Identification of a gene cluster encoding an arginine ATP-binding-cassette transporter in the genome of the thermophilic Gram-positive bacterium Geobacillus stearothermophilus strain DSMZ 13240

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A single gene cluster encoding components of a putative ATP-binding cassette (ABC) transporter for basic amino acids was identified in the incomplete genome sequence of the thermophilic Gram-positive bacterium Geobacillus stearothermophilus by BLAST searches. The cluster comprises three genes, and these were amplified from chromosomal DNA of G. stearothermophilus, ligated into plasmid vectors and expressed in Escherichia coli. The purified solute-binding protein (designated ArtJ) was demonstrated to bind L-arginine with high affinity (K_d = 0.39 ± 0.06 μM). Competition experiments revealed only partial inhibition by excess L-lysine (38%) and L-ornithine (46%), while no inhibition was observed with L-histidine or other amino acids tested. The membrane-associated transport complex, composed of a permease (designated ArtM) and an ATPase component (designated ArtP), was solubilized from E. coli membranes by decanoylsucrose and purified by metal-affinity chromatography. The ArtMP complex, when incorporated into liposomes formed from a crude extract of G. stearothermophilus lipids, displayed ATPase activity in the presence of ArtJ only. Addition of L-arginine further stimulated the activity twofold. ATP hydrolysis was optimal at 60°C and sensitive to the specific inhibitor vanadate. Analysis of kinetic parameters revealed a maximal velocity of ATP hydrolysis of 0.71 μmol P_i min"^"^-1 (mg protein)^"^-1 and a K_m (ATP) of 1.59 mM. Together, these results identify the ArtJMP complex as a high-affinity arginine ABC transporter.

INTRODUCTION

Amino acids are key intermediates in both carbon and nitrogen metabolism in bacteria. Some are also implicated in osmoregulation and pH homeostasis (Meng & Bennett, 1992; Sleator & Hill, 2001). Although bacteria are generally capable of synthesizing amino acids from metabolic intermediates, the uptake of amino acids from the environment is energetically favoured. Thus, it is hardly surprising that due to the chemical diversity of the side chains, a diverse number of transport systems have evolved. These belong to either the amino acid/polyamine/organocation (APC) superfamily or to the ATP-binding cassette (ABC) superfamily (Saier, 2000). ABC transporters share a common architectural organization comprising two hydrophilic transmembrane domains that form the translocation pathway and two hydrophilic nucleotide-binding (ABC) domains that hydrolyse ATP (Higgins, 1992). Members of the subclass of ABC import systems that is confined to prokaryotes require an additional extracellular substrate-binding protein (or solute receptor) for function (Van der Heide & Poolman, 2002).

ABC transporters mediating the uptake of amino acids have been classified in three separate subfamilies designated PAAT (polar amino acid transporters), HAAT (hydrophobic amino acid transporters) and MUT (methionine uptake transporters) (Hosie & Poole, 2001; Saier, 2000; Zhang et al., 2003). The PAAT family comprises most of the known transporters, including the well-studied histidine permease of Salmonella enterica serovar Typhimurium (S. typhimurium) (reviewed by Schneider, 2003). However, very few of the other family members are functionally characterized. In particular, this holds true for transport systems from thermophilic micro-organisms. For example, in the Gram-positive bacterium Bacillus (now Geobacillus)
stearothermophilus NUB36, which grows best at 55–60 °C, only two genes have been characterized so far, the products of which are involved in glutamine uptake (Wu & Welker, 1991). The proteins displayed significant sequence identities to the ABC subunits and solute-binding proteins, respectively, of glutamine, histidine and arginine transport systems in Escherichia coli/S. typhimurium. This observation is consistent with the result of computer-aided analyses of complete genome sequences that group ABC transporters for glutamine and basic amino acids in one subfamily (Dassa & Bouige, 2001). Thus, in order to obtain a more complete overview of the repertoire of ABC transporters for polar amino acids in G. stearothermophilus, we searched the incomplete genomic sequence of strain DSMZ 13240 (see http://www.genome.ou.edu/bstearch.html) by BLAST (at http://www.ncbi.nlm.nih.gov/) using the ATPase subunit HisP of the histidine/lysine-arginine-ornithine transporter (LAO/HisJ-QMP) of S. typhimurium (Schneider, 2003) as the seed sequence. The data revealed highest identity (52% compared to 37% for the next best hit) to a putative ABC transporter (YqiXYZ) of Bacillus subtilis (Segowska et al., 2001). However, attempts to more precisely predict the substrate by PSI-BLAST searches gave contradicting results. While the ATPase and membrane-integral subunits displayed highest similarities to glutamine transport components, the solute-binding protein was predicted to be most similar to the arginine-binding protein (ArtJ) of E. coli (Wissenbach et al., 1995). Thus, in order to clearly identify the physiological substrate(s) we have cloned and expressed the genes in E. coli and characterized the functions of their purified products by binding assays and in a reconstituted system. The results show that the gene cluster encodes a high-affinity arginine ABC transporter with low affinities for lysine and ornithine. In accordance with the E. coli nomenclature and supported by sequence alignments the genes are designated artJMP.

