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Functional reconstitution of a maltose ATP-binding cassette transporter from the thermoacidophilic gram-positive bacterium *Alicyclobacillus acidocaldarius*

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Abstract

The thermoacidophilic gram-positive bacterium *Alicyclobacillus acidocaldarius* grows at 60 °C and pH 2–3. The organism can utilize maltose and maltodextrins as energy source that are taken up by an ATP-binding cassette (ABC) import system. Genes encoding a maltose binding protein, MalE, and two membrane-integral subunits, MalF and MalG, are clustered on the chromosome but a *malK* gene translating into a cognate ATPase subunit is lacking. Here we report the cloning of *malK* from genomic DNA by using the *msiK* gene of *Streptomyces lividans* as a probe. Purified MalK exhibited a spontaneous ATPase activity with a V_{max} of 0.13 μmol P_i/min/mg and a K_m of 330 μM that was optimal at the growth temperature of the organism. Coexpression of *malK*, *malF* and *malG* in *Escherichia coli* resulted in the formation of a complex that could be coeluted from an affinity matrix after solubilization of membranes with dodecylmaltoside. Proteoliposomes prepared from the MalFGK complex and preformed phospholipid vesicles of *A. acidocaldarius* displayed a low intrinsic ATPase activity that was stimulated sevenfold by maltose-loaded MalE, thereby indicating coupling of ATP hydrolysis to substrate translocation. These results provide evidence for MalK being the physiological ATPase subunit of the *A. acidocaldarius* maltose transporter. Moreover, to our knowledge, this is the first report on the functional reconstitution of an ABC transport system from a thermophilic microorganism.

Keywords: ABC transporter; Maltose transport; Binding protein-dependent; *malK*; Proteoliposome; *Alicyclobacillus acidocaldarius*

1. Introduction

Starch is one of the major sources of carbon and energy available to heterotrophic bacteria and archaea. Since polysaccharides cannot penetrate the cell membrane, many microorganisms secrete amylases that produce maltose and maltodextrins (oligosaccharides with up to seven α-1,4 linked glucose units) as major degradation products of starch. The subsequent uptake of maltodextrins is usually mediated by a member of the superfamily of ATP-binding cassette (ABC) transport systems [1,2]. Maltose/maltodextrin ABC transporters are composed of a membrane-bound complex comprising the two hydrophobic permease subunits, MalF and MalG, and two copies of the ATPase (ABC) subunit, MalK. In addition, a cognate receptor, the maltose binding protein (MBP or MalE), is essential for this activity [3]. Substrate-loaded MalE interacts with the membrane integral subunits that form a translocation pore. This interaction stimulates the ATPase activity of the MalK components that provide the energy both for transport itself, and for overcoming the binding protein's tight interaction with the ligand [3].

In gram-negative bacteria, the binding proteins are situated in the periplasm between the outer and inner membrane, where they bind their ligands with high affinity. Gram-positive bacteria and archaea, which lack a periplasm, instead anchor the binding proteins to the outer surface of the cell membrane via an N-terminal lipid moiety [4], or alternatively, as observed for some archaea, an N-terminal hydrophobic helix [5].

Abbreviations: ABC, ATP-binding cassette; DM, *n*-dodecyl-β-D-maltoside; DTT, dithiothreitol; LB, Luria-Bertani; MOPS, 4-morpholinepropanesulfonic acid; 2-Me, 2-mercaptoethanol; NTA, nitrilo triacetic acid; PMSF, phenylmethylsulfonyl fluoride; SDS-PAGE, sodium dodecylsulfate polyacrylamide gel electrophoresis

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Based on sequence alignments, the maltose/maltodextrin ABC transporter belongs to the CUT1 subfamily of ABC import systems, which comprises transporters with specificity for di- and oligosaccharides, glycerol phosphate and polyols [6]. The ABC subunits of this family are further characterized by a C-terminal extension of about 150 residues. In case of *Escherichia coli/Salmonella typhimurium* MalK, a regulatory function of this subdomain has been demonstrated [3].

In gram-negative bacteria the genes encoding the maltose transport components are usually organized in two closely linked operons. In contrast, in gram-positives the *malK* gene is often not included in the respective gene clusters [7,8]. The most intriguing example was recently reported for the gut bacterium *Bifidobacterium longum*. Only two out of seven regions identified in the genome that comprise genes with homology to *malEFG* of *E. coli*, contain *malK* homologs [9]. In *Streptomyces*, the product of the *msiK* gene was demonstrated in whole cells to assist in cellobiose and maltose uptake [10]. These findings led to the hypothesis that unlinked ABC proteins may serve more than one member of the CUT1 subfamily as energy-providing module [10,11]. The underlying assumption that different translocation pores can form functional complexes with the same ABC domain is supported by the observation that the MalK subunits of *E. coli/S. typhimurium* are functionally exchangeable with other closely related family members, e.g. UgpC of *E. coli* [12,13] and LacK of *Agrobacterium radiobacter* [13].

The thermoacidophilic gram-positive bacterium *Alicyclobacillus acidocaldarius* that was first isolated from an acidic creek in Yellowstone National Park grows best at 57–60 °C and pH 2–3. The organism is further characterized by the presence of ω-alicyclic fatty acids in its cytoplasmic membrane [14] and can utilize starch as carbon and energy source [15] by action of a secreted α-amylase [16]. Uptake of the degradation products, maltodextrins and maltose, is mediated by a typical ABC transporter [17]. The respective binding protein was isolated from whole cells of *A. acidocaldarius* and demonstrated to bind maltose and maltodextrins with high affinity over a wide pH range (2.5 to 7) and up to 80 °C [17,18]. Its crystal structure was recently solved [19].

