pTWE341 by the two-step PCR method described by Link *et al.* (1997). First, a PCR product was generated containing a 9 nt linker with a *Bam*HI restriction site, nt 20–554 of *sapA*, a 21 nt linker, nt 291 of *sapF*–nt 79 of the gene 3' to *sapF* and a 9 nt linker with a *Bam*HI site. This DNA fragment, carrying the desired *sapABCD* deletion was treated with *Bam*HI and cloned into the *Bam*HI site of plasmid pKO3, giving plasmid pCH410. The Δ *sapABCD* deletion was then transferred from plasmid pCH410 to the chromosome of strain LB620, giving strain LB625, according to the procedure of Link *et al.* (1997), except that at 42 °C non-growing cells were enriched by a penicillin selection procedure. The correctness of the chromosomal Δ *sapA–F* deletion in this strain was checked by Southern hybridization. Strains were made Δ *atpB–C* by P1 transduction (Miller, 1972), using strain DK8 as a source of this deletion (Klionsky *et al.*, 1984).

Nucleotide sequencing. The nucleotide sequence of the 5.9 kb *E. coli* chromosomal insert of plasmid pTWE341 was determined by Eurogentec. Other sequences were determined by MWG-Biotech using the chain-termination method of Sanger *et al.* (1977). Nucleotide primers were from the latter company.

Detection of *sap* gene products. Plasmid-containing mini-cells of strain DK6 were isolated on a sucrose gradient and their Sap proteins were labelled with [³⁵S]methionine and made visible as described by Reeve (1984).

N-terminal sequence of Sap proteins from *E. coli* (EcSap). This sequence was determined for SapA, SapD and SapF,

Table 1. Strains and plasmids used in this study

Strain or plasmid	Genotype or plasmid feature	Reference	Source*
DH5 α	F ⁻ <i>lendA1 hsdR17</i> (<i>r_K⁻m_K⁺</i>) <i>supE44 thi-1 recA1 gyrA relA1</i> Δ (<i>lacIZYA-argF</i>) <i>U169 deoR</i> [ϕ 80dlac Δ (<i>lacZ</i>)M15]	Hanahan (1983)	Gibco-Life Science
DK6	F ⁻ <i>rpsL hsdR minAB purE pdxC his ilv met</i> Δ <i>atpBEFHAGDC</i>	Klionsky <i>et al.</i> (1984)	R. D. Simoni
DK8	HfrPO1 <i>bglR thi1 relA1 ilv::Tn10</i> Δ <i>atpBEFHAGDC</i>	Klionsky <i>et al.</i> (1984)	R. D. Simoni
LB620	TK1001 Δ <i>trkG::Km^R</i>	This work	
LB625	TK1001 Δ <i>trkG::Km^R ΔsapABCD</i>	This work	
LB627	TK1001 Δ <i>trkG::Km^R ΔsapABCD ΔatpB-C</i>	This work	
LB680	TK1001 Δ <i>trkH::Cm^R ΔsapABCD::Km^R</i>	Stumpe & Bakker (1997)	
LB682	TK1001 Δ <i>trkH::Cm^R ΔsapABCD::Km^R ΔatpB-C</i>	This work	
LB690	TK1001 Δ <i>trkG::Km^R ΔtrkH::Cm^R ΔsapABCD</i>	This work	
LB692	TK1001 Δ <i>trkG::Km^R ΔtrkH::Cm^R ΔsapABCD ΔatpB-C</i>	This work	
TK1001	F ⁻ <i>thi lacZ rha gal trkD1</i> (<i>kup1</i>) Δ <i>dkpFABC5</i>	Rhoads & Epstein (1997)	W. Epstein
pAS8	pT7-5 Ap ^R <i>trkG</i> under T7 promoter control	Schlösser <i>et al.</i> (1995)	
pCC1	pCH105, SapD Lys-46 $\rightarrow$ Gln, SapF Lys-53 $\rightarrow$ Gln; Ap ^R	This work	
pCC2	pCH105, SapD Lys-46 $\rightarrow$ Arg, SapF Lys-53 $\rightarrow$ Gln; Ap ^R	This work	
pCC3	pCH105, SapD Lys-46 $\rightarrow$ Met; Ap ^R	This work	
pCC4	pCH105, SapD Lys-46 $\rightarrow$ Ile; Ap ^R	This work	
pCC7	pCH105, SapD Lys-46 $\rightarrow$ Gln; Ap ^R	This work	
pCC8	pCH105, SapD Lys-46 $\rightarrow$ Gly; Ap ^R	This work	
pCC11	pCH105, SapD Lys-46 $\rightarrow$ Arg; Ap ^R	This work	
pCC15	pCH105, SapD Asp-183 $\rightarrow$ Glu; Ap ^R	This work	
pCC16	pCH105, SapD Asp-183 $\rightarrow$ Asn; Ap ^R	This work	
pCC17	pCH105, SapD Asp-183 $\rightarrow$ Lys; Ap ^R	This work	
pCC21	pCH105, SapF Lys-53 $\rightarrow$ Gln; Ap ^R	This work	
pCC31	pCH105, SapD Gly-162 $\rightarrow$ Ala	This work	
pCC32	pCH105, SapD Gln-165 $\rightarrow$ Leu	This work	
pCC101	pCH105, SapD Δ Lys-46	This work	
pCC111	pCH106, SapD Lys-46 $\rightarrow$ Gly; Ap ^R	This work	
pCH100	pTWE341 Δ <i>sapF</i> ; Ap ^R	This work	
pCH101	pTWE341 Δ <i>sapA</i> ; Ap ^R	This work	
pCH102	pTWE341 Δ <i>sapDF</i> ; Ap ^R	This work	
pCH103	pTWE341 Δ <i>sapADF</i> ; Ap ^R	This work	
pCH104	pTWE341 Δ <i>sapBCD</i> ; Ap ^R	This work	
pCH105	pTWE341 Δ <i>sapABC</i> ; Ap ^R	This work	
pCH106	pTWE341 Δ <i>sapABCF</i> ; Ap ^R	This work	
pCH107	pTWE341 Δ <i>sapABCD</i> ; Ap ^R	This work	
pCH410	pKO3 Δ <i>sapABCD</i>	This work	
pHG165	Cloning vector; Ap ^R	Stewart <i>et al.</i> (1986)	I. R. Booth
pKO3	<i>repA</i> (ts) M13ori <i>cat^R sacB⁺</i>	Link <i>et al.</i> (1997)	G. M. Church
pKT65	pHG165, containing <i>VatrkAH</i> ; Ap ^R	Nakamura <i>et al.</i> (1998a)	
pKT7	pHG165, containing <i>VasapBCD</i> ; Ap ^R	This work	
pKT70	pHG165, containing <i>VasapBCDF</i> ; Ap ^R	This work	
pKT72	pKT7, Δ <i>sapB</i> ; Ap ^R	This work	
pKT77	pKT7, Δ <i>sapBC</i> ; Ap ^R	This work	
pKT701	pKT70, Δ <i>sapBCD</i> ; Ap ^R	This work	
pTWE341	pT7-6 with the 5.9 kb <i>sap</i> operon inserted into its <i>Pst</i> I site; Ap ^R	Bossemeyer <i>et al.</i> (1989)	W. Epstein
pT7-6	Cloning vector, T7 promoter; Ap ^R	Tabor & Richardson (1985)	W. Epstein

