J. Biochem. **133**, 173–180 (2003)
DOI: 10.1093/jb/mvg021

A Novel Neutral Amino Acid Transporter from the Hyperthermophilic Archaeon *Thermococcus* sp. KS-1

Shohei Akahane, Hideaki Kamata, Hitoshi Yagisawa and Hajime Hirata*

Department of Life Science, Graduate School of Science, Himeji Institute of Technology, Hyogo 678-1297

Received October 15, 2002; accepted November 8, 2002

A novel gene encoding a small neutral amino acid transporter was cloned from the genome of the hyperthermophilic archaeon *Thermococcus* sp. KS-1 by functional cloning using *Escherichia coli* strain AK430, which is defective in transporting glycine and D-alanine. The cloned gene, *snatA*, encoded a protein of 216 amino acid residues, SnatA, and was predicted to be a membrane protein with six membrane-spanning segments. *E. coli* AK430 cells transformed with *snatA* transported glycine with an apparent K_t value of 24 μM, which was one order of magnitude higher than that of other known glycine/alanine transporters, including *cycA* of *E. coli* and *acp* of thermophilic bacterium PS3. Competition studies revealed that SnatA transported various L-type neutral amino acids, but its substrate specificity was different from that of CycA or ACP. The glycine transport was inhibited by a protonophore, FCCP, or valinomycin *plus* nigericin, indicating that the process is dependent on an electrochemical potential of H^+. Homology searches revealed no homology with any transporters known to date. However, several hypothetical genes in prokaryote cells enrolled in the gene bank showed significantly high homology scores, indicating that *snatA* and its homologues form a family of prokaryotes. To our knowledge, this is the first report on the cloning of a gene of an amino acid transporter from a hyperthermophilic archaeon.

Key words: archaeon, CycA, glycine, hyperthermophile, transporter.

Abbreviations: ACP, alanine carrier protein; FCCP, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone; SnatA, small neutral amino acid transporter A.

Most amino acid transporters belong to secondary transporter families driven by an electrochemical potential of H^+ or Na^+ formed by primary active transporters, and are composed of 400 to 700 amino acid residues with 10 to 12 membrane-spanning segments (for a recent review, see Ref. *1*). Among them, a typical protein family specific for transporting small neutral amino acids, such as alanine, glycine, and serine, includes products of the *cycA* gene in *Escherichia coli* (*2*, *3*), the *dagA* gene in *Alteromonas haloplanktis* (*4*) and the *acp* gene in thermophilic bacterium PS3 (*5*). Besides these, it has been shown that serine and threonine are specifically transported by a transporter family including TdcC (*6*, *7*) and SdaC (*8*). Although none of the transporter proteins have been crystallized or subjected to X-ray analysis, they are thought to be composed of two major hydrophobic domains, each consisting of 5 to 6 membrane-spanning segments, separated by a hydrophilic domain (*9–11*). Intriguingly, LysE in *Corynebacterium*, which is involved in the export of lysine, consists of 236 amino acids and is predicted to contain only 6 membrane-spanning segments based on hydrophathy analysis (*12*). Similarly, genes involved in resistance to homoserine (*rhtB*) or threonine (*rhtC*) in *E. coli* have been cloned and revealed to be transporters with six membrane-spanning segments (*13*, *14*). Although it is not known how the LysE family transporters work, it has been proposed that they form a dimer, analogous to mitochondrial carriers such as the ADP/ATP translocater (*15–17*), resulting in a functional unit similar to those with 10 to 12 membrane-spanning segments.

Microorganisms that can grow at and above 100°C were discovered a decade ago in geothermal heated environments such as deep-sea hydrothermal vents. Based on the sequence of the small subunit ribosomal RNA (16S rRNA) (*18*, *19*), all except two bacterial genera have been classified as archaea (for recent reviews, see Refs. *20* and *21*). A number of genes and proteins in these hyperthermophiles have been identified, isolated and characterized, and it has been suggested that proteins encoded by these genes work at extremely high temperatures (>100°C). However, they are built with ordinary amino acids and work with the same enzyme mechanistics as their mesophilic counterparts [for a review, see Ref. *22*]. Since both hyperthermophilic proteins and thermophilic proteins are generally stable against various physicochemical treatments (*22*, *23*), the identification and isolation of genes and proteins from hyperthermophiles will provide powerful information on the structure–function relationship of those from mesophiles. In this context, it should be noted that genome projects on several thermophilic archaea have been completed recently (*24*, *25*) and the genetic information is now available on the Web.

*To whom correspondence should be addressed. Tel: +81-791-58-0205, Fax: +81-791-58-0198, E-mail: hirata@sci.himeji-tech.ac.jp

Thermococcus sp. KS-1, which belongs to the *Thermococcaceae* group of Archaea (*26*), is a hyperthermophilic sulfur–dependent heterotroph archaeon that was isolated from a shallow submarine hydrothermal vent (*27*). It grows on proteinaceous mixtures, such as yeast extract, casein hydrolysate, or purified proteins (*e.g.*, casein and gelatin), but not on carbohydrates or organic acids as the sole carbon and energy source at 60 to 97°C, with the optimum temperature being between 85 and 90°C. Furthermore, it has been shown that nine amino acids (Thr, Leu, Ile, Val, Met, Phe, His, Tyr, and Arg) are essential for its growth (*27*). This clearly indicates that the organism imports amino acids *via* some kind of transporter. Although several genes and proteins from KS-1 have been identified, isolated and characterized (*28*, *29*), none of the amino acid transporters has been described yet. In this report, we describe the functional cloning of a gene encoding the glycine transporter from the KS-1 genome for the first time using a newly established *E. coli* strain defective in the glycine/D-alanine transporter (CycA).

MATERIALS AND METHODS

Materials—[U-^{14}C]Glycine (103 mCi/mmol) was obtained from Amersham (Buckinghamshire, UK). Restriction endonucleases and other DNA-modifying enzymes were purchased from Toyobo (Tokyo) and Takara Shuzo (Shiga). Amino acids were purchased from Nacalai Tesque (Kyoto). Carbonylcyanide p-trifluoromethoxyphenylhydrazone (FCCP) was obtained from Sigma (St. Louis, MO). Bacto-Tryptone and Bactoyeast extract were obtained from Difco Lab (Sparks, MD).