The genes coding for the MalE, MalF and MalG proteins are included in a cluster downstream of the *amyA* gene encoding the α-amylase and upstream of *malR cdaA glcA*, encoding a transcriptional regulator, a cyclomaltodextrinase [20] and an α-glucosidase, respectively [17]. The genes may constitute an operon governed by a putative promoter upstream of *amyA* [16] but there is evidence for a second promoter upstream of *malE* (Zielinski and Schneider, unpublished result). Like in other gram-positives, a gene encoding a cognate ABC subunit is lacking.

Here, we report the gene cloning and biochemical characterization of a MalK homolog of *A. acidocaldarius*. Moreover, we describe the purification of a MalFGK complex and its functional analysis in proteoliposomes, prepared from *A. acidocaldarius* lipids. To our knowledge, this is the first example of an ABC transporter from a thermophilic organism for which reconstitution into phospholipid vesicles has been demonstrated at the level of purified components.

2. Materials and methods

2.1. Bacterial strains and growth conditions

A. acidocaldarius ATCC27009 was grown as described [17]. *E. coli* strain JM109 (Stratagene) was used for cloning purposes and heterologous expression of *A. acidocaldarius* genes, respectively, and routinely grown in Luria-Bertani (LB) medium supplemented with antibiotics where required, at 30 or 37 °C.

2.2. Plasmids

Plasmid pFSA18 (Ap^r) harbors the *malK* gene under the control of the T5 promoter and was constructed by exchanging an *Nco*I–*Sal*I fragment of plasmid pGS91-1 [21] with a *malK*-containing fragment from pFSA22 (see below) obtained by PCR. The respective restriction sites were provided by the oligonucleotide primers.

Plasmid pFSA24 carrying the *malFG* genes under the control of the T5 promoter was constructed by ligating an *Xho*I–*Sal*I fragment of pFSA12 [17] with *Sal*I-digested pSU19 (P15A ori, Cm^r) [22]. The resulting translated MalF protein contained a 12-amino acid N-terminal extension ($MRGSH_6GS$).

In plasmid pFSA30 (Ap^r) the *malK* gene is placed under the control of the heat-inducible $\lambda p_R \lambda p_L$ promoters by ligating an *Nco*I–*Sal*I fragment of pFSA18 with expression vector pJLA502 [23].

Plasmid pFR1 is a derivative of expression vector pQE9 (pT5, Ap^r; Qiagen, Germany) that contains the *malE* gene lacking its signal sequence and with the wild-type initiation codon TGT (Cys) replaced by GCG (Ala). It was constructed by ligating a PCR-amplified *Bam*HI–*Bam*HI fragment from pAH18 [17] with linearized pQE9. The resulting translated protein has the sequence $MRGSH_6GS$ fused to the N-terminal alanine residue.

Plasmid pOFXtac-SL1 carries the *E. coli* genes *groESL* under the control of the tac promoter [24].

2.3. Cloning of the malK gene

Genomic DNA of *A. acidocaldarius* was digested with *Bam*HI and a fragment (3.9 kb) that hybridized with digoxigenin-labelled *msiK* of *S. lividans* was identified by Southern blotting. Isolation of the 3.9-kb fragment at a large scale, ligation into pUC18 (Roche), transformation of *E. coli* strain JM109 and subsequent colony hybridization with the probe were carried out as described in Ref. [17]. Plasmid DNA was prepared from one out of several positive clones and the

nucleotide sequence of both strands of the insert was determined commercially (Agowa, Berlin). The plasmid was designated pFSA22. Similarly, an *Eco*RI fragment of 3.5 kb was cloned from genomic DNA into pUC18 that contained the *malK* gene lacking 94 bp at the 5′ end, and additional 2 kb at its 3′ end. The resulting plasmid was named pFSA21 (see also Fig. 1).

2.4. Standard DNA techniques

Isolation of genomic DNA of *A. acidocaldarius*, preparation of plasmids, digestion by endonucleases, ligation reactions and PCR were performed as described previously [17].

2.5. Purification of MalK

MalK was purified as an N-terminal His_6-fusion protein from the cytosolic fraction of *E. coli* strain JM109(pFSA18, pOFXtac-SL1). Cells were grown in LB medium supplemented with ampicillin (100 μg/ml) and kanamycin (50 μg/ml) at 30 °C to OD_{650} = 0.5. Then, IPTG (0.5 mM) was added and growth continued for 2 h. Cells were harvested by centrifugation for 10 min at 8000 × *g* and resuspended in 50 mM Tris–SO_4, pH 8, 100 mM NaCl, 20 mM 2-Me, 0.5 mM EDTA, 7.5% glycerol (v/v), 5 mM ATP and 0.1 mM PMSF and desintegrated by two passages through a French Press (18,000 psi) followed by ultracentrifugation. The resulting supernatant was incubated with a Ni–NTA matrix for 1 h at 4 °C. The mixture was then transferred to a column and the resin was extensively washed with the same buffer containing 20 mM imidazole. MalK was eluted with buffer containing 50 mM imidazole, concentrated, dialyzed against buffer without imidazole and stored at − 80 °C until use. On average, ~ 10 mg of purified protein was obtained from a 1-l culture.

