

## Cloning of a Novel Na<sup>+</sup>-Dependent L-Serine Specific Symporter Gene from *Haemophilus influenzae* Rd and Characteristics of the Transporter

KIM, YOUNG-MOG<sup>1\*</sup>, IN-KOO RHEE<sup>2</sup>, AND TOMOFUSA TSUCHIYA<sup>3</sup>

<sup>1</sup>Institute of Agricultural Science & Technology, and <sup>2</sup>Department of Agricultural Chemistry, Kyungpook National University, Daegu 702-701, Korea

<sup>3</sup>Faculty of Pharmaceutical Science, Okayama University, Okayama 700-8530, Japan

Received: July 21, 2003

Accepted: April 24, 2004

**Abstract** A protein that exhibited a high similarity to a major serine transporter of *Escherichia coli*, SdaC, was found in *Haemophilus influenzae* Rd. Also, Na<sup>+</sup>-stimulated serine transport activity was detected in the cells. The *sdaC* of *H. influenzae* was cloned and the properties of the transporter were investigated. The activity of serine transport was stimulated by Na<sup>+</sup>. Uptake of Na<sup>+</sup> elicited by L-serine influx into cells was also observed, which supports the idea that L-serine is transported by a mechanism of Na<sup>+</sup>/serine symport. No uptake of H<sup>+</sup> elicited by L-serine influx was detected. This result was not consistent with that obtained with the homologous protein, SdaC of *E. coli*, which uses H<sup>+</sup> as a coupling cation. The serine transport via the SdaC of *H. influenzae* was not inhibited by other amino acids such as threonine or D-serine like the SdaC of *E. coli*. Thus, the SdaC of *H. influenzae* is a Na<sup>+</sup>-dependent L-serine specific symporter and an unusual natural mutant. The  $K_m$  and the  $V_{max}$  value for the serine transport in the SdaC of *H. influenzae* were 7.6  $\mu$ M and 22.9 nmol/min/mg protein, respectively.

**Key words:** *Haemophilus influenzae* Rd, Na<sup>+</sup>-dependent L-serine specific symporter

Four transport systems for serine have been identified in *E. coli*. The major system for the uptake of serine is the Na<sup>+</sup>/serine symporter, SstT, which is produced constitutively in *E. coli* K-12 [7, 14]. This system mediates the uptake of serine and threonine. The second system is a serine-specific SdaC, which is induced by leucine, and is an H<sup>+</sup>/serine symporter [8, 20]. The third system, the TdcC system, has been reported as a threonine transport system that also mediates serine transport [22]. This is also an H<sup>+</sup>-coupled symporter [17], and is not present when *E. coli*

cells are grown under aerobic conditions [22]. The fourth system is a leucine-isoleucine-valine system (LIV-1) [1]. A periplasmic binding protein is involved in this transporter and it is an ATP-driven system [1]. The presence of multiple transport systems for serine in *E. coli* made it difficult to isolate a serine-transport defective mutant, which would be very useful for cloning gene(s) encoding the serine transporter(s). There has been a success in isolating a mutant that lacks the principal serine transporter, SstT [17], making it easy to clone the serine transporter genes. In fact, there was success in cloning several serine transporter genes using the mutant as the cloning host [17, 18].

Venter and coworkers reported the complete nucleotide sequence of the genome from *Haemophilus influenzae* Rd [6]. A gene exhibiting a high similarity to *sdaC* of *E. coli* was identified, and L-serine specific transport activity in *H. influenzae* cells was also observed [13]. The L-serine transport system showed properties similar to SdaC of *E. coli*. However, the L-serine transport system seems to use Na<sup>+</sup> as a coupling ion while the SdaC of *E. coli* uses H<sup>+</sup> [8]. Thus, there is a possibility to obtain an unusual natural mutant type. For transporters, the comparison between family members that have different substrate selectivity, ion coupling stoichiometry, or kinetic properties provides important insights to understanding the structure-function relationships of protein. In this paper, the cloning of a novel Na<sup>+</sup>-dependent L-serine specific symporter gene from *H. influenzae* Rd and the characteristics of the transporter are reported.