Construction of cycA Mutant AK430 of E. coli—The chromosomal DNA fragment carrying the *cycA* gene, which encodes a specific transporter for glycine, alanine and serine, was isolated from a phage clone 7E9 in Kohara's genomic library of *E. coli* (*30*), and was cloned in a temperature-sensitive plasmid, pMAN031 (*31*). Then the kanamycin-resistant (*kan*^{r}) gene was inserted into the *cycA* gene at the *Pvu*II site in this plasmid. *E. coli* K-12 cells were then transformed with the resulting plasmid, which showed both an ampicillin- and kanamycin-resistant phenotype. Following incubation at 42°C for 12 h, we isolated a kanamycin-resistant but ampicillin-sensitive clone, which was generated by homologous recombination between chromosomal DNA and plasmid DNA. The disruption of the *cycA* gene in the chromosome was confirmed by Southern blot analysis. The *cycA*-disrupted *E. coli* strain thus obtained is named AK430.

Cloning of a Novel Glycine Transporter Gene from KS-1 Genome—Genomic DNA (150 μg) from *Thermococcus* sp. KS-1, kindly donated by Dr. T. Ohta (Tsukuba Univ), was partially digested with *Sau*3AI (2U) for 30 s at 37°C. The DNA fragments generated were fractionated by electrophoresis on 0.7% low melting agarose gel (SeaPlaque GTG Agarose, FMC, Rockland, ME), and fragments of between 2 and 8 kbp were recovered from the gel. The KS-1 genome library was constructed by ligating these DNA fragments to *Bam*HI-digested pUC19 vectors. The library generated contained 1.3×10^4 clones carrying KS-1 genome DNA fragments of an average size of about 3 kbp. The *cycA*-disrupted *E. coli* strain, AK430, was transformed with the library, and the transformed cells were incubated on plates of M9 medium containing 5 mg/ml D-alanine as a carbon source and 50 μg/ml ampicillin at 37°C for a week. Colonies grown on the plates were picked up and cultured in LB medium containing 50 μg/ml ampicillin. Plasmids were recovered from the cells and analyzed.

Southern Hybridization Analysis and DNA Sequencing—DNA fragments (5 μg) of the KS-1 genome digested with restriction enzymes were fractionated by electrophoresis on a 0.7% agarose gel and then transferred to a nylon filter (Hybond-N$^+$, Amersham, Buckinghamshire, UK) by capillary blotting. The filter was hybridized with a DNA probe for *snatA*, which was prepared by labeling with α-[^{32}P]dCTP using the Random Primer DNA Labeling Kit Ver. 2 (Takara Shuzo, Shiga). DNA sequences were determined by the dideoxy chain termination method using a DNA sequencer (ABI PRISM 377 DNA sequencing system, Applied Biosystems, Foster City, CA).

Glycine Transport Assay—E. coli cells grown in LB medium at 37°C under aerobic conditions were harvested by centrifugation and washed twice with buffer A (100 mM HEPES/Tris, pH 7.4, 2 mM MgSO4). Then the cells were suspended in buffer A supplemented with 50 μg/ml chloramphenicol, and the density of cells was adjusted to 1.6×10^9 cells per ml (OD_{600} = 2.0). The reaction was started by adding 200 μl of the cell suspension to 800 μl of buffer A containing 0.1 μCi [^{14}C]glycine and 4 mM Tris-lactate, pH 7.4, and allowed to proceed at 37°C with vigorous shaking. Aliquots of 200 μl of the suspension were withdrawn at appropriate time intervals, and the reaction was terminated by filtering through nitrocellulose membrane filters (pore size 0.45 μm) (Schleicher& Schuell, Dassel, Germany). The filters were immediately washed twice with buffer A, and the radioactivity retained on the filters was measured with a scintillation counter (Beckman LS 6000TA-2, Beckman, Fullerton, CA) in Clear-Sol II (Nacalai Tesque, Kyoto).

RESULTS

Construction of the E. coli cycA Mutant—E. coli cells transport small neutral amino acids such as glycine, alanine and serine by a specific transporter that is encoded by the *cycA* gene (*2*, *32*). A homology search of cycA revealed that its homologous genes are widely distributed among the genomes of various bacteria and archaea. However, we could not find any homologous genes in the genomes of *Pyrococcus horikoshii, Pyrococcus abyssi,* or *Pyrococcus furiosus*, which are classified in the *Thermococcales* group of Archaea (*26*). This prompted us to isolate a novel transporter gene from the genome of KS-1, which belongs to the *Pyrococcus* group, using a functional cloning technique. We first constructed a stable deletion mutant of *cycA* by homologous recombination using a temperature-sensitive replicon following a method as described (*31*). *E. coli* K12 was transformed with the plasmid encoding the disrupted *cycA* gene, in which a kanamycin-resistance gene had been inserted, and its franking regions. Then we isolated the *cycA* mutant by screening for kanamycin-resistant but ampicillin-sensitive cells (Fig. 1A). The disruption of the *cycA* gene on the chromosome was confirmed by Southern blot

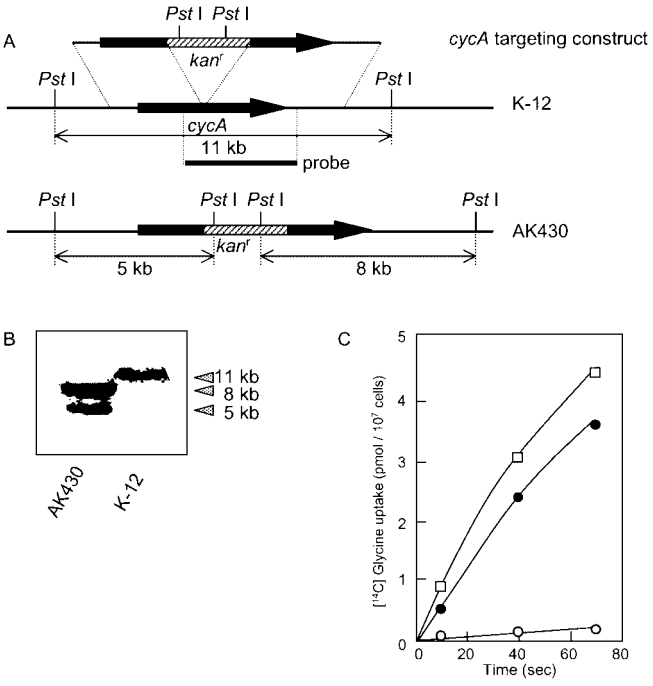

Fig. 1. **Isolation of the *cycA* mutant by homologous recombination.** A: The chromosomal 11-kbp DNA fragment carrying the *cycA* gene was cloned in a temperature-sensitive plasmid, and then the kanamycin-resistant (*kan*^{r}) gene was inserted into the *cycA* gene to generate a *cycA* targeting construct. *E. coli* K-12 cells were transformed with the resulting plasmid. Homologous recombination between the chromosome and the plasmid would insert the *kan*^{r} gene into chromosome, thus disrupting the *cycA* gene on the chromosome. The resulting disruption mutant was designated AK430. B: Southern blot analysis. Chromosomal DNA was digested with *Pst*I and fractionated on 0.8% agarose gel by electrophoresis. Southern blot hybridization analysis was performed using the DNA fragment encoding *cycA* gene as a probe. C: Glycine transport activity of *E. coli* K12 and AK430. The transport activity of *E. coli* K12 (closed circles), AK430 (open circles), and AK430 harboring the plasmid encoding *cycA* (open square) was assayed as described under "MATERIALS AND METHODS".