2.6. Purification of a MalFGK complex

The MalFGK complex was purified from strain JM109(pFSA24, pFSA30). Cells were grown in LB medium containing ampicillin (100 μg/ml) and chloramphenicol (20 μg/ml) at 30 °C to an OD_{650} = 1. Then, IPTG (0.5 mM) was added, the cells were shifted to 42 °C and growth continued for 4 h. Cells were subsequently harvested by centrifugation, resuspended in 50 mM Tris–HCl, pH 7.5, containing 0.1 mM EDTA, 2 mM DTT, 20% (v/v) glycerol, 0.1 mM PMSF and desintegrated by one passage through a French Press (14,000 psi). After removing cell debris by a low speed spin, the membrane fraction was recovered by ultracentrifugation for 1 h at 200,000 × *g*. Membranes were resuspended at 5 mg/ml in 50 mM Tris–HCl, pH 7.5, containing 5 mM $MgCl_2$, 20% glycerol, 0.1 mM PMSF and solubilized with 1.1% DM under gentle stirring on ice for 1 h. Following ultracentrifugation, the supernatant was diluted 1:2 with 50 mM Tris–HCl, pH 7.5, containing 5 mM $MgCl_2$, 20% glycerol, 0.1 mM PMSF, 0.01% (w/v) DM (column buffer) and mixed with Ni–NTA matrix. After incubation for 1 h at 4 °C on a rotary shaker the mixture was transferred to a column and the resin was extensively washed with column buffer containing 20 mM imidazole. MalFGK was eluted with column buffer routinely containing 150 mM imidazole, concentrated by ultrafiltration (Amicon, YM100), passed through a PD10 column (Amersham-Pharmacia) to remove imidazole and stored at − 80 °C until use. Routinely, ~ 400 μg of complex protein was obtained from a 1-l culture.

2.7. Purification of MalE

MalE was purified as an N-terminal His_6 fusion protein from strain JM109(pRF1) as described elsewhere [19].

2.8. Isolation of total lipids from A. acidocaldarius

Lipids were extracted from cells of *A. acidocaldarius* by a published method [25], dissolved in chloroform at a concentration of 20 mg/ml and stored under nitrogen at − 80 °C.

2.9. Preparation of proteoliposomes

The MalFGK complex was incorporated into liposomes essentially as described in Ref. [26]. Briefly, crude lipids from *A. acidocaldarius* (20 mg) were dried under a stream of nitrogen, and slowly redissolved in 50 mM MOPS–KOH, pH 7.5, containing 1% octly-β-D-glucopyranoside over a time period of 2 h. The mixture was then sonicated for 15 min with a BRANSON sonifier 250 under a stream of nitrogen. Subsequently, MalFGK (50 μg), MalE (120 μg) and maltose (2.2%, final) were added to the lipid–detergent mixture (125 μl) to give a final volume of 300 μl. Proteoliposomes were formed by removal of detergent by adsorption to Biobeads (100 mg; BioRad, München) at 4 °C overnight. After replacing the beads with a new batch incubation 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 MalE and maltose.

2.10. Analytical methods

Hydrolysis of ATP was assayed in microtiter plates essentially as described in Ref. [27]. Protein was assayed by using the BCA kit from BioRad. SDS-PAGE and immunoblot analyses were performed as described in Ref. [17].

2.11. Nucleotide sequence accession number

The *malK* sequence reported in this paper has been deposited in the EMBL database and assigned accession number AJ580946.

3. Results

3.1. Cloning and sequencing of the A. acidocaldarius malK gene

Since sequence analysis of additional 2.5 kb downstream of the *glcA* gene by primer walking did not provide evidence for a nearby localization of a *malK* gene (Ref. [17] and unpublished results), we used Southern hybridization instead to clone a candidate gene. To this end, genomic DNA of *A. acidocaldarius* digested either by *Bam*HI or *Eco*RI was probed with the *msiK* gene of *S. lividans*. This resulted in the identification of two fragments of 3.9 and 3.5 kb, respectively, that were subsequently cloned into pUC18, yielding pFSA22 and pFSA21, and sequenced. The data revealed an open reading frame with homology of its translated product to ABC proteins of the CUT1 subfamily (Fig. 1). Consequently, the *orf* was designated *malK*. A promotor region with a putative transcriptional start 18 base pairs upstream of the initiation codon (GTG) was predicted using NNPP 2.2 (http://www.fruitfly.org/seq_tools/promoter.html). Further upstream of *malK* two open reading frames were identified with homology to NAD^+-dependent malic enzyme of *Geobacillus stearothermophilus* (data base accession number P16468) (*orf1′*) and to a regulator of polyketide synthase expression of *Thermoanaerobacter tengcongensis* (NP_623553) (*orf2*). Downstream of *malK* three *orfs* were found that display highest sequence identity with an HPr-like protein of *Bacillus anthracis* (NP_653599) (*orf4*), a methyl-accepting chemotaxis protein of *B. cereus* (*orf5*) and a phosphoenolpyruvate carboxylase of *Xanthomonas campestris* (NP_636145) (*orf6′*). Thus, the data revealed no evidence for other ABC transporter genes, indicating that MalK is genetically unlinked to membrane integral ABC components.

The *malK* gene translates into a protein of 385 amino acids with a calculated molecular mass of. 42,977 Da. Analysis by BLASTP identified three ABC proteins from the gram-positive bacteria *T. tengcongensis*, *Clostridium perfringens* and *Bacillus firmus* as its closest relatives, displaying 67%, 66%, and 65% identical residues, respectively. Comparison with MalK proteins from the rather distantly related gram-negative bacterium *E. coli* and the hyperthermophilic archaeon *Thermococcus litoralis* still revealed 48% and 55% sequence identity, respectively. Moreover, sequence alignment with these proteins using CLUSTALW clearly confirmed that MalK of *A. acidocaldarius* is a true member of the CUT1 subfamily (Fig. 2): the C-terminal extension following the conserved 'switch' region of a consensus ABC domain [3] contains several peptide motifs and single amino acid residues by which family members are recognized. Thus, we concluded that the protein was a promising candidate for forming a functional transport complex with MalFG of *A. acidocaldarius*.