### MATERIALS AND METHODS

#### Materials

Radioactive [<sup>14</sup>C]serine was obtained from Amersham Life Science (Piscataway, NJ, U.S.A.). All other reagents were of reagent grade and purchased from commercial sources.

\*Corresponding author

Phone: 82-53-950-5718; Fax: 82-53-953-7233;

E-mail: ymkimchoi@bh.knu.ac.kr

### Gene Cloning and Sequencing

Chromosomal DNA was prepared from cells of *H. influenzae* Rd by the method of Berns and Thomas [2]. The chromosomal DNA was partially digested with *Sau3A*I and fragments of 2 to 4 kbp were separated by sucrose density gradient centrifugation. The DNA fragments were ligated into pSTV 28 (TaKaRa, Japan), which had been digested with *Bam*H1 and dephosphorylated with bacterial alkaline phosphatase by using T4 DNA ligase. Competent cells of *E. coli* WAT9 [17] were transformed with the ligated plasmids and spread on the minimal medium consisting of 40 mM serine, 1 mM glycine, 1 mM isoleucine, 1 mM threonine, 15  $\mu$ g of chloramphenicol per ml, and 1.5% agar. The plates were incubated at 37°C for 3 days. Plasmids were prepared from the transformants and the insert nucleotides were sequenced by dideoxy-chain-termination [19].

### Transport Assay

Transport of serine into bacterial cells was assayed as described elsewhere [4, 12] with minor modification. Cells were grown under aerobic conditions at 37°C in a minimal medium [18] supplemented with 40 mM potassium lactate. Cells were harvested at the late logarithmic phase of growth. Harvested cells were washed twice with buffer A (0.1 M MOPS-Tris, 2 mM  $Mg_2SO_4$ , pH 7.0). After harvesting and washing, the pellet was suspended in 2 ml of 0.1 M MOPS-Tris (pH 7.0) containing 50  $\mu$ g/ml chloramphenicol and 10 mM lactate. The assay mixture was buffer A containing 50  $\mu$ g/ml chloramphenicol and 10 mM lactate. A 100  $\mu$ l of cell suspension was added to 850  $\mu$ l of the assay mixture and incubated for 150 sec at 25°C. Transport assay was initiated by adding 50  $\mu$ l of various concentrations of [ $^{14}C$ ]serine (final 0.2  $\mu$ Ci/ml). When necessary, other amino acids were added to the assay mixture. A 100  $\mu$ l of the mixture was taken at indicated times, filtered on a membrane filter (0.45  $\mu$ m; ADVANTEC, Tokyo, Japan), and washed with 2 ml of buffer A. Transport assay was performed at 25°C and radioactivity was measured with a liquid scintillation counter.

### Measurement of $Na^+$ or $H^+$ Movement

To measure  $Na^+$  or  $H^+$  entry into cells elicited by serine influx, cells were grown in minimal medium [18] supplemented with 40 mM glycerol under aerobic conditions at 37°C. Ion movement was measured as described previously for a  $Na^+$  electrode [7] and the uptake of  $H^+$  elicited by the serine influx was measured as described previously for an  $H^+$  electrode [7].

### Protein Assay

Protein contents were determined by the Lowry method with bovine serum albumin as a standard [15].

## RESULTS AND DISCUSSION

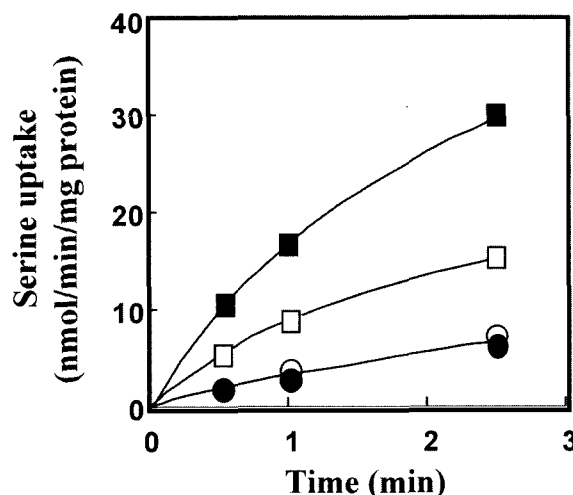
### Cloning of the L-Serine Specific Transporter Gene from *H. influenzae*