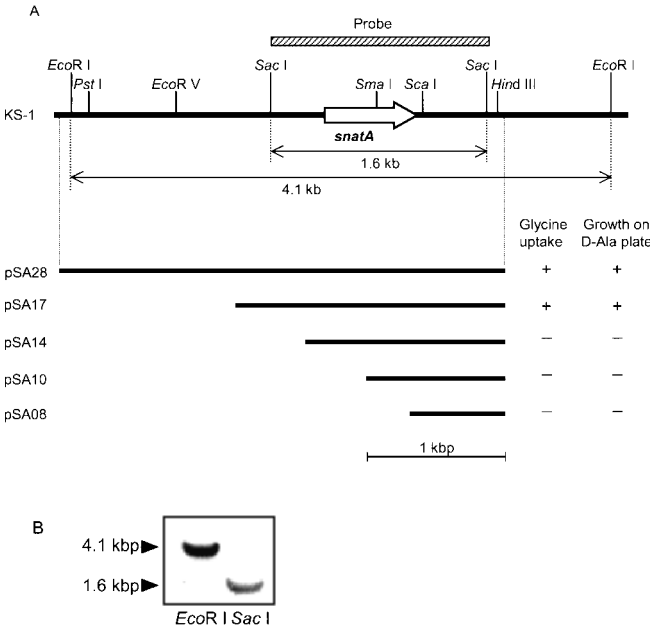

Fig. 2. **Physical map of plasmid pSA28 and its derivatives.** A: Physical map of *snatA* and its flanking region on the KS-1 chromosome and plasmids used in this study. The arrow represents ORF and the direction of transcription. The hatched box represents the DNA fragment probe used for Southern hybridization analysis. The lines below the chromosomal map represent the regions of DNA fragments carried by the plasmid. B: Southern hybridization analysis of the genome DNA of KS-1 using a DNA probe of *snatA*. KS-1 genome DNA (5 μg) was digested with *Eco*RI or *Sac*I, then fractionated on 0.8% agarose gel. The DNA was transferred to a filter, and the filter was hybridized with the DNA probe.

hybridization analysis using a DNA fragment encoding *cycA* gene as the probe (Fig. 1B). The *cycA* mutant thus obtained, designated AK430, was defective in glycine transport, and transformation of this strain with a plasmid encoding *cycA* restored the transporter activity (Fig. 1C).

Cloning of a Gene(s) for the Glycine Transporter from the KS-1 Genome Library—Using the AK430 strain, we cloned the gene(s) for the glycine transporter from the genome library of *Thermococcus* sp. KS-1. After transforming the library into AK430 cells, colonies formed on agar plates of minimal medium containing D-alanine as a carbon source were isolated and tested for glycine transport. It should be noted that it took a week to identify colonies grown on the plate as described under "MATERIALS AND METHODS", which in turn indicated that D-alanine served as a poor substrate for the transformed cells (see below). We obtained several clones capable of glycine transport and selected a single clone with the highest activity for further analysis. The plasmid pSA28, recovered from the clone, contained a DNA insert of about 2.8 kbp (Fig. 2A). Southern hybridization analysis revealed that it hybridized with either a 4.1-kbp fragment of *Eco*RI-digested or a 1.6-kbp fragment of *Sac*I-digested KS-1 genome (Fig. 2B). We next prepared four deletion plasmids (pSA17, pSA14, pSA10, and pSA08) from pSA28 and tested for both glycine transport activity and colony forming capability on minimal medium containing D-alanine as described. Of these plasmids, only pSA17 was facilitated both transport activity and colony formation (Fig. 2A). It was thus obvious that this plasmid contained the gene(s) for the glycine transporter of *Thermococcus* sp. KS-1.

Primary structure of the snatA Gene—Upon determination of the DNA sequence of pSA17, we found an open reading frame coding for 216 amino acids (*snatA*) (Fig. 3A). A Shine-Dalgarno box–like sequence was found upstream of an initiation codon ATG. The DNA sequence analysis of pSA14, which showed no transport activity, revealed that it started from 97 bp upstream of the initiation codon of *snatA* and contained its full open reading frame. This indicated that the *snatA* promoter is located more than 100 bp and less than 300 bp upstream of the initiation codon of *snatA*. Indeed, Pribnow box-like sequences (TTGAAT—AAGTAT and GTACCA–TATAAT) were found in this region. Although the reason was unclear, the [G+C] content of *snatA* was calculated to be 52.2%, which was significantly higher than that of the total genome of KS-1, 42% (*27*).