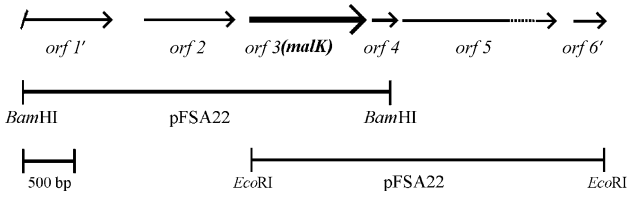


Fig. 1. Organization of the genomic region around *malK*. Shown is the *malK* region of the *A. acidocaldarius* chromosome (top line), encompassing 5.75 kb. *orfs* are represented by arrows in the direction of transcription. The region of *orf5* for which no sequence information is available is shown by a dashed line. Incomplete *orfs* are marked by an apostrophe. At the bottom, inserts of the constructed plasmids are drawn in relation to the *malK* gene with the restrictions sites used for ligation in the host vector.

3.2. Purification and characterization of MalK

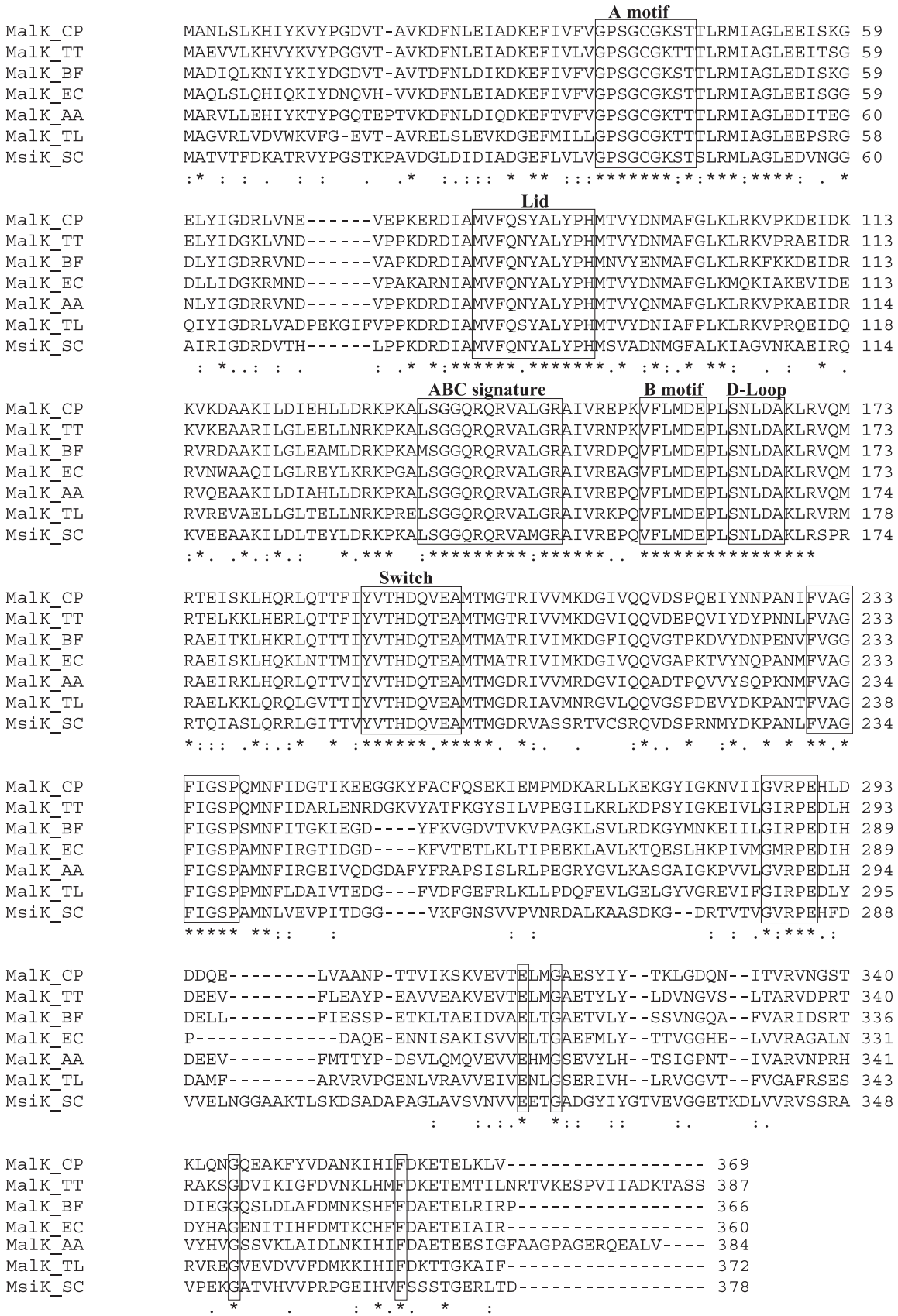
In order to investigate its basic biochemical properties, the MalK protein was purified to near homogeneity by Ni–NTA chromatography from the cytosolic fraction of *E. coli* strain JM109(pFSA18, pOFXtac-SL1) (Fig. 3, lane 2). Some aggregation of the protein found in initial experiments could be overcome by coexpression of *E. coli* chaperones GroEL and GroES, provided by plasmid pOFXtac-SL1. Furthermore, the presence of 5 mM ATP throughout the purification procedure turned out to be crucial for protein stability.

Purified MalK exhibited a spontaneous ATPase activity with a K_m of 330 μM and a maximal velocity of 0.15 μmol P_i/min/mg. In accordance with the optimum growth temperature of the organism, activity was highest at 60 °C and pH 7.5 (Fig. 4A and B).

