Identification of the ABC protein SapD as the subunit that confers ATP dependence to the K⁺-uptake systems \( \text{T} \text{rk}^\text{H} \) and \( \text{T} \text{rk}^\text{G} \) from \textit{Escherichia coli} K-12

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The activity of the two almost identical K⁺-uptake systems, \( \text{T} \text{rk}^\text{H} \) and \( \text{T} \text{rk}^\text{G} \), from \textit{Escherichia coli} K-12 depends completely and partially on the presence of the \( \text{trk}^\text{E} \) gene, respectively. \( \text{trk}^\text{E} \) maps inside the \( \text{sap}^\text{ABCDF} \) 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 \( \text{sap}^\text{ABCDF} \) gene products in the ATP dependence of the \( \text{E. coli} \) \( \text{T} \text{rk} \) systems. For this purpose \( \Delta\text{sapABCDF} \Delta\text{trk}^\text{G} \) and \( \Delta\text{sapABCDF} \Delta\text{trk}^\text{H} \) strains of \( \text{E. coli} \) containing plasmids with \( \text{sap} \) genes from either \( \text{E. coli} \) or \textit{Vibrio alginolyticus} were used. All five plasmid-encoded \( \text{E. coli} \) \( \text{sap} \) proteins were made in \( \text{E. coli} \) mini-cells. The presence of the ATP-binding \( \text{sap} \) protein from either \( \text{E. coli} \) or \textit{V. alginolyticus} alone was sufficient for stimulating the K⁺ transport activity of the \( \text{T} \text{rk}^\text{H} \) and \( \text{T} \text{rk}^\text{G} \) systems. K⁺-uptake experiments with \textit{Escherichia coli} cells containing \( \text{sap} \) 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 \( \text{sapD} \) rather than ATP hydrolysis by this subunit is required for the activity of the \( \text{E. coli} \) \( \text{T} \text{rk}^\text{H} \) system. K⁺ transport via two plasmid-encoded \( \text{T} \text{rk} \) systems in a \( \Delta\text{sapABCDF} \text{E. coli} \) strain remained dependent on both a high membrane potential and a high cytoplasmic ATP concentration, indicating that in \( \text{E. coli} \) ATP dependence of \( \text{T} \text{rk} \) activity can be independent of \( \text{sap} \) proteins. These data are interpreted to mean that \( \text{T} \text{rk} \) systems can interact with an ABC protein other than \( \text{sapD} \).

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

INTRODUCTION

Under most conditions of growth \textit{Escherichia coli} K-12 cells accumulate K⁺ via two almost identical high-rate, low-affinity systems, \( \text{T} \text{rk}^\text{H} \) and \( \text{T} \text{rk}^\text{G} \). The products of four non-linked genes, \( \text{trkA} \), \( \text{trkE} \), \( \text{trkG} \) and \( \text{trkH} \) contribute to the activity of these systems (Walderhaug \textit{et al.}, 1987; Dosch \textit{et al.}, 1991; Schlösser \textit{et al.}, 1995). The \( \text{T} \text{rk}^\text{H} \) and \( \text{T} \text{rk}^\text{G} \) gene products (\( \text{T} \text{rkH} \) and \( \text{T} \text{rkG} \), respectively) are the K⁺-translocating subunits of each system. They are similar proteins, sharing 41% identical amino acid residues (Schlösser \textit{et al.}, 1995). The reason that \( \text{E. coli} \) K-12 possesses two \( \text{T} \text{rk} \) systems appears to be that \( \text{trk}^\text{G} \), a second copy of \( \text{trk}^\text{H} \), has been inserted with the prophage \( \text{rac} \) into the chromosome (Schlösser \textit{et al.}, 1992, 1995). However, genome data show that it is an exception for prokaryotes to contain two \( \text{trk} \)-like genes (Durell \textit{et al.}, 1999). \( \text{T} \text{rk}^\text{H} \) and \( \text{T} \text{rk}^\text{G} \) belong to a superfamily of K⁺-transporters with members in plants.

Abbreviations: ABC, ATP-binding cassette; EcTrk, VaTrk, Trk from \textit{E. coli} and \textit{V. alginolyticus}, respectively; EcSac, VaSac, Sap proteins and sap genes, respectively, from \textit{E. coli}; pmf, proton-motive force; VaSac and Vasap, Sap proteins and sap genes, respectively, from \textit{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; 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\(^0\) 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 ΔtrakE* strain (Nakamura et al., 1998a). In both *S. typhimurium* and *E. coli* trkE maps inside the sapABCDF 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 sapABCDF 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 SapABCDF 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 sapABCDF 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 sapABCDF 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 sapABCDF 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\(^0\) 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 ΔatpB-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 carbencillin \(l^{-1}\) and 20 mM glucose.