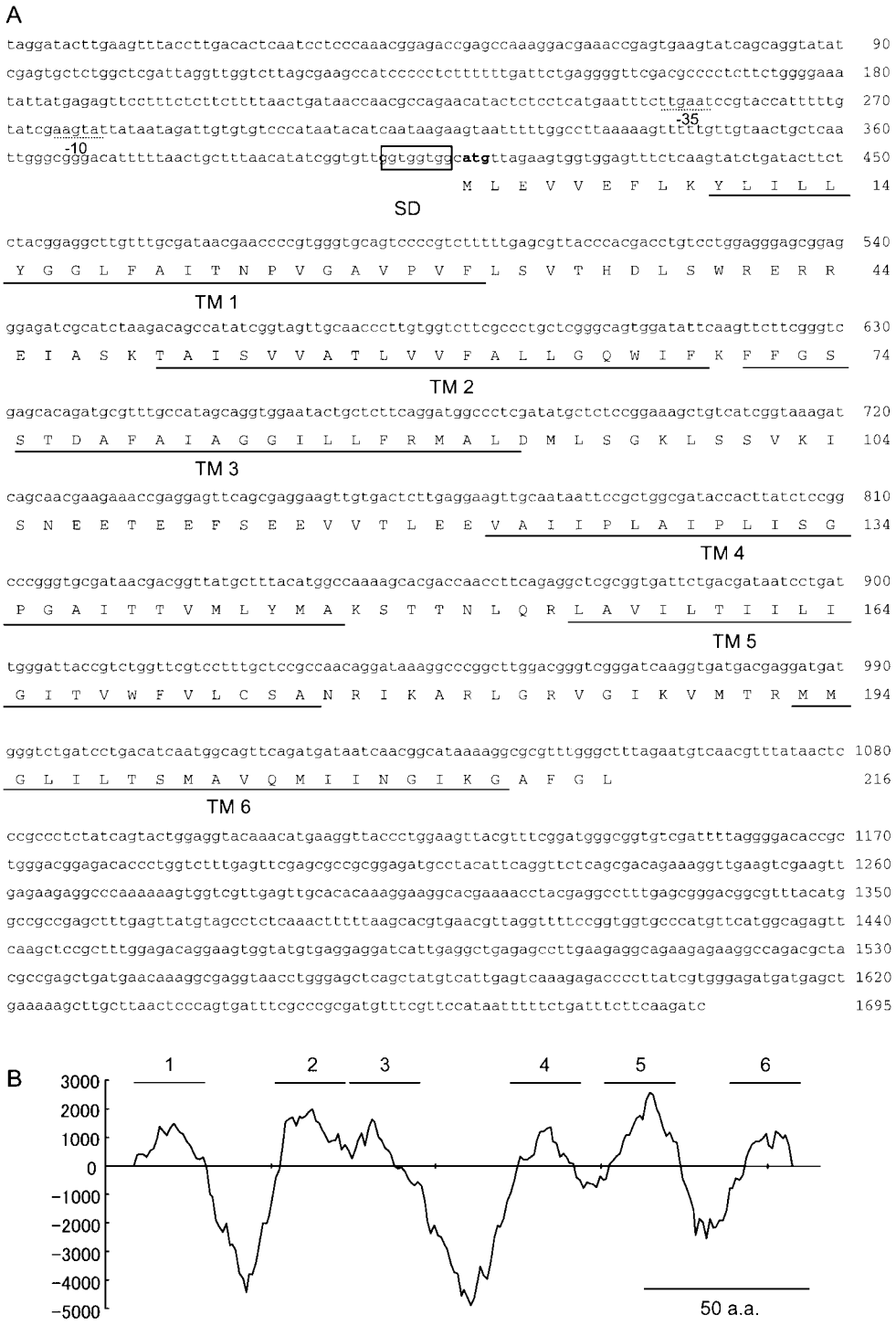

Fig. 3. **Nucleotide and predicted amino acid sequences of *snatA*.** A: Nucleotide sequence of the DNA fragment carried by pSA17 plasmid and the predicted amino acid sequence of *snatA*. Pribnow box-like sequences and the Shine-Dalgarno sequence (SD) are shown by dotted lines and a box, respectively. The deduced amino acid sequences of SnatA are shown below the DNA sequence. Bold letters represent the start codon of *snatA*. The predicted membrane-spanning segments, TM1-TM6, are underlined. B: Hydropathy plot of SnatA. The amino acid sequence of SnatA was analyzed using the TMpred program. The numbers on the profile represent the predicted membrane-spanning segments.

The apparent molecular mass of SnatA was 23,500 Da, and hydropathy analysis using a TMpred program (ISREC TMpred server) (*33*) revealed that it was a membrane protein consisting of six membrane-spanning segments with both the N- and C-terminals facing the cytoplasmic side of the membrane (Fig. 3B).

Characteristics of Glycine Transport by SnatA—AK430 cells harboring the plasmid pSA28 were used for characterization of SnatA-mediated glycine transport. As shown in Fig. 4A, glycine transport was severely suppressed when an uncoupling agent, carbonylcyanide *p*-trifluoromethoxyphenylhydrazone (FCCP), was present. It was not affected by the K^+ ionophore valinomycin, but it was inhibited when nigericin, a K^+/H^+ exchanger, was added together with valinomycin (Fig. 4B). It was also not influenced by Na^+ or K^+ (data not shown). This suggested that the glycine transport mediated by SnatA in *E. coli* cells is energy dependent and driven by an electrochemical potential of H^+ across the membrane.

Kinetic studies (Table 1) revealed that the apparent Kt value for glycine is 24 μM, which is up to one order of magnitude higher than those of CycA-mediated trans-

Table 1. **Apparent K_t values of SnatA, CycA, and ACP.**

Transporter	K_t for glycine (μM)
SnatA	24.0
CycA	2.4
ACP	4.0

The transport assays were carried out using various concentrations of glycine as described under "MATERIALS AND METHODS". The initial velocities were determined by sampling at 30 s after the addition of [^{14}C]glycine at 37°C. Apparent K_t values (apparent affinity constants of transport) obtained by Lineweaver-Burk plotting are shown.

Table 2. **Substrate specificities of SnatA, CycA, and ACP.**

	Inhibition (%)		
Competitor added	SnatA	CycA	ACP
Glycine	100	100	100
D-Alanine	15	92	80
L-Alanine	84	76	85
L-Cysteine	95	10	n.d.
L-Threonine	100	22	51
L-Serine	90	0	86
L-Methionine	65	0	51
L-Tryptophan	60	0	19
L-Phenylalanine	37	0	17
L-Proline	24	0	0
L-Leucine	35	0	0
L-Isoleucine	20	0	0
L-Valine	18	0	14
L-Tyrosine	22	0	0
L-Asparagine	20	0	0
L-Glutamine	0	44	37
L-Aspartic acid	0	0	70
L-Glutamic acid	0	0	66
L-Lysine	0	0	0
L-Histidine	14	0	73
L-Arginine	0	n.d.	n.d.