3.3. Synthesis of MalK in A. acidocaldarius grown on different carbon sources

As a candidate for the ABC subunit that assembles with MalFG into a maltose transport complex in vivo, MalK should be synthesized under similar growth conditions as the MalEFG proteins. Thus, we analyzed production of MalK and MalE in cells of *A. acidocaldarius* grown in minmal salt medium with different carbon sources by immunoblotting. As shown in Fig. 5, both proteins are

Fig. 2. Sequence alignment of MalK of *A. acidocaldarius* (MalK_AA) and related proteins. The proteins considered are: MalK_CP (*C. perfringens*, acc. no. NP_563261), MalK_TT (*T. tengcongensis*, acc. no. NP_623554), MalK_BF (*B. firmus*, acc. no. AAC62409), MalK_EC (*E. coli*, acc. no. NP_418459), MalK_TL (*T. litoralis*, acc. no. AF121946) and MsiK_SC (*S. coelicolor*, acc. no. NP_628414). Conserved sequence motifs and amino acid residues are boxed. Those that are conserved throughout the ABC superfamily are indicated above the respective box by their nicknames. Others are confined to CUT1 family members.


A motif
Lid
ABC signature
B motif
D-Loop
Switch
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MalK_TT MAEVVLKHVYKVYPGGVT-AVKDFNLEIADKEFIVLVGPSGCGKTTTLRMIAGLEEITSG 59
MalK_BF MADIQLKNIYKIYDGDVT-AVTDFNLDIKDKEFIVFVGPSGCGKSTTLRMIAGLEDISKG 59
MalK_EC MAQLSLQHIQKIYDNQVH-VVKDFNLEIADKEFIVFVGPSGCGKSTTLRMIAGLEEISGG 59
MalK_AA MARVLLEHIYKTYPGQTEPTVKDFNLDIQDKEFTVFVGPSGCGKTTTLRMIAGLEDITEG 60
MalK_TL MAGVRLVDVWKVFG-EVT-AVRELSLEVKDGEFMILLGPSGCGKTTTLRMIAGLEEPSRG 58
MsiK_SC MATVTFDKATRVYPGSTKPAVDGLDIDIADGEFLVLVGPSGCGKSTSLRMLAGLEDVNGG 60
MalK_CP ELYIGDRLVNE------VEPKERDIAMVFQSYALYPHMTVYDNMAFGLKLRKVPKDEIDK 113
MalK_TT ELYIDGKLVND------VPPKDRDIAMVFQNYALYPHMTVYDNMAFGLKLRKVPRAEIDR 113
MalK_BF DLYIGDRRVND------VAPKDRDIAMVFQNYALYPHMNVYENMAFGLKLRKFKKDEIDR 113
MalK_EC DLLIDGKRMND------VPAKARNIAMVFQNYALYPHMTVYDNMAFGLKMQKIAKEVIDE 113
MalK_AA NLYIGDRRVND------VPPKDRDIAMVFQNYALYPHMTVYQNMAFGLKLRKVPKAEIDR 114
MalK_TL QIYIGDRLVADPEKGIFVPPKDRDIAMVFQSYALYPHMTVYDNIAFPLKLRKVPRQEIDQ 118
MsiK_SC AIRIGDRDVTH------LPPKDRDIAMVFQNYALYPHMSVADNMGFALKIAGVNKAEIRQ 114
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MalK_TT KVKEAARILGLEELLNRKPKALSGGQRQRVALGRAIVRNPKVFLMDEPLSNLDAKLRVQM 173
MalK_BF RVRDAAKILGLEAMLDRKPKAMSGGQRQRVALGRAIVRDPQVFLMDEPLSNLDAKLRVQM 173
MalK_EC RVNWAAQILGLREYLKRKPGALSGGQRQRVALGRAIVREAGVFLMDEPLSNLDAKLRVQM 173
MalK_AA RVQEAAKILDIAHLLDRKPKALSGGQRQRVALGRAIVREPQVFLMDEPLSNLDAKLRVQM 174
MalK_TL RVREVAELLGLTELLNRKPRELSGGQRQRVALGRAIVRKPQVFLMDEPLSNLDAKLRVRM 178
MsiK_SC KVEEAAKILDLTEYLDRKPKALSGGQRQRVAMGRAIVREPQVFLMDEPLSNLDAKLRSPR 174
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MalK_TT RTELKKLHERLQTTFIYVTHDQTEAMTMGTRIVVMKDGVIQQVDEPQVIYDYPNNLFVAG 233
MalK_BF RAEITKLHKRLQTTTIYVTHDQTEAMTMATRIVIMKDGFIQQVGTPKDVYDNPENVFVGG 233
MalK_EC RAEISKLHQKLNTTMIYVTHDQTEAMTMATRIVIMKDGIVQQVGAPKTVYNQPANMFVAG 233
MalK_AA RAEIRKLHQRLQTTVIYVTHDQTEAMTMGDRIVVMRDGVIQQADTPQVVYSQPKNMFVAG 234
MalK_TL RAELKKLQRQLGVTTIYVTHDQVEAMTMGDRIAVMNRGVLQQVGSPDEVYDKPANTFVAG 238
MsiK_SC RTQIASLQRRLGITTVYVTHDQVEAMTMGDRVASSRTVCSRQVDSPRNMYDKPANLFVAG 234
MalK_CP FIGSPQMNFIDGTIKEEGGKYFACFQSEKIEMPMDKARLLKEKGYIGKNVIIGVRPEHLD 293
MalK_TT FIGSPQMNFIDARLENRDGKVYATFKGYSILVPEGILKRLKDPSYIGKEIVLGIRPEDLH 293
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MalK_TT DEEV--------FLEAYP-EAVVEAKVEVTELMGAETYLY--LDVNGVS--LTARVDPRT 340
MalK_BF DELL--------FIESSP-ETKLTAEIDVAELTGAETVLY--SSVNGQA--FVARIDSRT 336
MalK_EC P-------------DAQE-ENNISAKISVVELTGAEFMLY--TTVGGHE--LVVRAGALN 331
MalK_AA DEEV--------FMTTYP-DSVLQMQVEVVEHMGSEVYLH--TSIGPNT--IVARVNPRH 341
MalK_TL DAMF--------ARVRVPGENLVRAVVEIVENLGSERIVH--LRVGGVT--FVGAFRSES 343
MsiK_SC VVELNGGAAKTLSKDSADAPAGLAVSVNVVEETGADGYIYGTVEVGGETKDLVVRVSSRA 348
MalK_CP KLQNGQEAKFYVDANKIHIFDKETELKLV------------------ 369
MalK_TT RAKSGDVIKIGFDVNKLHMFDKETEMTILNRTVKESPVIIADKTASS 387
MalK_BF DIEGGQSLDLAFDMNKSHFFDAETELRIRP----------------- 366
MalK_EC DYHAGENITIHFDMTKCHFFDAETEIAIR------------------ 360
MalK_AA VYHVGSSVKLAIDLNKIHIFDAETEESIGFAAGPAGERQEALV---- 384
MalK_TL RVREGVEVDVVFDMKKIHIFDKTTGKAIF------------------ 372
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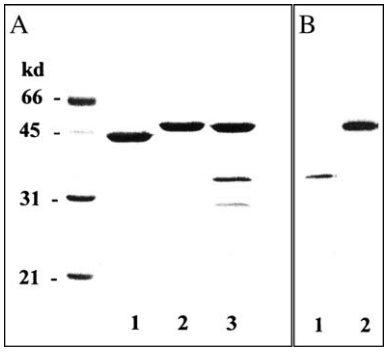