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encoded by plasmid pTWE341 or one of its derivatives (Fig. 1a). To this end these proteins were overproduced with the aid of the T7 promoter in front of the *sapABCD* operon, the cells

were broken by sonication, membranes were collected by ultracentrifugation and membrane proteins were separated by SDS-PAGE. SapA, SapD or SapF were cut out from the gel and

Fig. 1. *sap* deletion plasmids. (a) Plasmids derived from pTWE341, which contains the complete *EcsapABCDF* operon (line 1); (b) plasmids containing *Vasap* genes. Restriction sites used to generate the deletion plasmids and complete genes present in these plasmids are indicated above and below the plasmids, respectively. Arrows indicate the direction of transcription. Plasmid promoters are indicated in the first lines of both panels.

their N-terminal sequence was determined by automatic Edman degradation in an Applied Biosystems 473A apparatus.

Transport experiments and ATP content. All of these experiments were done at 20–22 °C and were repeated at least two times. Figs 3, 4 and 6 give mean values of three independent experiments. Figs 5, 7, 8 and Table 2 give results from a single experiment, of which all of its elements were repeated at least once, but not necessarily in a single experiment. For the calculation of the cell content of K^+ , [^{14}C]proline, [^{14}C]glutamine and ATP, a cell suspension with an OD_{578} of 1.0 was taken to be equivalent to 0.33 mg cell dry wt ml $^{-1}$ (Bakker & Mangerich, 1981).

E. coli cells were depleted of K^+ by treating them with Tris/EDTA as described previously (Bakker & Mangerich, 1981). Net K^+ uptake by K^+ -depleted cells in experiments in which the role of Sap proteins in K^+ transport was measured at 1 mg dry wt cells (ml 200 mM HEPES buffer, pH 7.5) $^{-1}$. Glucose (10 mM) and 1 mM KCl were added at $t = -10$ min and 0 min, respectively. At different time points cells were centrifuged through silicone oil and the K^+ content of the cell pellet was determined by flame photometry (Bakker & Mangerich, 1981).

K^+ and energy reserves of cells of *atp* $^+$ or Δ *atpB-C* strains were depleted by shaking them for 1.75 h at 37 °C in the presence of 5 mM 2,4-dinitrophenol in minimal mineral growth medium plus 0.15 mM chloramphenicol, but without K^+ , glucose, thiamine and other antibiotics. The protonophore was re-

moved by washing the cells three times with cold buffer A, containing 75 mM sodium phosphate, 0.4 mM $MgSO_4$ and 0.15 mM chloramphenicol, pH 7.0. Subsequently, cells were suspended in buffer A at a concentration of 10–20 mg dry wt ml $^{-1}$ and kept on ice for up to 3 h before the start of the transport experiment. For uptake experiments of K^+ and radioactive compounds cells were suspended at 1 and 0.67 mg dry wt (ml buffer A) $^{-1}$, respectively. Glucose (10 mM) or 20 mM sodium succinate was added at $t = -15$ and -30 min, respectively, and the cell suspension was shaken at 160 r.p.m. At $t = 0$ min, 2 mM KCl, 2 μ M [^{14}C]glutamine (40 nCi ml $^{-1}$) or 4 μ M [^{14}C]proline (100 nCi ml $^{-1}$) was added and the suspension was shaken as described above. For the [^{14}C]proline-uptake experiments the buffer contained 2 mM KCl. Samples for cell K^+ determination were taken and treated as described above. For the measurement of radioactivity in the cells 0.3 ml samples of the suspension were filtered through 2.5 cm diameter glass fibre GF-5 filters (pore size 0.45 μ m; Macherey & Nagel). The cells on the filter were washed twice with 100 mM LiCl solution, the filters were dried and their radioactivity was measured by liquid-scintillation counting. Samples for ATP determinations in the cells were withdrawn from the same suspension as the one used for measuring K^+ content of the cells. After cell lysis by cold 5 mM H_3PO_4 containing 12% perchloric acid, readjustment of the suspension to pH 7.0 with a solution containing 2 M KOH plus 0.3 M MOPS and removal of $KClO_4$ and protein by centrifugation, ATP was determined in the supernatant by using a luminescence assay (Kimmich *et al.*, 1975).