SnatA: AK430 cells harboring plasmid pSA28 were incubated for 60 min with 4 μM [^{14}C]glycine in the presence of each competitor at a concentration of 830 μM. CycA: AK430 cells harboring plasmid pYN5108, which encodes *cycA*, were incubated for 40 sec with 4 μM [^{14}C]glycine in the presence of each competitor at a concentration of 500 μM. ACP: AK430 cells harboring plasmid pAC5629 (*60*) were incubated for 40 s with 4 μM [^{14}C]glycine in the presence of each competitor at a concentration of 500 μM. The inhibitory effect of each amino acid on glycine transport is shown as a percentage that of glycine. n.d.: not determined.

port (2.4 μM) and ACP-mediated transport (4 μM). The substrate specificity of the glycine transport by SnatA was determined by means of competition studies and compared with those of CycA and ACP (Table 2). More than 80% inhibition was observed when L-alanine, L-cysteine, L-threonine, or L-serine was used as competitor. Also, L-methionine and L-tryptophan showed significant inhibitory effects (more than 50%), indicating that SnatA has a relatively broad substrate specificity toward neutral amino acids. A similar broad substrate specificity toward neutral amino acids was observed in AK430 cells transformed with the *acp* gene, while CycA showed rather strict substrate specificity (Table 2). Both SnatA and ACP transported glycine, L-alanine, L-threonine, and L-serine effectively, but SnatA was unable to transport acidic amino acids, while ACP transported them fairly

Table 3. **Expression of *snatA* caused growth inhibition of WAT9.**

	Carbon source	
	Serine (TS)	Lactose (TL)
WAT9/pUC119	+	++
WAT9/pSA28 (*snatA*)	–	++
WAT9/pMST321 (*sstT*)	–	++
W3133-2	–	++

A serine transport–defective mutant WAT9 was transformed with plasmid pUC119, pSA28, or pMST321. W3133-2 is the wild-type strain. Cells were grown on a minimal agar plate containing 5 mg/ml L-serine (TS) or 40 mM lactose (TL) as carbon source for 24 h at 37°C. (–) indicates no growth and (+) indicates of good growth of cells.

well. Furthermore, the mode of competition by D-alanine was different among these three glycine transporters: D-alanine showed strong competition with glycine in the ACP or CycA system, indicating that D-alanine behaves as a good substrate for either transporter, while it is a poor substrate for SnatA.

It is intriguing that both serine and threonine served as good substrates for either SnatA or ACP but not for CycA (Table 2). In *E. coli*, it has been shown that the transport of serine/threonine is catalyzed by the Na^+-specific transporter SstT (*34*). Here, we used an *sstT* deletion mutant WAT9 to test how efficiently SnatA transports serine: efficient transport should suppress growth, because the accumulation of serine through SstT results in severe feedback inhibition of the serine/threonine metabolism, thereby suppressing the growth of cells on minimal medium with L-serine as a carbon source. As shown in Table 3, while WAT9 was able to form colonies on minimal medium containing L-serine, wild-type cells (W3133–2) as well as WAT9 cells transformed with either pSA28 or pMST321 carrying the *sstT* gene (*34*) failed to grow on the medium. This indicates that SnatA mediates serine transport as efficiently as SstT in *E. coli* cells.

DISCUSSION

In this report we describe for the first time, to our knowledge, the cloning of the amino acid transporter gene from the hyperthermophilic Archaeon *Thermococcus* sp. KS-1 by means of functional cloning using the newly established *E. coli* mutant AK430, which is defective in glycine/alanine transport. A numbers of genes and proteins have been isolated and characterized from hyperthermophilic archaea and bacteria (for review see Ref. *22*). However, except for a few proteins related to energy metabolism (*35–39*) and sugar transporters (*40–42*), membrane proteins have not yet been sufficiently analyzed. In particular, none of the proteins involved in the secondary active transport of amino acid has been identified in hyperthermophiles.

The cloned gene *snatA* facilitates glycine transport in AK430 cells and encodes a postulated hydrophobic membrane protein with six membrane-spanning segments. The majority of molecules involved in the active transport of sugars and amino acids are structurally similar in having 10 to 12 membrane-spanning segments. However, there are several exceptions, such as carriers in mitochondrial inner membranes (*43–45*) and some of the

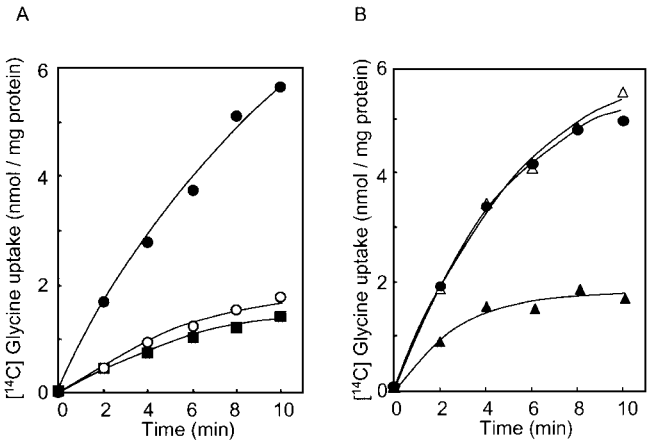

Fig. 4. **Characterization of the glycine transport activity of SnatA expressed in AK430 cells.** A: The transport activity of AK430 cells harboring plasmid pSA28 (AK430/pSA28) (closed circles), AK430 harboring control vector pUC119 (open squares) and AK430/pSA28 cells in the presence of 10 μg/ml FCCP (open circles) was assayed as described under "MATERIALS AND METHODS". B: Effect of valinomycin and nigericin on glycine transport by SnatA. AK430/pSA28 cells were incubated in the absence (closed circles) or presence of valinomycin (open triangles) or valinomycin *plus* nigericin (closed triangles).

transporters containing the ATP-binding cassette (ABC) transporters (*46*, *47*). The latter proteins consist of about half of the amino acid residues of the former and are postulated to span the membrane five to six times. For this reason, ABC transporters with 6 transmembrane segments are called "half transporters" and are thought to form dimers when functioning (*48–50*). Similar findings have been reported with enzyme II of the phosphoenolpyruvate : sugar phosphotransferase system specific for mannitol transport (mannitol permease) in *E. coli* (*51–54*). Although no direct evidence is available at present, it is plausible that SnatA forms a dimer to function as a glycine transporter.

The transport of glycine by SnatA seems to be driven by an electrochemical potential of H^+, since it was abolished by the addition of either the protonophore FCCP or valinomycin together with nigericin (Fig. 4, A and B) and was not influenced by the presence of Na^+ (data not shown). This is in contrast to ACP in the thermophilic bacterium PS3, which is driven by an electrochemical potential of Na^+ (*55*, *56*). Furthermore, this seems to be somewhat contradictory to the report that *Thermococcus* sp. KS-1 was isolated from a submarine hydrothermal vent and strictly requires Na^+ for growth (*27*). It is possible that the electrochemical potential of Na^+ is converted to one of H^+ by an Na^+/H^+ exchanger, which may be present in KS-1membranes.