Two proteins similar to two major serine transporters of *E. coli*, SstT and SdaC, were found in *H. influenzae* [13]. In *H. influenzae* cells, we observed a  $Na^+$ -stimulated L-serine transport system, which showed properties similar to the SdaC of *E. coli* [13]. However, the L-serine transport system seems to use  $Na^+$  as a coupling ion while the SdaC of *E. coli* uses  $H^+$  [8, 13]. This result supports the idea that an unusual natural mutant type might exist in *H. influenzae*.

*E. coli* WAT9 was unable to grow on serine as a major carbon source since its principal serine uptake system, the  $Na^+$ /serine symporter (SstT), was lacking [17]. As described in Materials and Methods, this strain was used as a host cell to clone the serine transporter gene(s) from *H. influenzae* and five recombinant candidate plasmids were obtained which enabled WAT9 cells to grow on serine as a carbon source. These plasmids carried a common DNA insert of 2.0 kb and the nucleotides of the inserted DNA were sequenced. The sequenced data were consistent with a region registered in the GenBank database with the accession number HI0289, *sdaC*. Finally, a candidate recombinant plasmid was obtained and designated as pSHC4.

### Properties of the Transporter Derived from pSHC4

The serine transport activity was measured with WAT9/pSHC4 cells as described in Materials and Methods. As



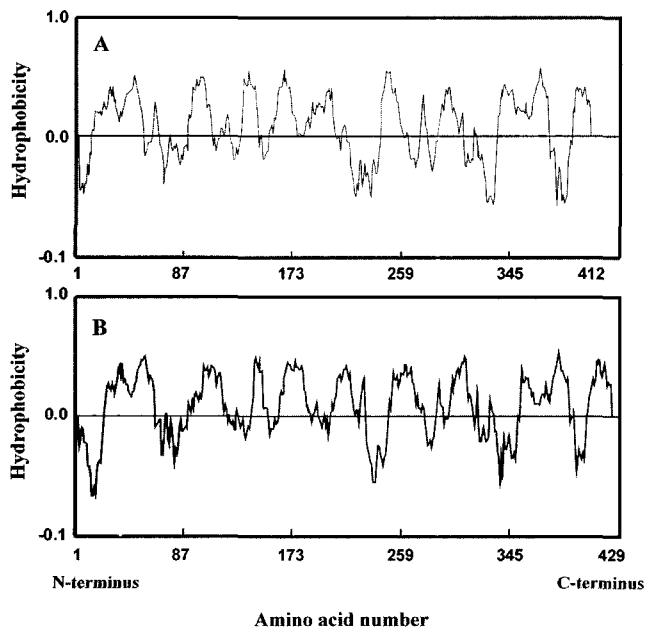
**Fig. 1.** Serine transport in WAT9/pSTV28 and WAT9/pSHC4 cells.

The transport assay was performed as described in Materials and Methods. The transport assay was initiated by the addition of [ $^{14}C$ ]serine (final 0.1 mM, 0.2  $\mu$ Ci/ml). Each experimental point is the average of three determinations. ○, WAT9/pSTV28 in the absence of  $NaCl$ ; ●, WAT9/pSTV28 in the presence of 10 mM  $NaCl$ ; □, WAT9/pSHC4 in the absence of  $NaCl$ ; ■, WAT9/pSHC4 in the presence of 10 mM  $NaCl$ .

shown in Fig. 1, high levels of serine transport activity were observed in WAT9/ pSHC4 cells compared with that of control cells, WAT9/pSTV28. In order to determine whether the Na<sup>+</sup>-stimulated system is used for serine transport, the serine transport assay was performed in the presence or absence of NaCl. A 2–3-fold more stimulation was shown by the addition of 10 mM NaCl (Fig. 1). Considerable serine uptake was also detected in the absence of NaCl such as other Na<sup>+</sup>-stimulated secondary transporters. This seems to be due to contaminating Na<sup>+</sup> in the assay mixture [3, 7], because the control cells, WAT9/pSTV29, were not affected by NaCl addition. In view of the above results, it suggested that the transporter derived from the gene carried a Na<sup>+</sup>-stimulated serine transporter called pSHC4. In addition, the relationship between serine transport and NaCl concentration was investigated. Although a large stimulation was obtained at a considerably low concentration of NaCl (a few mM), the optimum NaCl concentration for the stimulation was 10 mM (data not shown).