RESULTS

Nucleotide sequence of the *sapABCD* operons from *E. coli* and *V. alginolyticus*

Plasmid pTWE341 (Fig. 1) contains the *E. coli trkE* gene under control of a T7 promoter (Bossemeyer *et al.*, 1989). The nucleotide sequence of the 5.9 kb chromosomal insert of plasmid pTWE341 was determined (EMBL accession no. X97282). It contained the complete *sapABCD* region and differs at three positions to that from accession no. D90767 (nt C-355, G-337 and C-2599, respectively in X97282) and at one position with that reported in Blattner *et al.* (1997) (nt C-2599 in X97282). By determining the N-terminal sequence of SapA we found that codons 34 and 35 of *sapA* translate as serine and glycine residues, respectively, confirming the sequence AGCGGT reported by both Blattner *et al.* (1997) and us for nt 333–338 in X97282. This suggests that the sequence AGGGTT for these codons (which would translate into arginine and valine) reported in D90767 is wrong. By contrast, our nucleotide C-2599 might be a T as reported in both Blattner *et al.* (1997) and in accession no. D90767. This change does not affect the primary sequence of SapB, since both ATC and ATT codons at positions 2597–2599 encode an isoleucine residue for position 242 in SapB. In conclusion, the nucleotide sequence of the *sap* region as reported in the *E. coli* K-12 genome sequence (Blattner *et al.*, 1997) is correct.

Since the action of VaTrk in *E. coli* is independent of the *EcsapABCD* operon (Nakamura *et al.*, 1998a) it was important to determine whether *V. alginolyticus* also contains a *sapABCD* operon. Clones containing most of *sapA* and complete *sapBCD* of this organism were obtained (Fig. 1b). Their nucleotide sequence was determined and filed under number AB015765 at the DDBJ database. The percentage of identical residues in *E. coli* and *V. alginolyticus* varies between 43% for SapA and 64% for SapD. By contrast, the Sap proteins of *E. coli* and *S. typhimurium* have highly identical sequences, varying between 90% for SapA, and 96 and 98% for the ATP-binding subunits SapD and SapF, respectively.

sap gene products in mini-cells

To obtain more information on the function of *sap* genes, deletion plasmids were constructed from plasmids pTWE341, pKT7 and pKT70 (Fig. 1). The *Ecsap* plasmids, derived from plasmid pTWE341, were brought into cells of the mini-cell-producing *E. coli* strain DK6 (Klionsky *et al.*, 1984). These cells made all five plasmid-encoded EcSap proteins (Fig. 2) under conditions at which the T7 promoter should not be active, suggesting that in this system *sap* genes are transcribed from their own promoter(s). However, with *sapA* DNA as a probe in Northern hybridization experiments, it was observed that plasmid pTWE341 made very large transcripts of up to 9 kb (results not shown), indicating that under these conditions

sapABCD transcription was not necessarily directed by its own promoter(s).

Role of *sap* gene products in K⁺ transport via EcTrk^H

For this purpose strain LB625 ($\Delta sapABCD$ $\Delta trkG::Km^R$) was constructed. The effect of plasmids pTWE341, pKT7 and pKT70 and their derivatives (Fig. 1) on net K⁺ uptake by K⁺-depleted cells of this strain was investigated (Fig. 3). Since all five plasmid-encoded EcSap proteins are made in *E. coli* mini-cells of strain DK6, and deletion of *sap* genes at the 5' end of the operon did not cause polar effects on the synthesis of products of *sap* genes located 3' to them (Fig. 2), we assumed that the effect of individual EcSap proteins on K⁺ transport via Trk^H of strain LB625, containing the plasmids in Fig. 1(a), could be tested. In accordance with Dosch *et al.* (1991) we observed that Trk^H had a very low activity in the absence of EcSap gene products (Fig. 3a). The presence of the ATP-binding protein SapD was sufficient for restoring K⁺ uptake and this effect was slightly enhanced by ATP-binding protein SapF [c.f. plasmids pCH106 (*sapD*⁺) and pCH100 (*sapABCD*⁺) with plasmids pCH105 (*sapDF*⁺) and pTWE341 (*sapABCD*⁺), respectively (Fig. 3a)]. By contrast, SapF alone was inactive [plasmids pCH107 (*sapF*⁺; Fig. 3a) and pCH104 (*sapAF*⁺; results not shown), respectively].

K⁺ uptake via Trk^H in *V. alginolyticus* (Va) *sapBCD*-plasmid containing cells of strain LB625 was stimulated by growing the cells in the presence of 0.3 mM IPTG. Under these conditions maximal Trk^H activity was comparable to that of cells containing *E. coli sapDF* genes on the plasmid (Fig. 3b and 3a, respectively). VaSapD stimulated Trk^H activity to some extent (plasmids pKT72 and pKT77; Fig. 3b) and VaSapF alone was inactive (pKT701; Fig. 3b). However, in contrast to the situation with EcSapD, plasmids encoding both of the integral membrane proteins VaSapBC supported the VaSapD function, since the stimulation of K⁺ uptake by cells containing plasmid pKT7 (*VasapBCD*⁺; Fig. 3b) was larger than that of cells containing either plasmid pKT72 (*VasapCD*⁺; Fig. 3b) or plasmid pKT77 (*VasapD*⁺; Fig. 3b). Finally, only plasmid pKT70, containing *VasapBCDF*, exerted the maximal effect (Fig. 3b), suggesting that VaSapF also supports the function of VaSapBCD in this test. Despite the fact that these plasmids directed the synthesis of approximately equal amounts of VaSapD in *E. coli* mini-cells (results not shown), these effects might still be due to differences in *VasapD* gene expression by the different plasmids in strain LB625.