**METHODS**

**Bacterial strains, plasmids and growth conditions.** All bacterial strains and plasmids used in this study are listed in Table 1.

**G. stearothermophilus** was grown in peptone broth (medium 220, DSMZ, Braunschweig, Germany) at 55 °C. E. coli strains were grown in LB medium (Miller, 1972) at 37 °C. When required, ampicillin was added at 100 μg ml⁻¹. Growth of all bacterial strains was followed by measuring the OD₆₅₀.

**Standard DNA techniques.** Isolation of genomic DNA of G. stearothermophilus, preparation of plasmids, digestion by endonucleases, ligation reactions and PCR were performed as described previously (Hülsmann et al., 2000).

**Cloning of artJ.** The artJ gene was amplified by PCR using the Taq platinum polymerase (Invitrogen), and G. stearothermophilus chromosomal DNA as the template. The primers (5’-CGC TCC TCG AGG CGG GCA AGT CAA CGG AAA AAA CAA G-3’; 5’-GAT CCA GAA TTC TCA CCT TCT TCA AAC AAA AAA TGG TAA CCT GC-3’) were designed in such a way that the amplified fragment lacked the 5’ sequence encoding the signal peptide and that in the translated mature polypeptide, alanine substituted for the N-terminal cysteine residue. The amplified fragment was ligated with pGEM-T, resulting in plasmid pAW10. To overexpress artJ in E. coli the gene was further subcloned (as an XhoI-EcoRI fragment) into expression vector pBAD/HisA. The resulting plasmid was designated pAW11.

**Cloning of artMP.** The artMP genes were co-amplified by PCR as one fragment. The primers (5’-AGA ACG GGA TCC GAT TTT

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<tr>
<td>Strains</td>
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<tr>
<td>G. stearothermophilus DSM13240</td>
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<tr>
<td>E. coli</td>
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<td>JM109</td>
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<td>pRF2</td>
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*DSMZ, Deutsche Sammlung von Mikroorganismen und Zellkulturen, Braunschweig, Germany.*
CGT TTT GAT ATT ATT GTC-3'; 5'-GAG TGA GAT CTA AAT ACT TTT GAC AAA AAC GCT TTT G-3') were designed in such a way that the start codon of artM was replaced by a BamHII site and that a BglII site substituted for the stop codon of artP. The resultant 1.4 kb fragment was cloned into expression vector pQE60, yielding plasmid pRF2.

**Purification of ArtJ.** E. coli strain TOP10(pAW11) was grown in LB, containing ampicillin, to an OD_{600} of 0.5 and supplemented with 0.02% arabinose to induce artJ expression, and growth was continued for 4 h. Cells were harvested, resuspended in 50 mM Tris/HCl, pH 8, 5% (v/v) glycerol, 0.1 mM PMSF and disintegrated by one passage through a French press at 14000 p.s.i. (96-6 MPa). After ultracentrifugation (45 min at 200000 g), ArtJ was recovered in the supernatant and subsequently purified by immobilized metal-affinity chromatography using a Co^{2+}-loaded resin (TALON, Clontech).