Fig. 3. SDS-PAGE (A) and Western blot analysis (B) of purified maltose transport components. (A) Five micrograms of each protein sample was run on an SDS gel and subsequently stained with Coomassie blue R250. Lanes: 1, MalE; 2, MalK; 3, MalFGK. (B) MalFGK was separated by SDS-PAGE, electrotransferred to nitrocellulose and probed with RGS-His-antibodies to identify His_6-MalF (lane 1) or a polyclonal antiserum raised against MalK (lane 2).

absent in cells grown in the presence of glucose, glycerol or cellobiose but their synthesis was induced in the presence of starch, maltodextrins or maltose. Moreover, MalK but not MalE was detected in cells grown on trehalose. These results indicate that *A. acidocaldarius* cells growing on

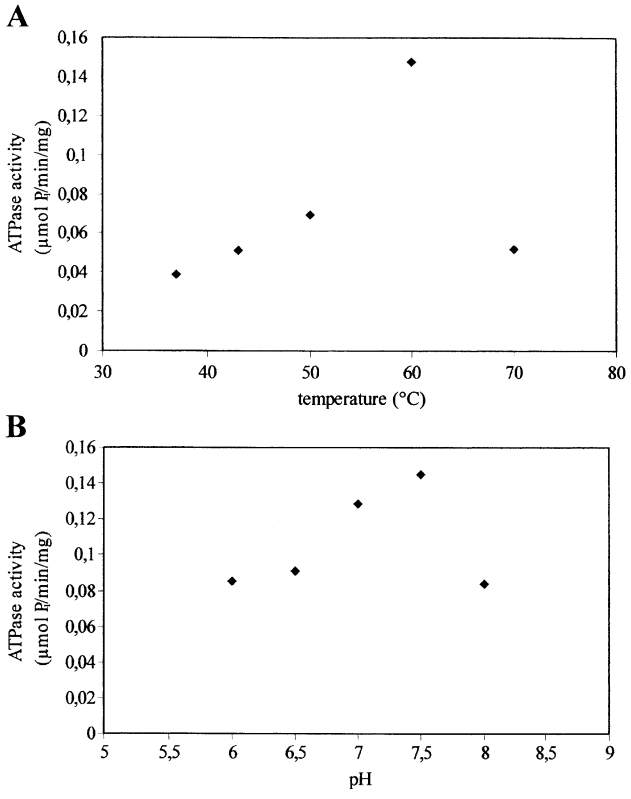

Fig. 4. Temperature (A) and pH optima (B) of MalK-ATPase. (A) ATPase activity was assayed in 50 mM MOPS–NaOH, pH 7.5, 150 mM NaCl, at the temperatures indicated. (B) To determine the pH optimum, ATPase activity was assayed at 57 °C in 50 mM MOPS–NaOH, 150 mM NaCl (for pH values from 6 to 7.5) or 50 mM Tris–HCl, 150 mM NaCl (for pH values 7.5 and 8).

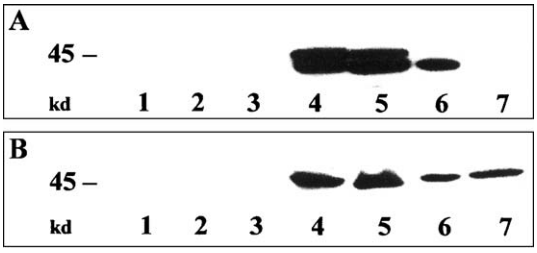

Fig. 5. Synthesis of MalE (A) and MalK (B) in the presence of different sugars. Cells of *A. acidocaldarius* were grown for 6 h in minimal salt medium [17] supplemented with the following sugars as carbon and energy sources: cellobiose (10 mM) (lane 1), glucose (10 mM) (lane 2), glycerol (10 mM) (lane 3), maltodextrins (0.2% w/v) (lane 4), maltose (10 mM) (lane 5), starch (0.2% w/v) (lane 6) and trehalose (10 mM) (lane 7). Proteins from whole cell extracts were separated by SDS-PAGE and subsequently subjected to immunoblot analysis. In A, the double band observed in lanes 4 and 5 corresponds to full-length (upper) and proteolytically processed forms (lower) of MalE [17,18].

substrates of the maltose/maltodextrin ABC transporter [17] also synthesize the MalK protein.