K⁺ transport via Trk^G

For these experiments we used strain LB680 ($\Delta sapABCD::Km^R$ $\Delta trkH::Cm^R$) containing the plasmids in Fig. 1. The same type of experiments were carried out as in Fig. 3. The Trk^G system exhibited about 30% residual activity in the absence of *sap* genes (Fig. 4a), confirming earlier data (Dosch *et al.*, 1991). How-

Fig. 2. All five EcSap proteins are synthesized in mini-cells. Plasmid-encoded proteins were labelled with [35 S]methionine in mini-cells from *E. coli* strain DK6. The proteins were separated by SDS-PAGE and detected on the dried gel by autoradiography. The positions of the Sap proteins and the molecular mass of marker proteins are indicated. Lanes: 1, pT7-6 (control); 2, pTWE341 (SapABCDF); 3, pCH100 (SapABCD); 4, pCH101 (SapBCDF); 5, pCH102 (SapABC); 6, pCH103 (SapBC); 7, pCH104 (SapAF); 8, pCH105 (SapDF); 9, pCH106 (SapD); 10, plasmid pCH107 (SapF).

Fig. 3. ATP-binding proteins SapDF are sufficient for K^+ uptake via the *E. coli* Trk^H system. *E. coli* strain LB625 ($\Delta trkG \Delta sapABCD$) contained *Ecsap* (a) and *Vasap* plasmids (b). Net K^+ uptake by K^+ -depleted cells was assayed as described in Methods. K^+ uptake by glucose-metabolizing cells was initiated at $t = 0$ by the addition of 1 mM KCl. Cells containing *Vasap* plasmids (b) were grown in the presence of 0.3 mM IPTG. (a) $\square$, no plasmid; $\triangle$, pTWE341 (*EcsapABCDF* $^+$); $\bullet$, pCH100 (*EcsapABCD* $^+$); $\blacktriangle$, pCH102 (*EcsapABC* $^+$); $\blacksquare$, pCH105 (*EcsapDF* $^+$); $\blacklozenge$, pCH106 (*EcsapD* $^+$); $\blacktriangledown$, pCH107 (*EcsapF* $^+$). pCH101 (*EcsapBCDF* $^+$) gave a result identical to that of pTWE341 (*EcsapABCDF* $^+$); pCH103 (*EcsapBC* $^+$) and pCH104 (*EcsapAF* $^+$) gave results identical to cells without a plasmid (not shown). (b) $\circ$, pHG165 (vector); $\diamond$, pKT70 (*VasapBCDF* $^+$); $\triangle$, pTWE341 (*EcsapABCDF* $^+$, control); $\bullet$, pKT7 (*VasapBCD* $^+$); $\blacksquare$, pKT72 (*VasapCD* $^+$); $\blacklozenge$, pKT77 (*VasapD* $^+$); $\blacktriangledown$, pKT701 (*VasapF* $^+$).

ever, in contrast to the latter, we observed that this effect was due to a reduction of V_{max} for K^+ of Trk^G (results not shown). With respect to stimulation of K^+ uptake by plasmid-encoded EcSap proteins, the results with Trk^G differed from those of Trk^H , in that (i) plasmid-encoded SapD alone was sufficient for full stimulation of K^+ transport, (ii) the presence of plasmid pCH100, encoding SapABCD was without effect (Fig. 4a) and (iii) the additional presence of SapF gave the full effect (pTWE341; Fig. 4a).

Fig. 4. Effect of plasmid-encoded Sap proteins on the *E. coli* Trk^G system. *E. coli* strain LB680 ($\Delta trkH \Delta sapABCD$) contained *Ecsap* (a) or *Vasap* plasmids (b). Cells containing *Vasap* plasmids (b) were grown in the presence of 0.3 mM IPTG. Net K^+ uptake by K^+ -depleted cells was assayed as described in Methods. K^+ uptake by glucose-metabolizing cells was initiated at $t = 0$ by the addition of 1 mM KCl. Symbols are as described in the legend to Fig. 3 except: (a) $\circ$, plasmid pT7-6 (vector).

With one exception, the effects of *Vasap* plasmids on Trk^G were similar to those of their *E. coli* counterparts (Fig. 4b and 4a, respectively): the presence of plasmid pKT7 (*VasapBCD* $^+$) inhibited the intrinsic K^+ -uptake activity of strain LB680 by as much as 70 % (Fig. 4b). We will return to the results of Figs 3 and 4 in the Discussion.

Protein variants of the EcSapD Walker A box residue Lys-46

SapDF are the ATP-binding cassette (ABC) subunits of the SapABCDF complex and in analogy with other bacterial systems, we assume that through ATP hydrolysis they energize the uptake by the cells of the unknown substrate via the SapABCDF complex (Schneider & Hunke, 1998). These ABC subunits

Fig. 5. Effect of EcSapD protein variants on Trk^H activity. (a) Variants of the Walker A box SapD residue Lys-46; (b) variants of the Walker B box SapD residue Asp-183; (c) changes in residues Gly-162 and Gln-165 of the SapD signature motif region. K⁺ uptake by K⁺-depleted cells of strain LB625 ($\Delta trkG \Delta sapABCDF$) containing plasmid pCH105 (*EcsapDF*⁺) or one of its derivatives was measured as described in Methods. K⁺ uptake by glucose-metabolizing cells was initiated at $t = 0$ by the addition of 1 mM KCl. (a–c) $\circ$, pT7-6 (vector control); $\square$, pCH105 (*EcsapDF*⁺, positive control). (a) ∇ , pCC3 (*EcsapD*-46Met, *EcSapF*); $\blacktriangle$, pCC4 (*EcsapD*-46Ile, *EcSapF*); $\blacklozenge$, pCC7 (*EcsapD*-46Gln, *EcSapF*); $\blacksquare$, pCC8 (*EcsapD*-46Gly, *EcSapF*); $\bullet$, pCC11 (*EcsapD*-46Arg, *EcSapF*); $\bullet$, pCC101 (*EcsapD*- Δ Lys46, *EcSapF*). (b) $\blacktriangle$, pCC15 (*EcsapD*-183Glu, *EcSapF*); $\blacksquare$, pCC16 (*EcsapD*-183Asn, *EcSapF*); $\bullet$, pCC17 (*EcsapD*-183Lys, *EcSapF*). (c) $\bullet$, pCH31 (*EcsapD*-162Ala, *EcSapF*); $\blacksquare$, pCH32 (*EcsapD*-165Leu, *SapF*).