**Purification of ArtMP.** E. coli strain JM109(pRF2) was grown under the same conditions as TOP10(pAW11) but adding 0.5 mM IPTG for induction of gene expression. For purification of ArtMP, the membrane fraction obtained after cell disruption and ultracentrifugation (1 h at 200000 g) was resuspended in 50 mM Tris/HCl, pH 8, 5% (v/v) glycerol, 0.1 mM PMSF and solubilized with 1% 2-mercaptoethanol. After ultracentrifugation (30 min at 200000 g) the supernatant was adjusted to 10 mM ATP and 20 mM 2-mercaptoethanol and purified on a Co^{2+}-loaded affinity matrix in the presence of 0.1% 2-mercaptoethanol.

**Isolation of total lipids from G. stearothermophilus.** Lipids were extracted from cells of *G. stearothermophilus* by a published method (Folch *et al.*, 1957). Briefly, cells (10 g wet weight) were washed once with 50 mM Tris/HCl, pH 8, homogenized in 20 vols chloroform/methanol (2:1, v/v) and stirred for 18 h at room temperature. Undissolved material was removed by filtration and the filtrate was extracted with 0.2 vols double-distilled water. After separation into two phases, the lower (organic) phase was evaporated to dryness and washed with acetone for 1 h at room temperature. After removing acetone with a pipette, the sediment was dried under a stream of nitrogen, dissolved to 20 mg ml^{-1} in chloroform and stored at -20 °C.

**Preparation of proteoliposomes.** The ArtMP complex was incorporated into liposomes essentially as described previously (Scheffel *et al.*, 2004). Lipids (20 mg) were dried under a stream of nitrogen, and slowly redissolved in 50 mM MOPS/KOH, pH 7-5, containing 1% octyl ß-D-glucopyranoside. The mixture was then sonicated for 15 min, and then ArtMP (50 µg), ArtJ (120 µg) and L-arginine (1 mM, final concentration) were added to the lipid/detergent mixture. These values correspond to a 7.6-fold molar excess of binding protein over transport complex, which is in the range of what has been proven optimal for the maltose and histidine ABC transporters (Landmesser *et al.*, 2002; Hall *et al.*, 1998; Liu *et al.*, 1997). Proteoliposomes were formed by removal of detergent by adsorption to Biobeads (100 mg; Bio-Rad) at 4°C overnight. After replacing the beads with a new batch incubation was continued for 1 h. The mixture was subsequently centrifuged for 1 min at 300 g to pellet the beads and the supernatant was assayed for ATPase activity. As controls proteoliposomes were prepared by the same procedure but omitting ArtJ/L-arginine or L-arginine.

**Binding assay.** Binding of L-[^14]C]arginine to purified ArtJ was performed by the method of Richarme & Kepes (1983). Standard assay mixtures (100 µl) containing 50 mM MOPS/KOH, pH 7-5, 150 mM NaCl, 5% (v/v) glycerol and purified ArtJ (3 µg) were preheated at 60 °C for 1 min prior to the addition of L-[^14]C]arginine (Hartmann Analytic; 264 mCi mmol^{-1}; 9.77 GBq; 5 µM final concentration). After 1 min, the reaction was terminated by adding 2 ml of an ice-cold saturated (NH_{4})_{2}SO_{4} solution and immediately filtered through a nitrocellulose filter (0.45 µm). The filter was washed once with the same solution, followed by distilled water, and retained radioactivity was determined in a liquid scintillation counter. In competition experiments, unlabelled L-amino acids (0.5 mM) were added 1 min prior to the addition of L-[^14]C]arginine. To determine the binding constant, ArtJ was first subjected to a denaturation/renaturation procedure using guanidine hydrochloride (6 M) to remove excess bound unlabelled substrate (Hall *et al.*, 1998).