Another feature of SnatA is that its substrate specificity is different from those of ACP and CycA (Table 2). While glycine transport by ACP and CycA is efficiently inhibited by both L-alanine and D-alanine, glycine transport by SnatA is only partially inhibited by D-alanine, indicating that the substrate specificity of SnatA is stereo-specific. Thus, SnatA is a novel type of alanine/glycine transporter. Indeed, *snatA* does not show significant homology to *acp* or *cycA*. Enzymes derived from hyperthermophiles are generally not only thermostable (resistant to irreversible inactivation at high temperatures >80°C) but also optimally active at high temperatures and almost inactive below 40°C (*22*). Several enzymes isolated from *Thermococcus* sp. KS-1 have been shown to be most active at temperatures >70°C (*28*, *29*). In this context, it is interesting that AK430 cells transformed with *snatA* show significant glycine transport activity at the optimal temperature for *E. coli* growth (37°C). Preliminary studies revealed that SnatA in *E. coli* cells seemed to be more active at higher temperatures and permissive for growth below 45°C (data not shown). However, it is obviously necessary to establish a hyperthermophilic system for genetic manipulation and/or an *in vitro* artificial system to clarify these points.

Homology searches of SnatA using BLAST [Basic Local Alignment Search Tool (*57*)] have revealed that SnatA is a novel protein without any homologous protein enrolled in the protein bank to date. However, several hypothetical proteins proposed recently in genome projects of various organisms have shown high homology scores with SnatA: E82125 (VC2035) of *Vibrio cholerae* (*58*), YchE of *E. coli* (*59*), Y760 (PH0760) of *Pyrococcus horikoshii* (*25*), PF0745 of *Pyrococcus furios* (published on http://www.genome.utah.edu/sequence.html), Y863 of *Pyrococcus abyssi* (Heilig, R., unpublished), and YG77 (MJ1677) of *Methanococcus jannaschii* (*24*). The latter four organisms are classified as hyperthermophilic archaea (*22*), and these four hypothetical proteins show significantly high homology scores with more than 40% identity in the amino acid sequences. Furthermore, there are several homologues of these proteins found in the respective genomes (Y214 of *P. horikoshii*, and YhgN and MarC in *E. coli*), indicating that these, including SnatA, form a family of prokaryote cells. Characterization of homologues in *E. coli* is now under investigation.

We are grateful to Drs. T. Maruyama and T. Hoaki of the Marine Biotechnology Institute, Iwate, and Dr. T. Ohta of Tsukuba University, Ibaraki, for providing us with the genome of *Thermococcus* sp. KS-1. We also thank Drs. W. Ogawa and T. Tsuchiya of Okayama University, Okayama, for providing us with *E. coli* strains WAT9 and W3133-2 and the plasmid pMST321, and Dr. Y. Kohara of the National Institute of Genetics, Shizuoka, for providing us with the λ7E9 phage.

Nucleotide sequence data reported are available in the DDBJ/EMBL/GenBank Databases under the accession number AB093585.

REFERENCES

1. Saier, M.H., Jr. (2000) Families of transmembrane transporters selective for amino acids and their derivatives. *Microbiology* **146**, (Pt 8), 1775–1795
2. Robbins, J.C. and Oxender, D.L. (1973) Transport systems for alanine, serine, and glycine in *Escherichia coli* K-12. *J. Bacteriol.* **116**, 12–18
3. Ghrist, A.C. and Stauffer, G.V. (1995) The *Escherichia coli* glycine transport system and its role in the regulation of the glycine cleavage enzyme system. *Microbiology* **141**, (Pt 1), 133–140
4. MacLeod, P.R. and MacLeod, R.A. (1992) Identification and sequence of a Na(+)-linked gene from the marine bacterium *Alteromonas haloplanktis* which functionally complements the dagA gene of *Escherichia coli*. *Mol. Microbiol.* **6**, 2673–2681