contain conserved sequences essential for ATP binding and/or ATP hydrolysis (Walker *et al.*, 1982). The Walker A box consists of the conserved sequence GXXGXGKT/S (Walker *et al.*, 1982; Boos & Lucht, 1996; Schneider & Hunke, 1998). It reads as 40-GESGSGKS-47 in both EcSapD and VaSapD. In HisP, for which a crystal structure is available (Hung *et al.*, 1998), the charged group of the side-chain Walker A box lysine residue interacts with the β -phosphate group of ATP.

To determine the effect of adenine nucleotides via EcSapD on K⁺ uptake via EcTrkH, the Walker A box lysine codon 46 of *sapD* in plasmid pCH105 (*sapDF*⁺) was replaced by codons for arginine, glutamine, methionine, isoleucine or glycine (plasmids pCC11, pCC7, pCC3, pCC4 and pCC8, respectively) and the effect of these changes on K⁺ uptake by strain LB625 containing these plasmids was determined (Fig. 5a). Remarkably, in none of these strains was K⁺ transport activity inhibited completely. Even with plasmid pCC8 (encoding SapD variant Gly-46, which showed the largest effect) K⁺ uptake still occurred at a rate of about 25% of the control. Only the presence of plasmid pCC101, containing *sapDF* in which the codon for Lys-46 of *sapD* had been deleted, hardly showed any stimulation of net K⁺-uptake activity by strain LB625 above background (Fig. 5a). This effect is unlikely to be due to lack of *sapDF* gene expression, since the pCC101-encoded SapD 'mutin' was made in normal amounts in *E. coli* mini-cells (results not shown).

Similar results (not shown) were obtained with cells that contained both variants of SapD residue Lys-46 and of

the similar Walker A box SapF residue Lys-53 [plasmids pCC1 (SapD-46Gln; SapF-53Gln) or pCC2 (SapD-46Arg; SapF-53Gln)] or with cells containing plasmid pCC111, which was derived from pCH106 (only *sapD*, Fig. 1a), encoding the variant SapD-46Gly: none of these cells showed a complete inhibition of transport activity.

Protein variants of the EcSapD Walker B box residue Asp-183

The Walker B box of ABC proteins consists of the conserved sequence hhhhD, in which h represents an apolar residue and D is an aspartate residue (Walker *et al.*, 1982; Boos & Lucht, 1996; Schneider & Hunke, 1998). It reads as 179-Leu-Leu-Ile-Ala-Asp-183 in both EcSapD and VaSapD. In HisP the side chain of the conserved Walker B box residue aspartate interacts via a water molecule with both the β - and γ -phosphate group of ATP (Hung *et al.*, 1998). The residue Asp-183 of SapD in plasmid pCH105 (*sapDF*⁺) was replaced by glutamate, asparagine or lysine (plasmids pCC15, pCC16 and pCC17, respectively). The effect of these changes on K⁺ uptake via Trk^H was determined (Fig. 5b). The strongest inhibition (85%) was observed for the plasmid encoding the SapD variant Lys-183 (Fig. 5b).

Protein variants in the EcSapD signature motif residues Gly-162 and Gln-165

In ABC proteins the conserved 'signature motif' is thought to play a role in energy coupling between ATP hydrolysis and transport. In both HisP and MalK this region is located relatively far away from the nucleotide-

Fig. 6. Sap-independent K^+ transport by Trk systems in *E. coli*. K^+ uptake by K^+ -depleted cells was measured as described in Methods. K^+ uptake was initiated by the addition of 1 mM KCl to the cell suspension at $t = 0$. Δ , LB625 ($\Delta trkG \Delta sapABCD$); $\square$, LB680 ($\Delta trkH \Delta sapABCD$); $\circ$, LB690 ($\Delta trkG \Delta trkH \Delta sapABCD$); $\blacktriangle$, LB625(pAS8) ($trkG^+$); $\blacksquare$, LB680(pAS8) ($trkG^+$); $\bullet$, LB690(pAS8) ($trkG^+$); $\blacklozenge$, LB625(pTWE341) ($sapABCD^+$, positive control); $\blacktriangledown$, LB625(pKT65) ($VatrAH^+$).

binding site in a region that might interact with the integral membrane proteins of the transport complex (Hung *et al.*, 1998; Diederichs *et al.*, 2000). For MalK from *S. typhimurium*, Schmees *et al.* (1999) have characterized several protein variants of linker region residues that still bind the ATP analogue 8-azido-ATP, but for which the MalEFGK₂ complex has become inactive in maltose transport. We observed that the similar signature motif SapD variants Ala-162 (instead of glycine) and Leu-165 (instead of glutamine) exhibited a 20 and 40% inhibition of K^+ -uptake activity of the Trk^H system, respectively (Fig. 5c).

The most likely explanation for the data in Fig. 5 is that ATP binding to SapD rather than its ATP hydrolysis is required for TrkH activity, confirming an early conclusion that ATP activates the *E. coli* Trk system (Stewart *et al.*, 1985).