Plasmids pCH100–pCH107 were generated from plasmid pTWE341 by deleting parts of the *E. coli* sapABCDF operon with the restriction enzymes indicated in Fig. 1(a). Plasmids pK17 and pKT70 contain Sau3AI restriction fragments with chromosomal *V. alginolyticus* sap genes, cloned into the BamHI site of plasmid pHG165 (Stewart et al., 1986). These plasmids were identified by complementation of the K\(^+\) transport defect of *E. coli ΔsapABCDF* strains. Plasmids of the pKT7 series were generated from plasmids pKT17 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 sapABCDF 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 BamHI 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 BamHI site. This DNA fragment, carrying the desired sapABCDF deletion was treated with BamHI and cloned into the BamHI site of plasmid pKO3, giving plasmid pCH410. The ΔsapABCDF 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 ΔsapA–F deletion in this strain was checked by Southern hybridization. Strains were made ΔatpB-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 \(^{35}\)S)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,
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
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⁺, [¹⁴C]proline, [¹⁴C]glutamine and ATP, a cell suspension with an OD_{600} 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 was determined by the method of Bakker & Mangerich (1981). K⁺ and energy reserves of cells of atp⁺ or ΔatpB-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 removed by washing the cells three times with cold buffer A, containing 75 mM sodium phosphate, 0·4 mM MgSO₄ 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 = 10 min and 0·67 min, respectively, and the cell suspension was shaken at 160 r.p.m. At t = 0 min, 2 mM KCl, 2 µM [¹⁴C]glutamine (40 nCi ml⁻¹) or 4 µM [¹⁴C]proline (100 nCi ml⁻¹) was added and the suspension was shaken as described above. For the [¹⁴C]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 µm; 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 H₃PO₄ 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 KClO₄ and protein by centrifugation, ATP was determined in the supernatant by using a luminescence assay (Kimmich et al., 1975).
RESULTS

Nucleotide sequence of the sapABCDF 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 sapABCDF 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 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 EcsapABCDF operon (Nakamura et al., 1998a) it was important to determine whether V. alginolyticus also contains a sapABCDF operon. Clones containing most of sapA and complete sapBCDF 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 sapABCDF transcription was not necessarily directed by its own promoter(s).

Role of sap gene products in K⁺ transport via EcTrkH

For this purpose strain LB625 (ΔsapABCDF ΔtrkG::KmH) 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 TrkH of strain LB625, containing the plasmids in Fig. 1(a), could be tested. In accordance with Dosch et al. (1991) we observed that TrkH 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 (sapABC+)] with plasmids pCH105 (sapDF+) and pTWE341 (sapABCDF+), respectively (Fig. 3a)]. By contrast, SapF alone was inactive [plasmids pCH107 (sapF+; Fig. 3a) and pCH104 (sapAF+; results not shown), respectively].

K⁺ uptake via TrkH in V. alginolyticus (Va) sapBCDF-plasmid containing cells of strain LB625 was stimulated by growing the cells in the presence of 0.3 mM IPTG. Under these conditions maximal TrkH activity was comparable to that of cells containing E. coli sapDF genes on the plasmid (Fig. 3b and 3a, respectively). VaSapD stimulated TrkH 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 TrkE

For these experiments we used strain LB680 (ΔsapABCDF::KmH ΔtrkH::CmH) containing the plasmids in Fig. 1. The same type of experiments were carried out as in Fig. 3. The TrkE system exhibited about 30% residual activity in the absence of sap genes (Fig. 4a), confirming earlier data (Dosch et al., 1991). How-
SapD alone was sufficient for full stimulation of K\textsuperscript{+} uptake via the E. coli Trk\textsuperscript{K} system. E. coli strain LB625 (ΔtrkG ΔsapABCD) contained Ecsap (a) and Vasap plasmids (b). Net K\textsuperscript{+} uptake by K\textsuperscript{+}-depleted cells was assayed as described in Methods. K\textsuperscript{+} 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) □, no plasmid; △, pTWE341 (EcsapABCD); ●, pCH100 (EcsapABC); ▲, pCH102 (EcsapABC); ■, pCH105 (EcsapDF); ●, pCH106 (EcsapD); ▼, pCH107 (EcsapF). pCH101 (EcsapBCDF) gave a result identical to that of pTWE341 (EcsapABCD); pCH103 (EcsapBC) and pCH104 (EcsapAF) gave results identical to cells without a plasmid (not shown). (b) ○, pHG165 (vector); ○, pKT70 (VasapBCDF); △, pTWE341 (EcsapABCD, control); ●, pKT7 (VasapBC); ■, pKT72 (VasapCD); ●, pKT77 (VasapD); ▼, pKT701 (VasapF).

Fig. 2. All five EcSap proteins are synthesized in mini-cells. Plasmid-encoded proteins were labelled with [\textsuperscript{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 (SapABCD); 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).