**Analytical methods.** Hydrolysis of ATP was assayed in microtitre plates essentially as described by Landmesser *et al.*, (2002). Protein was assayed by using the BCA kit from Bio-Rad. SDS-PAGE and immunoblot analyses were performed as described by Hülsmann *et al.* (2000).

## RESULTS AND DISCUSSION

### Identification of the substrate specificity of ArtJ

Substrate specificity of an ABC importer is primarily determined by the extracellular solute-binding protein. In Gram-positive bacteria, binding proteins are anchored to the cytoplasmic membrane by fatty acids that are covalently linked to the N-terminal cysteine residue (Sutcliffe & Russell, 1995). No evidence exists that the lipid modification is crucial for substrate binding or transport (Hülsmann *et al.*, 2000; Horlacher *et al.*, 1998). However, when genes encoding solute-binding proteins are expressed in *E. coli*, overproduction is often poor (Horlacher *et al.*, 1998) or can have deleterious effects on the host cells (Sutcliffe & Russell, 1995). Low protein yields were also observed when genes encoding lipo-binding proteins were expressed with their natural signal sequence, which is required for translocation across the cytoplasmic membrane via the Sec apparatus (Horlacher *et al.*, 1998; Kempf *et al.*, 1997). Thus, to avoid any of these potential problems and to assure the availability of sufficient amounts of purified protein for the intended binding and reconstitution studies, we changed the N-terminal cysteine residue to alanine and deleted the signal sequence (see Methods for details). As a consequence, when overproduced as an N-terminal fusion with six consecutive histidine residues using strain TOP10(pAW11), ArtJ was recovered with the cytosolic fraction. Purification was achieved by using a Co^{2+}-loaded affinity matrix and was verified by SDS-PAGE (Fig. 1a).

In order to identify the substrate(s) of ArtJ, binding assays using radiolabelled amino acids were performed at 60 °C. Initial experiments identified L-arginine as the most likely ligand. To further explore the arginine-binding activity the protein was first subjected to a denaturation–renaturation procedure using 6 M guanidine hydrochloride in order to remove bound unlabelled amino acids. Subsequent binding experiments at increasing concentrations of L-[^14]C]arginine and analysis of the data by a Scatchard plot revealed a dissociation constant (K_d) of 0.39 ± 0.06 µM. This value is in good agreement with the dissociation constant reported for the arginine-binding protein ArtJ of *E. coli* (0.4 µM; Wissenbach *et al.*, 1995) but about 20 times higher than...
that of the lysine-arginine-ornithine-binding protein (LAO) of S. typhimurium (Nikaido & Ames, 1992). When binding assays were performed in the presence of 100-fold excess of unlabelled amino acids, only L-lysine (38 %) and L-ornithine (46 %) partially inhibited binding of L-[14C]arginine (5 μM) to ArtJ. In contrast, histidine or glutamine had no effect (Table 2). These data clearly identified ArtJ as a high-affinity arginine-binding protein with low affinities for lysine and ornithine, thereby suggesting that the complete gene cluster encodes a transporter with preference for arginine. In order to demonstrate this in vitro we also purified the ArtMP proteins.

**Functional characterization of the ArtJMP transport system in proteoliposomes**

The ArtMP proteins were overproduced from E. coli strain JM109(pRF2). After solubilization of the membrane transport components, Protein samples were run on an SDS gel and subsequently stained with Coomassie blue R250. (a) Purification of His6-ArtJ. Lanes: 1, standard proteins; 2, cytoplasmic fraction of strain TOP10(pAW11); 3, pooled fractions eluted from TALON matrix with 100 mM imidazole (4-6 μg); 4, denatured and renatured ArtJ (3-7 μg). (b) Purification of ArtMP-His6. Lanes: 1, standard proteins; 2, cytoplasmic-membrane fraction of strain JM109(pRF2); 3, proteins solubilized with 1-2 % decanoylsucrose; 4, pooled fractions eluted from TALON matrix with 100 mM imidazole (9 μg). The upper band was identified as ArtP by immunoblottting using an anti-His-tag antibody.