3.4. Coexpression of malK and malFG in E. coli and purification of a complex

The above results suggest but do not prove that MalK forms a functional complex with MalFG. To demonstrate complex formation, we constructed two compatible plasmids to allow coexpression of *malFG* and *malK* in *E. coli*. To this end, *malK* was placed under the control of the heat-inducible $\lambda p_R \lambda p_L$-promoters in plasmid pJLA502 (resulting in pFSA30) while the *malFG* genes governed by the pT5 promoter were cloned into pSU19 (resulting in pFSA24). For purification purposes, MalF is translated as an N-terminal fusion with six consecutive histidine residues. Cells of *E. coli* strain JM109(pFSA24, pFSA30) were induced at early log phase for *malK* and *malFG* expression by shifting the temperature from 30 to 42 °C and by the addition of IPTG. Membranes prepared from the resulting cells were solubilized with DM and the supernatant was subjected to Ni–NTA chromatography. As shown in Fig. 6, coelution of three proteins with apparent molecular masses of 45, 33 and 28 kDa, respectively, was achieved by the addition of 50 mM imidazole. Immunoblot analysis of pooled and concentrated fractions (Fig. 3A, lane 3) identified the 45-kDa and the 33-kDa proteins as MalK and MalF, respectively (Fig. 3B). No antiserum raised against MalG is currently available. However, two lines of evidence support the notion that the 31-kDa protein is indeed MalG: (i) the coding regions of *malF* and *malG* overlap thus suggesting coupled translation [17], and (ii) a protein of the respective molecular mass could not be isolated from the solubilized membrane fraction of cells from JM109(pFSA30), lacking the *malFG* containing plasmid. Thus, these results clearly imply that MalK forms a stable complex with MalFG. In this context, it should be noted that CysA, an ABC protein from *A.*

acidocaldarius that displays 29% identity to MalK (data base accession no. AJ252160), failed to coelute with MalFG from the Ni–NTA matrix (not shown). This finding further indicates that interaction of MalK with MalFG is specific.

3.5. Reconstitution of a functional MalFGK transport complex in proteoliposomes

In order to demonstrate that the coeluted MalFGK proteins are functionally assembled in a complex, we analyzed ATPase activity in detergent solution and in proteoliposomes in the presence and absence of maltose-loaded MalE. For other ABC importers, like the maltose transporter of *E. coli/S. typhimurium* [27–29] and the histidine transporter of *S. typhimurium* [30], it was demonstrated that the purified complexes exhibit a low intrinsic enzymatic activity that, unlike the ATPase activity of the soluble ABC subunit, is stimulated severalfold by the cognate liganded substrate binding protein. In proteoliposomes the increase in ATPase activity occurs concomitantly with substrate translocation [31]. Thus, monitoring ATPase activity of the complex in the presence of substrate-loaded binding protein provides a measure of complex integrity. In detergent solution, the MalFGK complex of *A. acidocaldarius* displayed an ATPase activity of 0.4 μmol P_i/min/mg that, however, was only marginally increased in the presence of purified MalE and maltose (Fig. 7). Thus, the result might still be explained by the spontaneous ATPase activity of MalK itself. In contrast, when incorporated into liposomes preformed from a crude lipid extract of *A. acidocaldarius* cells, a sevenfold stimulation of ATPase activity was observed (Fig. 7). These data strongly suggest that the enzymatic activity is coupled to transport. It should be noted that proteoliposomes prepared with lipids from another thermophile, *G. stearothermophilus*, were completely inactive (not shown).

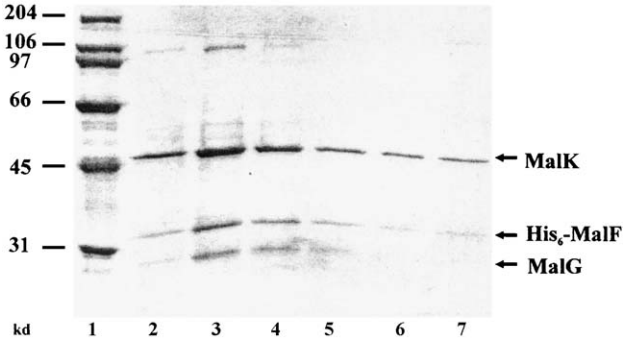

Fig. 6. Coelution of MalK with His_6-MalFMalG from Ni–NTA matrix. MalFGK was solubilized with DM from membranes of JM109(pFSA24, pFSA30) and subjected to Ni–NTA chromatography as described in Materials and methods. After several washing steps, protein was successively eluted with 50 and 100 mM imidazole. Aliquots of individual fractions were loaded on an SDS gel, electrophoresed and stained. Lanes: 1, molecular mass standards; 2–5, fractions eluted with 50 mM imidazole; 6–7, fractions eluted with 100 mM imidazole.

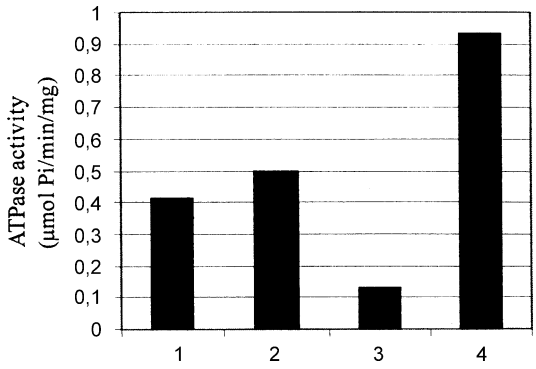

Fig. 7. ATPase activity of the purified MalFGK complex. ATPase activity was assayed under standard conditions at pH 7.5 and 57 °C in detergent solution (1, 2) or in proteoliposomes (3, 4) in the absence (1, 3) or presence (2, 4) of MalE/maltose. For details see Materials and methods.

4. Discussion

In this publication we describe the cloning of *malK* from the thermoacidophilic gram-positive bacterium *A. acidocaldarius*, its coexpression with the genetically unlinked *malFG* genes in *E. coli* and the functional reconstitution of the purified MalFGK complex in proteoliposomes.