5. Kamata, H., Akiyama, S., Morosawa, H., Ohta, T., Hamamoto, T., Kambe, T., Kagawa, Y., and Hirata, H. (1992) Primary structure of the alanine carrier protein of thermophilic bacterium PS3. *J. Biol. Chem.* **267**, 21650–21655
6. Sumantran, V.N., Schweizer, H.P., and Datta, P. (1990) A novel membrane-associated threonine permease encoded by the tdcC gene of *Escherichia coli*. *J. Bacteriol.* **172**, 4288–4294
7. Ogawa, W., Kayahara, T., Tsuda, M., Mizushima, T., and Tsuchiya, T. (1997) Isolation and characterization of an *Escherichia coli* mutant lacking the major serine transporter, and cloning of a serine transporter gene. *J. Biochem.* **122**, 1241–1245
8. Shao, Z., Lin, R.T., and Newman, E.B. (1994) Sequencing and characterization of the sdaC gene and identification of the sdaCB operon in *Escherichia coli* K12. *Eur. J. Biochem.* **222**, 901–907
9. Nikaido, H. and Saier, M.H., Jr. (1992) Transport proteins in bacteria: common themes in their design. *Science* **258**, 936–942
10. Poolman, B. and Konings, W.N. (1993) Secondary solute transport in bacteria. *Biochim. Biophys Acta* **1183**, 5–39
11. Reizer, J., Reizer, A., and Saier, M.H., Jr. (1994) A functional superfamily of sodium/solute symporters. *Biochim. Biophys Acta* **1197**, 133–166
12. Vrljic, M., Sahm, H., and Eggeling, L. (1996) A new type of transporter with a new type of cellular function: L-lysine export from *Corynebacterium glutamicum*. *Mol. Microbiol.* **22**, 815–826
13. Zakataeva, N.P., Aleshin, V.V., Tokmakova, I.L., Troshin, P.V., and Livshits, V.A. (1999) The novel transmembrane *Escherichia coli* proteins involved in the amino acid efflux. *FEBS Lett.* **452**, 228–232
14. Vrljic, M., Garg, J., Bellmann, A., Wachi, S., Freudl, R., Malecki, M.J., Sahm, H., Kozina, V.J., Eggeling, L., and Saier, M.H., Jr. (1999) The LysE superfamily: topology of the lysine exporter LysE of *Corynebacterium glutamicum*, a paradyme for a novel superfamily of transmembrane solute translocators. *J. Mol. Microbiol. Biotechnol.* **1**, 327–36
15. Runswick, M.J., Powell, S.J., Nyren, P., and Walker, J.E. (1987) Sequence of the bovine mitochondrial phosphate carrier protein: structural relationship to ADP/ATP translocase and the brown fat mitochondria uncoupling protein. *EMBO J.* **6**, 1367–1373
16. Palmieri, F. (1994) Mitochondrial carrier proteins. *FEBS Lett.* **346**, 48–54
17. Palmieri, L., Runswick, M.J., Fiermonte, G., Walker, J.E., and Palmieri, F. (2000) Yeast mitochondrial carriers: bacterial expression, biochemical identification and metabolic significance. *J. Bioenerg. Biomembr.* **32**, 67–77
18. Woese, C.R. and Fox, G.E. (1977) Phylogenetic structure of the prokaryotic domain: the primary kingdoms. *Proc. Natl Acad. Sci. USA* **74**, 5088–5090
19. Fox, G.E., Stackebrandt, E., Hespell, R.B., Gibson, J., Maniloff, J., Dyer, T.A., Wolfe, R.S., Balch, W.E., Tanner, R.S., Magrum, L.J., Zablen, L.B., Blakemore, R., Gupta, R., Bonen, L., Lewis, B.J., Stahl, D.A., Luehrsen, K.R., Chen, K.N., and Woese, C.R. (1980) The phylogeny of prokaryotes. *Science* **209**, 457–463
20. Noll, K.M. and Vargas, M. (1997) Recent advances in genetic analyses of hyperthermophilic archaea and bacteria. *Arch. Microbiol.* **168**, 73–80
21. Stetter, K.O. (1999) Extremophiles and their adaptation to hot environments. *FEBS Lett.* **452**, 22–25
22. Vieille, C. and Zeikus, G.J. (2001) Hyperthermophilic enzymes: sources, uses, and molecular mechanisms for thermostability. *Microbiol. Mol. Biol. Rev.* **65**, 1–43
23. Kagawa, Y., Sone, N., Hirata, H., and Yoshida, M. (1979) Structure and function of H^+-ATPase. *J. Bioenerg. Biomembr.* **11**, 39–78
24. Bult, C.J., White, O., Olsen, G.J., Zhou, L., Fleischmann, R.D., Sutton, G.G., Blake, J.A., FitzGerald, L.M., Clayton, R.A., Gocayne, J.D., Kerlavage, A.R., Dougherty, B.A., Tomb, J.F., Adams, M.D., Reich, C.I., Overbeek, R., Kirkness, E.F., Weinstock, K.G., Merrick, J.M., Glodek, A., Scott, J.L., Geoghagen, N.S., and Venter, J.C. (1996) Complete genome sequence of the methanogenic archaeon, *Methanococcus jannaschii*. *Science* **273**, 1058–1073
25. Kawarabayasi, Y., Sawada, M., Horikawa, H., Haikawa, Y., Hino, Y., Yamamoto, S., Sekine, M., Baba, S., Kosugi, H., Hosoyama, A., Nagai, Y., Sakai, M., Ogura, K., Otsuka, R., Nakazawa, H., Takamiya, M., Ohfuku, Y., Funahashi, T., Tanaka, T., Kudoh, Y., Yamazaki, J., Kushida, N., Oguchi, A., Aoki, K., and Kikuchi, H. (1998) Complete sequence and gene organization of the genome of a hyper-thermophilic archaebacterium, *Pyrococcus horikoshii* OT3 (supplement). *DNA Res.* **5**, 147–155
26. Zillig, W. and Reysenbach, A.-L. (2001) Class IV *Thermococci* class. nov. in *Bergey's Manual of Systematic Bacteriology* (Boone, D.R., Castenholz, R.W., and Garrity, G.M., eds) Vol. **1**, pp. 341–348, Springer, New York
27. Hoaki, T., Nishijima, M., Kato, M., Adachi, K., Mizobuchi, S., Hanzawa, N., and Maruyama, T. (1994) Growth requirements of hyperthermophilic sulfur-dependent heterotrophic archaea isolated from a shallow submarine geothermal system with reference to their essential amino acids. *Appl. Environ. Microbiol.* **60**, 2898–2904
28. Yoshida, T., Yohda, M., Iida, T., Maruyama, T., Taguchi, H., Yazaki, K., Ohta, T., Odaka, M., Endo, I., and Kagawa, Y. (1997) Structural and functional characterization of homo-oligomeric complexes of alpha and beta chaperonin subunits from the hyperthermophilic archaeum *Thermococcus* strain KS-1. *J. Mol. Biol.* **273**, 635–645
29. Iida, T., Furutani, M., Nishida, F., and Maruyama, T. (1998) FKBP-type peptidyl-prolyl cis-trans isomerase from a sulfur-dependent hyperthermophilic archaeon, *Thermococcus* sp. KS-1. *Gene* **222**, 249–255
30. Kohara, Y., Akiyama, K., and Isono, K. (1987) The physical map of the whole *E. coli* chromosome: application of a new strategy for rapid analysis and sorting of a large genomic library. *Cell* **50**, 495–508
31. Matsuyama, S. and Mizushima, S. (1985) Construction and characterization of a deletion mutant lacking *micF*, a proposed regulatory gene for OmpF synthesis in *Escherichia coli*. *J. Bacteriol.* **162**, 1196–1202
32. Lee, M., Robbins, J.C., and Oxender, D.L. (1975) Transport properties of merodiploids covering the *dagA* locus in *Escherichia coli* K-12. *J. Bacteriol.* **122**, 1001–1005
33. Hofmann, K. and Stoffel, W. (1992) PROFILEGRAPH: an interactive graphical tool for protein sequence analysis. *Comput. Appl. Biosci.* **8**, 331–337
34. Ogawa, W., Kim, Y.M., Mizushima, T., and Tsuchiya, T. (1998) Cloning and expression of the gene for the Na^+-coupled serine transporter from *Escherichia coli* and characteristics of the transporter. *J. Bacteriol.* **180**, 6749–6752
35. Iida, T., Hoaki, T., Kamino, K., Inatomi, K., Kamagata, Y., and Maruyama, T. (1996) Vacuolar-type ATPase in a hyperthermophilic archaeum, *Thermococcus* sp. KI. *Biochem. Biophys. Res. Commun.* **229**, 559–564
36. Dirmeier, R., Keller, M., Frey, G., Huber, H., and Stetter, K.O. (1998) Purification and properties of an extremely thermostable membrane-bound sulfur-reducing complex from the hyperthermophilic *Pyrodictium abyssi*. *Eur. J. Biochem.* **252**, 486–491
37. Nubel, T., Klughammer, C., Huber, R., Hauska, G., and Schutz, M. (2000) Sulfide:quinone oxidoreductase in membranes of the hyperthermophilic bacterium *Aquifex aeolicus* (VF5). *Arch. Microbiol.* **173**, 233–244
38. Perez-Castineira, J.R., Lopez-Marques, R.L., Losada, M., and Serrano, A. (2001) A thermostable K(+)-stimulated vacuolar-type pyrophosphatase from the hyperthermophilic bacterium *Thermotoga maritima*. *FEBS Lett.* **496**, 6–11
39. Scheide, D., Huber, R., and Friedrich, T. (2002) The proton-pumping NADH:ubiquinone oxidoreductase (complex I) of *Aquifex aeolicus*. *FEBS Lett.* **512**, 80–84
40. Cusdin, F.S., Robinson, M.J., Holman, G.D., Hough, D.W., and Danson, M.J. (1996) Characterisation of glucose transport in