**Table 2.** Arginine-binding activity of ArtJ in the presence of competing substrates

<table>
<thead>
<tr>
<th>Addition</th>
<th>Binding of L-[14C]arginine (% of control)</th>
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<tr>
<td>None</td>
<td>100*</td>
</tr>
<tr>
<td>Arginine</td>
<td>1-6</td>
</tr>
<tr>
<td>Asparagine</td>
<td>99-6</td>
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<tr>
<td>Aspartate</td>
<td>97-9</td>
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<tr>
<td>Cysteine</td>
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<td>Glutamate</td>
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<tr>
<td>Glutamine</td>
<td>93-1</td>
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<tr>
<td>Histidine</td>
<td>94-5</td>
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<tr>
<td>Lysine</td>
<td>62</td>
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<tr>
<td>Ornithine</td>
<td>54-1</td>
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*The control value was 12-1 pmol arginine (μg ArtJ)⁻¹.

ABC transporter functions of ArtJMP were subsequently studied in proteoliposomes by assaying the following well-established criteria: (i) stimulation of ATPase activity of the complex by the cognate binding protein (Davidsson et al., 1992; Liu et al., 1997; Landmesser et al., 2002; Scheffel et al., 2004), and (ii) sensitivity of this activity to the specific inhibitor orthovanadate (Landmesser et al., 2002; Sharma & Davidson, 2000). To this end, proteoliposomes were prepared from purified ArtMP and a crude total lipid extract of G. stearothermophilus (mainly composed of phosphatidylethanolamine, diphasphatidylglycerol and phosphatidylglycerol; Jurado et al., 1991) in the presence of L-arginine (1 mM) and/or ArtJ. By this procedure, substrate and binding protein were enclosed in the lumen of the proteoliposomes. Assuming that incorporation of complex molecules occurs randomly with respect to orientation, as has been demonstrated for example for the maltose and histidine ABC transporters of S. typhimurium (Landmesser et al., 2002; Liu & Ames, 1997), only proteoliposomes with the ArtP subunits facing the medium would contribute to ATPase activity. As shown in Fig. 2, proteoliposomes prepared in the absence of ArtJ and arginine exhibited only marginal ATPase activity; however, the activity was markedly stimulated by ArtJ. Moreover, loading the proteoliposomes with ArtJ and L-arginine further increased the initial rate of ATP hydrolysis by a factor of 1-6. Similar rates of substrate-dependent stimulation of ATPase activity were reported for the maltose (Landmesser et al., 2002) and histidine (Liu et al., 1997) transporters of S. typhimurium. Consistent with the growth conditions of...
G. stearothermophilus, ATPase activity was optimal at a temperature of 60°C (not shown). Analysis of kinetic parameters revealed a maximal velocity of 0.71 µmol P_i min^{-1} per mg protein (of total complex) and a $K_m$ (ATP) of 1.59 mM. Furthermore, the binding-protein-stimulated catalytic activity was sensitive to the specific inhibitor orthovanadate, thereby indicating functional coupling of ATP hydrolysis to substrate transport (Hunke et al., 1995). Vanadate inhibition is caused by trapping of ADP in the binding pocket after hydrolysis of the Vanadate inhibition is caused by trapping of ADP in the ATP hydrolysis to substrate transport (Hunke et al., 1995). Half-maximal inhibition was observed at a concentration of 40 µM, which confirms data reported for other ABC importers (Schneider, 2003) (data not shown).

Conclusions

We have demonstrated at the level of purified protein components that the artJMP gene cluster of the thermophilic Gram-positive bacterium G. stearothermophilus encodes a high-affinity arginine ABC-transport complex with low affinity for lysine and ornithine. Our data confirm the prediction from PSI-BLAST analysis based on the sequence of ArtJ but disprove the results obtained with ArtMP. Thus, the sequence of the cognate binding protein rather than those of the ATPase and/or the hydrophobic subunits of a putative ABC transporter appears to be more reliable for predicting the substrate specificity. This finding could be useful for a more precise annotation of binding-protein-dependent ABC transporters in available genomes.

REFERENCES


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