### Characteristics of the Gene Product

According to the deduced amino acid sequence of SdaC of *H. influenzae*, the protein consists of 412 amino acid residues with a calculated molecular mass of 45,894 Da. A search for sequence similarity between the SdaC and other proteins was conducted using standard protein-protein



**Fig. 2.** Hydropathy patterns of SdaC from *Haemophilus influenzae* Rd (A) and *E. coli* (B).

Hydropathy values were calculated by the method of Eisenberg *et al.* [5] along with the deduced amino acid sequence of both SdaC. Portions above and below the midpoint line indicate hydrophobic and hydrophilic regions, respectively.

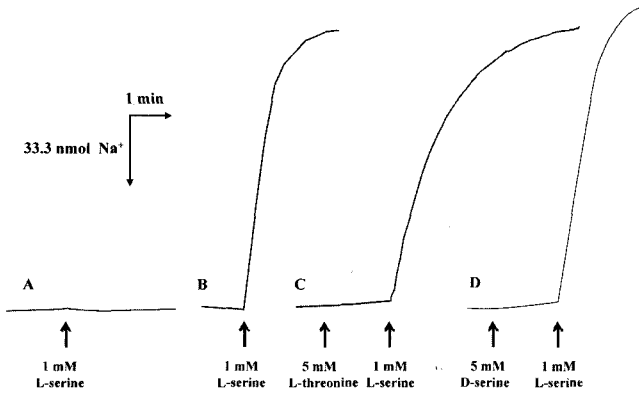
BLAST. The SdaC of *E. coli* showed high sequence similarity throughout the entire sequence: 59% identity and 73% similarity. Many of the putative serine transporters registered in the GenBank database also showed similar levels of identity and similarity to the SdaC of *H. influenzae* (data not shown). Some other amino acid transporters of bacteria (TyrP and TnaB) showed some extent of identity and similarity [6, 21].

Hydropathy values were calculated by the method of Eisenberg *et al.* [5] and plotted along the deduced amino acid sequence of the SdaC of *H. influenzae* (Fig. 2A). There were 10 to 11 hydrophobic domains with sufficient length to span the membrane. The hydropathy pattern was similar to those of the SdaC of *E. coli* (Fig. 2B).

It was presumed that the protein encoded by the *sdaC* gene of *H. influenzae* will be homologous to the SdaC of *E. coli* because of both high similarity and their similar hydropathy patterns. Indeed, a serine transport activity was detected in WAT9 cells harboring the plasmid carrying the *sdaC* gene from *H. influenzae* (Fig. 1). However, a Na<sup>+</sup>-stimulated serine activity was observed in WAT9/pSHC4 cells, though the SdaC of *E. coli* is the H<sup>+</sup>-coupled serine transport system. In order to study this issue in detail, ion movement elicited by substrate influx was investigated as described in Material and Methods.

### Na<sup>+</sup> Uptake Elicited by L-Serine Influx

From the above results, it was supposed that the SdaC of *H. influenzae* uses Na<sup>+</sup> as a coupling cation while the SdaC of *E. coli* uses H<sup>+</sup> [8]. The most convincing evidence of this issue was the finding of Na<sup>+</sup> uptake into cells elicited by substrate influx. It was confirmed that the addition of serine to a cell suspension of WAT9/ pSHS2 elicited Na<sup>+</sup> uptake into cells (Fig. 3B) but not control cells, WAT9/pSTV28 (Fig. 3A). These results support the idea that L-serine is transported by a mechanism of Na<sup>+</sup>/serine symport in the SdaC of *H. influenzae*. The effect of L-threonine or D-serine on Na<sup>+</sup> fluxes into cells elicited by L-serine (Fig. 3C and 3D) was also tested. Na<sup>+</sup> uptake elicited by L-serine was not affected in the presence of excess L-threonine or D-serine. A possibility of an H<sup>+</sup>/L-serine symport system in the SdaC of *H. influenzae* was also investigated. However, no uptake of H<sup>+</sup> elicited by L-serine influx was detected (data not shown). On the other hand, a large H<sup>+</sup> exclusion in the presence of NaCl was observed (data not shown). This observation is consistent with the view that a membrane potential is established by an electrogenic Na<sup>+</sup>/serine symporter mechanism, which produces secondary electrophoretic H<sup>+</sup> extrusion. A similar phenomenon has been reported for the Na<sup>+</sup>/substrate symport via the melibiose transporter [23] and the serine transporters [7, 13]. Considering the above results, it was presumed that the SdaC of *H. influenzae* is a Na<sup>+</sup>-coupled L-serine specific symporter.