Cloning of the *malK* gene was achieved by probing genomic DNA with the *msiK* gene of *S. lividans*. Earlier attempts employing oligonucleotides derived from conserved sequence motifs resulted in the cloning of several 'ABC' genes, none of which, however, encoding a CUT1 family member. Thus, the *msiK* gene might have provided family-specific 'earmarks'. In addition, *msiK* also might have been advantageous due to its similarly high GC content (66%) as compared to *malK* (61%).

The kinetic parameters of ATP hydrolysis displayed by the isolated MalK protein are well comparable to those reported for other thermophilic members of CUT1 family, e.g. the MalK protein of *T. litoralis* [32] and GlcV of *Sulfolobus solfataricus* [5]. Moreover, they also lie in the same range as those of the well-studied MalK protein from the mesophilic bacteria *E. coli/S. typhimurium* [3]. Together with the result from sequence alignment (Fig. 2), these data made MalK a likely candidate for the ABC subunit that forms a complex with MalFG. This was subsequently demonstrated by coexpression in *E. coli* of the respective plasmid-bourne genes and by coelution of a stable complex from the Ni–NTA matrix. Moreover, and most importantly, the MalFGK complex, when incorporated into liposomes, displayed a low ATPase activity that could be stimulated severalfold by liganded maltose binding protein. Such a stimulation is taken as a measure to demonstrate coupling of ATP hydrolysis to substrate translocation and, thus, functionality of the system [27,29,30]. To our knowledge, this

has not yet been reported for an ABC import system from a thermophilic organism.

A MalFGK complex was purified from *T. litoralis* but characterized only in detergent solution [33]. Similar to our results, no stimulation of ATPase activity by the cognate maltose/trehalose binding protein was observed. This is in marked contrast to data reported for the maltose and histidine ABC transporters, respectively, from *E. coli/S. typhimurium* demonstrating activation of complex ATPase activity by the respective binding protein even in solution [27–29]. In one report, an effect of the detergent on the coupling mechanism of the transporter was discussed as a possible explanation [28]. Alternatively, a similar effect of endogenous lipids cosolubilized with the transport complex from the membrane of the overproducing *E. coli* strain may be considered. While in case of the *E. coli/Salmonella* transporters such lipid molecules would originate from their native membranes, this does not hold true for the thermophilic systems. *T. litoralis*, an archaeon, contains ether lipids while *A. acidocaldarius* membranes are enriched in glycolipids and the predominant phospholipids are diglycerolphosphate and glycerophosphate [34]. Thus, *E. coli* lipids, mainly composed of phosphatidyl ethanolamine, by no means mimic the natural environment of the thermophilic transporters. A strong dependency of the *A. acidocaldarius* MalFGK complex on its natural lipids is also indicated by the observation that reconstitution with lipids extracted from the thermophile *G. stearothermophilus* did not result in an active transporter. The lipid composition of *G. stearothermophilus* is similar to *Bacillus* species but contains in addition glucocardiolipins [35].

We have demonstrated that MalK is capable of forming a functional complex with MalFG in vitro but does this finding also reflect the situation in vivo? Unfortunately, mutational analysis to answer the question is not feasible because *A. acidocaldarius* cannot yet be manipulated by genetic means. However, the observed simultaneous induction of *malE* and *malK* in the presence of starch or its degradation products argues in favor of MalK being the physiological ABC subunit of the transporter. It appears highly unlikely that more than one ABC protein interacting with MalFG would be synthesized in the cell under the same conditions.

Synthesis of MalE is absent in glucose-grown cells of *A. acidocaldarius* but occurred in the presence of glucose and maltose which argues for the existence of a repressor that is released from its binding site by an inducing molecule [17]. The product of the *malR* gene immediately downstream of *malG* is a good candidate for such an activity. MalR was identified by BLASTP as a putative transcriptional regulator belonging to the LacI-GalR family [17]. Preliminary results from gel shift assays indeed indicated that MalR binds to two DNA fragments with homology to a consensus LacI binding motif located upstream of *amyA* and of *malE*, respectively. Moreover, maltose and maltotriose were found to release the protein (Zielinski and Schneider, unpublished data). Since MalK was synthesized in the presence of maltose or maltodextrins, it is tempting to speculate that expression of the *malK* gene might also be controlled by MalR. However, no binding motif for a LacI-type repressor could be identified. Instead, a palindromic sequence (TGAAATCGCTTTCT) with similarity to *cre* (catabolite responsive element) is present in the putative promoter region. *cre* sequences bind the catabolite control protein CcpA in the process of carbon catabolite repression in *Bacilli* and other gram-positive bacteria with low-GC content [36]. Thus, we hypothesize that transcription of *malK* is controlled by catabolite repression. Our result that MalK was absent in cells grown on either glucose or glycerol is in line with this notion.

In *Streptomyces*, a defective *msiK* gene was shown to abolish transport of both maltose and cellobiose by separate ABC transporters. Growth on either one of these disaccharides was restored by providing a copy of *msiK* in trans [10]. Moreover, in a subsequent study, Schlösser [37] demonstrated that MsiK was also induced by trehalose in *S. reticuli*. Based on these findings, it was proposed that in gram-positives genetically unlinked sugar ABC proteins may generally serve more than one transporter. Our finding that *A. acidocaldarius* cells grown in the presence of trehalose synthesized MalK but not MalE might thus well be taken as a first clue in favor of such a dual function of MalK. *A. acidocaldarius* can utilize trehalose as sole source of carbon and energy. The absence of MalE under these conditions and the failure of purified MalE to bind trehalose [17] argue against a role of the MalEFG system in trehalose uptake but for the existence of a yet to be identified separate trehalose transporter in this organism.

Acknowledgements

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