With one exception, the effects of Vasap plasmids on Trk\textsuperscript{K} were similar to those of their E. coli counterparts (Fig. 4b and 4a, respectively): the presence of plasmid pKT7 (VasapBCDF) inhibited the intrinsic K\textsuperscript{+}-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
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 ‘mutein’ 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 pCC11, 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 TrkH 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-
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 MalEFGKα complex becomes 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 TrkH 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 ΔsapABCDF background K⁺-uptake activity by TrkH activity was low, but not zero as is the case for ΔtrkH ΔtrkG ΔsapABCDF strain LB690 (Fig. 6). Hence, both TrkH and TrkG are partially active in a ΔsapABCDF background (Figs 4 and 6). To obtain more information on this phenomenon, we investigated the influence of plasmid-encoded VaTrk in E. coli was independent of sapABCDF (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 Vmax values for K⁺ of the different systems.

**Energy coupling to K⁺ uptake via Trk in E. coli ΔsapABCDF strains**

The easiest way to explain the data in Fig. 6 is to assume that K⁺ uptake via EcTrkG 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 (ΔatpB-C ΔsapABCDF ΔtrkG), LB682 (ΔatpB-C ΔsapABCDF ΔtrkH) and LB692 (ΔatpB-C ΔsapABCDF ΔtrkG) 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 cytoplasmatic 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 cytoplasmatic ATP concentration. However, at that time it was not known that in this E. coli strain Trk consists of the two separate TrkH and TrkG 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 TrkH and strains LB680(pTWE341) and LB682(pTWE341) for TrkG], for TrkG in a ΔsapABCDF background [strains LB680(pAS8) and LB682(pAS8)] and for plasmid-encoded VaTrk in an E. coli ΔsapABCDF 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 cytoplasmatic 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
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 ΔsapABCDF background [strain LB682(pAS8)], and plasmid-encoded VaTrk in an E. coli ΔsapABCDF 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 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 *atp* 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.
Similar results were obtained with the chromosomally encoded EcTrkH 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 TrkH and TrkG (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 EcTrkG and VaTrk in E. coli ΔsapABCDF strains (Table 2). The results were very similar to those obtained with E. coli TrkH and TrkG 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 ΔsapABCDF background in E. coli; as observed in Fig. 6, occurred due to a lack of ATP dependence of this process.

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

<table>
<thead>
<tr>
<th>System</th>
<th>Strain</th>
<th>atp genotype</th>
<th>Substrate</th>
<th>K⁺ transport (nmol min⁻¹ mg⁻¹)</th>
<th>[ATP]ₘ₉ (nmol mg⁻¹)</th>
<th>[¹⁴C]Proline transport (nmol min⁻¹ mg⁻¹)</th>
<th>[¹⁴C]Glutamine transport (nmol min⁻¹ mg⁻¹)</th>
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</thead>
<tbody>
<tr>
<td>EcTrkH</td>
<td>LB625(pTWE341) WT</td>
<td>Glucose</td>
<td>112</td>
<td>4-0</td>
<td>3-8</td>
<td>1-2</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Succinate</td>
<td>82</td>
<td>5-5</td>
<td>1-4</td>
<td>0-5</td>
<td></td>
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<tr>
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<td>Glucose</td>
<td>169</td>
<td>3-9</td>
<td>1-5</td>
<td>0-7</td>
<td></td>
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<tr>
<td></td>
<td></td>
<td>Succinate</td>
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<td>0-02</td>
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<tr>
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<td>2-3</td>
<td>2-4</td>
<td></td>
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<td></td>
<td></td>
<td>Succinate</td>
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<td>1-0</td>
<td>1-1</td>
<td></td>
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<tr>
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<td>Glucose</td>
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<td>1-7</td>
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<td>Succinate</td>
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<td>0-5</td>
<td>0-03</td>
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<td>3-1</td>
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<td>1-1</td>
<td>1-0</td>
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<td>EcTrkG</td>
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<td>57</td>
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<td>8</td>
<td>0-4</td>
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<td>LB690(pKT65) WT</td>
<td>Glucose</td>
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<td>125</td>
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<td>0-7</td>
<td>0-4</td>
<td></td>
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<tr>
<td>VaTrkAH</td>
<td>LB692(pKT65) ΔatpB-C</td>
<td>Glucose</td>
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<td>9</td>
<td>0-9</td>
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and ΔsapABCDF 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 TrkH 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 TrkH 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 MalEFGK 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

**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 sapABCD 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 ΔtrkH, ΔtrkG

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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 TrkH activity was only partially inhibited (Fig. 5a), support the notion that ATP binding to SapD is sufficient for TrkH 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 TrkH 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 TrkG and VaTrk are active in an ATP-dependent manner in E. coli ∆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 TrkG and TrkO exert such a different K+ transport activity in a ∆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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