Trk activity in the absence of sap gene products

Fig. 6 shows that in a $\Delta sapABCD$ background K^+ -uptake activity by Trk^H activity was low, but not zero as is the case for $\Delta trkH \Delta trkG \Delta sapABCD$ strain LB690 (Fig. 6). Hence, both Trk^H and Trk^G are partially active in a $\Delta sapABCD$ background (Figs 4 and 6). To obtain more information on this phenomenon, we investigated the influence of plasmid pAS8-encoded TrkG on K^+ transport by strains LB625 ($\Delta trkG \Delta sapABCD$), LB680 ($\Delta trkH \Delta sapABCD$) and LB690. All three strains took

up K^+ about twice as rapidly as did strain LB680 without the plasmid (Fig. 6), indicating that *trkG* exerts a gene dose effect in this process. As reported previously (Nakamura *et al.*, 1998a), K^+ uptake via plasmid-encoded VaTrk in *E. coli* was independent of *sapABCD* (Fig. 6) and even exceeded K^+ -uptake activity by strain LB625(pTWE341) (Fig. 6). Additional results (not shown) indicated that the differences in transport activity observed in Fig. 6 are due to differences in V_{max} values for K^+ of the different systems.

Energy coupling to K^+ uptake via Trk in *E. coli* $\Delta sapABCD$ strains

The easiest way to explain the data in Fig. 6 is to assume that K^+ uptake via EcTrk^G and VaTrk is independent of ATP. Hence, the mode of energy coupling to K^+ uptake by these strains was investigated. For this purpose strains LB627 ($\Delta atpB-C \Delta sapABCD \Delta trkG$), LB682 ($\Delta atpB-C \Delta sapABCD \Delta trkH$) and LB692 ($\Delta atpB-C \Delta sapABCD \Delta trkH \Delta trkG$) were constructed by P1 transduction from strains LB625, LB680 and LB690, respectively. In contrast to wild-type strains, ATP levels and the pmf can be influenced independently from each other in an *atp* (formerly *unc*) strain. This is achieved by: (i) glucose metabolism in the presence of a protonophore, like 2,4-dinitrophenol, resulting in a high ATP level in the absence of a pmf; (ii) succinate respiration by energy-starved cells, leading to a high pmf and a low ATP level in the cells; and (iii) glucose metabolism in the absence of a protonophore, giving rise to both a high cytoplasmic ATP concentration and a high pmf (Berger, 1973; Rhoads & Epstein, 1977). Such experiments on the mode of energy coupling to K^+ transport have been carried out with the *E. coli* Trk system (Rhoads & Epstein, 1977). Their results suggested that Trk activity depends both on a high pmf and a high cytoplasmic ATP concentration. However, at that time it was not known that in this *E. coli* strain Trk consists of the two separate Trk^H and Trk^G systems, each of which contribute approximately equally to total Trk activity (Dosch *et al.*, 1991; Schlösser *et al.*, 1995). We determined the mode of energy coupling to K^+ transport for both EcTrk systems [strains LB625(pTWE341) and LB627(pTWE341) for Trk^H and strains LB680(pTWE341) and LB682(pTWE341) for Trk^G], for Trk^G in a $\Delta sapABCD$ background [strains LB680(pAS8) and LB682(pAS8)] and for plasmid-encoded VaTrk in an *E. coli* $\Delta sapABCD$ background [strains LB690(pKT65) and LB692(pKT65)]. The results on K^+ uptake were compared with transport of proline and glutamine by these cells, which give an indication of the presence of a high membrane potential (which is the main driving force for proline uptake under these conditions) and a high cytoplasmic ATP concentration (ATP is the driving force for glutamine uptake via an *E. coli* ABC transporter), respectively (Berger, 1973; Rhoads & Epstein, 1977). The ATP content of the cells was also measured.

In glucose-metabolizing cells of the Δatp strain LB627(pTWE341) the dependence of the initial rates of

Fig. 7. K⁺ uptake via Trk^H depends on the membrane potential. Cells of strain LB627 ($\Delta trkG \Delta sapABCDF \Delta atpB-C$) containing plasmid pTWE341 ($EcsapABCDF^+$) were depleted of energy reserves and of K⁺ as described in Methods. Glucose (10 mM) and 2,4-dinitrophenol at the concentration indicated on the abscissa were added at $t = -10$ min. KCl (1 mM, ○), 4 μ M [¹⁴C]proline (△) or 2 μ M [¹⁴C]glutamine (□) were added at $t = 0$ and the initial rates of uptake of these compounds by the cells, as well as cell ATP content (■) were determined. One hundred percent values [expressed in nmol min⁻¹ (mg dry wt cells)⁻¹] were 210 for K⁺ uptake, 3 for proline uptake and 1.5 for glutamine uptake, respectively. The 100% value for ATP content was 4.6 nmol (mg dry wt cells)⁻¹.

K⁺ uptake via the Trk^H system, of proline uptake and of glutamine uptake were measured as a function of 2,4-dinitrophenol concentration (Fig. 7). Both the uptake of K⁺ and that of proline were strongly inhibited by the protonophore under conditions at which both the ATP level in the cells and the rate of glutamine uptake remained high (Fig. 7). Similar results were obtained with the EcTrk^G system [strain LB682(pTWE341)], chromosome and plasmid-encoded Trk^G in a $\Delta sapABCDF$ background [strain LB682(pAS8)], and plasmid-encoded VaTrk in an *E. coli* $\Delta sapABCDF$ background [(strain LB692(pKT65))] (results not shown). These data indicate (i) that K⁺ uptake via all four systems depends on the membrane potential and (ii) that in contrast to glutamine transport a high cytoplasmic ATP concentration alone is not sufficient to drive K⁺ transport via these systems.