the hyperthermophilic Archaeon *Sulfolobus solfataricus*. *FEBS Lett.* **387**, 193–195
41. Horlacher, R., Xavier, K.B., Santos, H., DiRuggiero, J., Kossmann, M., and Boos, W. (1998) Archaeal binding protein-dependent ABC transporter: molecular and biochemical analysis of the trehalose/maltose transport system of the hyperthermophilic archaeon *Thermococcus litoralis*. *J. Bacteriol.* **180**, 680–689
42. Greller, G., Horlacher, R., DiRuggiero, J., and Boos, W. (1999) Molecular and biochemical analysis of MalK, the ATP-hydrolyzing subunit of the trehalose/maltose transport system of the hyperthermophilic archaeon *Thermococcus litoralis*. *J. Biol. Chem.* **274**, 20259–20264
43. Kramer, R. (1996) Structural and functional aspects of the phosphate carrier from mitochondria. *Kidney Int.* **49**, 947–952
44. Schroers, A., Burkovski, A., Wohlrab, H., and Kramer, R. (1998) The phosphate carrier from yeast mitochondria. Dimerization is a prerequisite for function. *J. Biol. Chem.* **273**, 14269–14276
45. Trezeguet, V., Le Saux, A., David, C., Gourdet, C., Fiore, C., Dianoux, A., Brandolin, G., and Lauquin, G.J. (2000) A covalent tandem dimer of the mitochondrial ADP/ATP carrier is functional *in vivo*. *Biochim. Biophys Acta* **1457**, 81–93
46. Klein, I., Sarkadi, B., and Varadi, A. (1999) An inventory of the human ABC proteins. *Biochim. Biophys Acta* **1461**, 237–262
47. Kerr, I.D. (2002) Structure and association of ATP-binding cassette transporter nucleotide-binding domains. *Biochim. Biophys Acta* **1561**, 47–64
48. Croop, J.M. (1998) Evolutionary relationships among ABC transporters. *Methods Enzymol.* **292**, 101–116
49. Liu, L.X., Janvier, K., Berteaux-Lecellier, V., Cartier, N., Benarous, R., and Aubourg, P. (1999) Homo- and heterodimerization of peroxisomal ATP-binding cassette half-transporters. *J. Biol. Chem.* **274**, 32738–32743
50. Bates, S.E., Robey, R., Miyake, K., Rao, K., Ross, D.D., and Litman, T. (2001) The role of half-transporters in multidrug resistance. *J. Bioenerg. Biomembr.* **33**, 503–511
51. Roossien, F.F. and Robillard, G.T. (1984) Mannitol-specific carrier protein from the *Escherichia coli* phosphoenolpyruvate-dependent phosphotransferase system can be extracted as a dimer from the membrane. *Biochemistry* **23**, 5682–5685
52. Postma, P.W., Lengeler, J.W., and Jacobson, G.R. (1993) Phosphoenolpyruvate:carbohydrate phosphotransferase systems of bacteria. *Microbiol. Rev.* **57**, 543–594
53. Saraceni-Richards, C.A. and Jacobson, G.R. (1997) Subunit and amino acid interactions in the *Escherichia coli* mannitol permease: a functional complementation study of coexpressed mutant permease proteins. *J. Bacteriol.* **179**, 5171–5177
54. Koning, R.I., Keegstra, W., Oostergetel, G.T., Schuurman-Wolters, G., Robillard, G.T., and Brisson, A. (1999) The 5 A projection structure of the transmembrane domain of the mannitol transporter enzyme II. *J. Mol. Biol.* **287**, 845–851
55. Hirata, H., Kambe, T., and Kagawa, Y. (1984) A purified alanine carrier composed of a single polypeptide from thermophilic bacterium PS3 driven by either proton or sodium ion gradient. *J. Biol. Chem.* **259**, 10653–10656
56. MacLeod, P.R. and MacLeod, R.A. (1986) Cloning in *Escherichia coli* K-12 of a Na^+-dependent transport system from a marine bacterium. *J. Bacteriol.* **165**, 825–830
57. Altschul, S.F., Madden, T.L., Schaffer, A.A., Zhang, J., Zhang, Z., Miller, W., and Lipman, D.J. (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucleic Acids Res.* **25**, 3389–3402
58. Heidelberg, J.F., Eisen, J.A., Nelson, W.C., Clayton, R.A., Gwinn, M.L., Dodson, R.J., Haft, D.H., Hickey, E.K., Peterson, J.D., Umayam, L., Gill, S.R., Nelson, K.E., Read, T.D., Tettelin, H., Richardson, D., Ermolaeva, M.D., Vamathevan, J., Bass, S., Qin, H., Dragoi, I., Sellers, P., McDonald, L., Utterback, T., Fleishmann, R.D., Nierman, W.C., and White, O. (2000) DNA sequence of both chromosomes of the cholera pathogen *Vibrio cholerae*. *Nature* **406**, 477–483
59. Blattner, F.R., Plunkett, G., 3rd, Bloch, C.A., Perna, N.T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., Gregor, J., Davis, N.W., Kirkpatrick, H.A., Goeden, M.A., Rose, D.J., Mau, B. and Shao, Y. (1997) The complete genome sequence of *Escherichia coli* K-12. *Science* **277**, 1453–1474
60. Murai, N., Kamata, H., Nagashima, Y., Yagisawa, H., and Hirata, H. (1995) A novel insertion sequence (IS)-like element of the thermophilic bacterium PS3 promotes expression of the alanine carrier protein-encoding gene. *Gene* **163**, 103–107