Next it was investigated whether ATP is also required. Fig. 8 shows such an experiment for EcTrk^H. It was observed (i) that the uptake of both K⁺ and glutamine occurred in the *atp* wild-type strain LB625(pTWE341) with either glucose or succinate as the substrate; (ii) that these compounds were also taken up by the *atp* strain LB627(pTWE341) in the presence of glucose, but not in

Fig. 8. K⁺ uptake via Trk^H depends on ATP. Cells of either the *atp*⁺ strain LB625 ($\Delta trkG \Delta sapABCDF$) containing plasmid pTWE341 ($sapABCDF^+$) or of strain LB627 $\Delta trkG \Delta sapABCDF \Delta atpB-C$ containing plasmid pTWE341 were depleted of energy reserves and of K⁺ as described in Methods. Glucose (10 mM) or succinate (20 mM) were added as an energy source at $t = -15$ and -30 min, respectively. KCl (2 mM), 4 μ M [¹⁴C]proline or 2 μ M [¹⁴C]glutamine were added at $t = 0$ and the uptake of these compounds by the cells, as well as cell ATP content were determined as a function of time. (a) K⁺ uptake; (b) ATP content; (c) glutamine uptake; (d) proline uptake. ○, △, LB625(pTWE341); ●, ▲, LB627(pTWE341); ○, ●, substrate glucose; △, ▲, substrate succinate.

the presence of succinate (Fig. 8a, c); (iii) that proline was taken up under all four conditions (Fig. 8d); and (iv) that succinate-metabolizing cells of the Δatp strain were not able to generate a high cytoplasmic ATP level (Fig. 8b). These data show a positive correlation between Trk^H activity and the cytoplasmic ATP concentration.

Table 2. Energy coupling to K⁺ transport by Trk systems

System	Strain	<i>atp</i> genotype	Substrate	K ⁺ transport (nmol min ⁻¹ mg ⁻¹)	[ATP] _{in} (nmol mg ⁻¹)	[¹⁴ C]Proline transport (nmol min ⁻¹ mg ⁻¹)	[¹⁴ C]Glutamine transport (nmol min ⁻¹ mg ⁻¹)
EcTrk ^H	LB625(pTWE341)	WT	Glucose	112	4.0	3.8	1.2
			Succinate	82	5.5	1.4	0.5
	LB627(pTWE341)	$\Delta atpB$ -C	Glucose	169	3.9	1.5	0.7
			Succinate	20	0.5	0.52	0.02
EcTrk ^G	LB680(pTWE341)	WT	Glucose	102	2.8	2.3	2.4
			Succinate	66	3.8	1.0	1.1
	LB682(pTWE341)	$\Delta atpB$ -C	Glucose	99	3.1	1.7	1.0
			Succinate	4	0.4	0.5	0.03
EcTrk ^G ($\Delta sapABCDF$)	LB680(pAS8)	WT	Glucose	45	3.1	2.3	2.0
			Succinate	48	4.5	1.1	1.0
	LB682(pAS8)	$\Delta atpB$ -C	Glucose	57	2.6	2.4	1.2
			Succinate	8	0.4	1.2	0.03
VaTrkAH ($\Delta sapABCDF$)	LB690(pKT65)	WT	Glucose	174	3.5	1.2	0.9
			Succinate	125	2.7	0.7	0.4
	LB692(pKT65)	$\Delta atpB$ -C	Glucose	170	4.0	1.7	1.1
			Succinate	9	0.9	0.6	0.03

Similar results were obtained with the chromosomally encoded EcTrk^G system (Table 2). Together these data support the notion that the activity of the *E. coli* Trk system [which is now known to consist of the systems Trk^H and Trk^G (Dosch *et al.*, 1991)] requires both a pmf and ATP for activity (Rhoads & Epstein, 1977; Stewart *et al.*, 1985).

It was then investigated whether this also holds true for K⁺ transport via EcTrk^G and VaTrk in *E. coli* $\Delta sapABCDF$ strains (Table 2). The results were very similar to those obtained with *E. coli* Trk^H and Trk^G systems (Fig. 8, Table 2), suggesting that energy coupling to K⁺ transport was similar in all four situations. Hence, these data argue against the notion that K⁺ uptake via Trk systems in a $\Delta sapABCDF$ background in *E. coli*, as observed in Fig. 6, occurred due to a lack of ATP dependence of this process.

DISCUSSION

More than 20 years ago Rhoads & Epstein (1977) reported that the *E. coli* Trk system has an unusual mode of energy coupling in that both ATP and a high transmembrane pmf are required for its activity. Subsequent work suggested that ATP activates the system (Stewart *et al.*, 1985). The data reported here support the latter notion. Essential for our work was that Groisman and his coworkers had established that in *S. typhimurium* a gene essential for protamine resistance maps inside the *sapABCDF* operon and that *sap* genes of this organism are identical to *trk* genes in *E. coli* (Groisman *et al.*, 1992; Parra-Lopez *et al.*, 1993, 1994). Subsequently, the *EctrkE* locus was found to map inside the *sapABCDF* operon, presumably at *sapD* (W. Epstein, personal communication). With *E. coli* $\Delta trkH$, $\Delta trkG$

and $\Delta sapABCDF$ strains and deletion plasmids derived from either the *E. coli* *sapABCDF* plasmid pTWE341 (Bossemeyer *et al.*, 1989) or the *V. alginolyticus* *sapBCDF* plasmids pKT7 and pKT70 (Fig. 1) we established that the ATP-binding protein SapD was sufficient for transport activity of the two EcTrk systems (Figs 3 and 4). This result supplies a molecular basis for the observation that Trk activity is ATP-dependent (Rhoads & Epstein, 1977; Stewart *et al.*, 1985). Further information was then sought about the nature of the effect of SapD on K⁺ transport via Trk^H by changing residues in three conserved regions of ABC proteins (Walker A box, Walker B box and the signature motif, respectively). Amazingly, only in the case where the conserved residue Lys-46 of SapD was deleted was K⁺ uptake inhibited completely, whereas with all other SapD protein variants the system showed considerable residual activity (Fig. 5).

In accordance with what is known about other ABC systems (Schneider & Hunke, 1998; Schmees *et al.*, 1999), we conclude that rather aspecific effects such as observed in Fig. 5 suggest that ATP binding to SapD rather than its hydrolysis by the ABC protein is essential for activity of the Trk^H system. More specifically, first, Schmees *et al.* (1999) have described exactly such effects for the signature motif region protein variants Gly-137 → Ala and Gln-140 → Leu of the ATP-binding protein MalK from the maltose-uptake system MalEFGK₂ from *S. typhimurium*: ATP binding to the isolated variant MalK proteins still occurs, but these proteins do not hydrolyse ATP and transport activity of the complex is inhibited. Our result that cells with the similar SapD variants Gly-162 → Ala and Gln-165 → Leu were still active in K⁺ transport via TrkH (Fig. 5c) suggests that ATP still binds to these protein variants and that this

binding is sufficient for activity. Second, a common effect of mutations in the Walker A box lysine codon is that ATP hydrolysis, but not ATP binding is impaired. Our data that with these protein variants Trk^H activity was only partially inhibited (Fig. 5a), support the notion that ATP binding to SapD is sufficient for Trk^H activity. Finally, many mutations in the conserved Walker B box aspartate codon abolish both ATP hydrolysis and ATP binding. Since some mutations in the *sapD* Walker B box aspartate codon had relatively large effects on K⁺ transport (Fig. 5b), these data suggest that at least ATP binding to SapD is required for Trk^H activity. However, it should be realized that all of our experiments have been carried out with intact cells, in which under energized conditions the ATP concentration amounts to 3–5 mM (Table 2 and Schleyer *et al.*, 1993). This concentration is three orders of magnitude higher than that used to analyse effects of 8-azido-ATP binding to isolated variants of ABC proteins (Schmees *et al.*, 1999). Hence, it is difficult to compare the results of Fig. 5 with those of isolated proteins. Nevertheless, our data support the older notion that ATP plays a regulatory role in K⁺ uptake via Trk (Stewart *et al.*, 1985).

Kir-SUR is a eukaryotic ABC system in which adenine nucleotide binding to SUR regulates the activity of the small K⁺ channel Kir (Bryan & Aguilar-Bryan, 1999). SUR contains two membrane domains equivalent to the integral membrane protein subunits SapB and SapC from SapABCDF and these domains are thought to confer adenine nucleotide dependence to the channel (Bryan & Aguilar-Bryan, 1999). Kir-SUR resembles Sap-Trk in the following aspect: Kir contains only two transmembrane helices and thereby resembles the bacterial K⁺ channel KcsA. The Kir active complex is thought to be a tetramer surrounded by four SUR subunits, forming a huge supra-molecular membrane complex (Shyng & Nichols, 1997; Inagaki *et al.*, 1997; Clement *et al.*, 1997; Babenko *et al.*, 1998). The main part of the TrkH protein consists of a domain composed of eight transmembrane helices that is likely to have evolved from four covalently linked K⁺-channel subunits of the KcsA/Kir type (Durell *et al.*, 1999; Durell & Guy, 1999). By analogy with the Kir-SUR complex we would have expected the Sap-TrkH complex to contain at least the SapBCDF proteins, and that four SapB or SapC proteins interact with one TrkH or TrkG subunit by helix-helix interaction in the apolar part of the membrane. However, we have found no direct evidence for this, since most of our results with plasmid-encoded Sap proteins from *E. coli* and *V. alginolyticus* suggest that SapD is sufficient for K⁺ transport via the EcTrk systems (Figs 3 and 4). The fact that some of these data indicate that other subunits than SapD also support the activity of the Trk systems is most easily explained by assuming differences in *sapD* gene expression in the different cells.

The best interpretation of the observation that Trk^G and VaTrk are active in an ATP-dependent manner in *E. coli* Δ *sapABCDF* strains (Figs 6–8, Table 2) is to assume that these Trk systems can also interact with an ABC protein

other than SapD. This would also explain the apparent contradiction that TrkH systems are widely spread among prokaryotes (Durell *et al.*, 1999), but that *sapABCDF* operons are limited to a the γ -proteobacteria (results of our genome analyses for Sap proteins are not shown). The ATP dependence of Trk systems from organisms outside this group [e.g. *Enterococcus hirae* (Bakker & Harold, 1980)] would then be conferred by interaction with an unknown ABC protein.

An alternative interpretation of these data was also considered. One relatively simple explanation would be that EcTrkAG and VaTrkAH contain an additional adenine nucleotide binding site and that binding of ATP to this site stimulates K⁺ transport. Such a model would once again be similar to that of the K⁺ channel Kir, the activity of which has been shown to be inhibited by ATP in the absence of SUR and which has been shown to bind 8-azido-ATP (reviewed by Ueda *et al.*, 1999). Isolated TrkA binds dinucleotides rather than ATP (Schlösser *et al.*, 1993), leaving TrkH/TrkG as the subunits that should bind the nucleotide. However, at present it is not known whether these subunits contain such a site. Moreover, this model presents difficulties in explaining why Trk^H and Trk^G exert such a different K⁺ transport activity in a Δ *sapABCDF* background. Therefore, we prefer the above explanation that Trk systems can interact with an ABC protein other than SapD.

It remains unclear what the other function of SapABCDF is besides its role in K⁺ uptake in *E. coli*. Our preliminary results indicate that *sap* transcripts are made in cells grown in a mineral salt medium and that neither the presence of oligopeptides, as present in tryptone, nor treatment of the cells with protamine significantly influences the amount of transcript. Moreover, the peptide-uptake subgroup of bacterial ABC systems, to which SapABCDF belongs, covers systems with a broad substrate specificity, including a Ni²⁺ transporter (Navarro *et al.*, 1993) and a haem-binding subunit from *Haemophilus influenzae* (Hanson *et al.*, 1992). Hence it is impossible to predict from primary structure data what type of substrate is transported by SapABCDF.

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