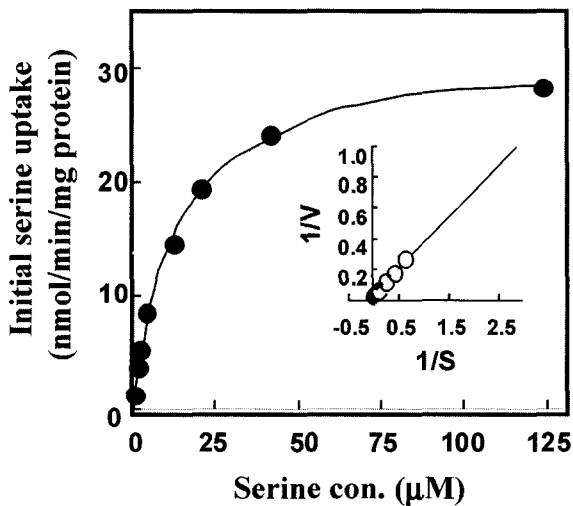


**Fig. 3.** Uptake of  $\text{Na}^+$  elicited by amino acids influx on WAT9/pSTV28 (A) and WAT9/pSHC4 (B, C, and D).

$\text{Na}^+$  movement was measured as described in Materials and Methods. The indicated amino acids were added to the assay mixture. The upward deflection in the chart represents the entry of  $\text{Na}^+$  into cells.

### Kinetics of L-Serine Transport

The relationship between serine concentration and serine transport upon the initial rates of [ $^{14}\text{C}$ ]serine uptake was investigated (Fig. 4). Serine uptake is a saturatable process displaying a hyperbolic curve. The kinetics of L-serine transport, by preparing a double-reciprocal plot of serine concentration versus initial serine uptakes, was also investigated (Fig. 4, inset). The  $K_m$  value and the  $V_{\max}$  were  $7.6 \mu\text{M}$  and  $22.9 \text{ nmol/min/mg protein}$ , respectively. It has been reported that there are two distinct pathways for serine transport in *H. influenzae* Rd cells [13]. One is a



**Fig. 4.** Effect of L-serine concentration on serine transport.

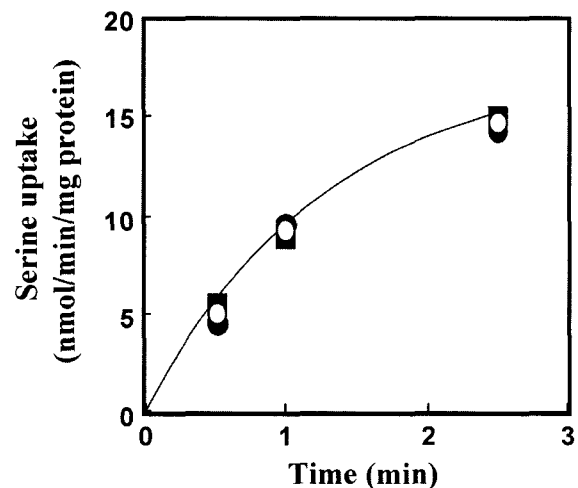
The transport assay was performed as described in Materials and Methods. Various concentrations of [ $^{14}\text{C}$ ]serine (final  $0.2 \mu\text{Ci/ml}$ ) were added to the assay mixture containing  $10 \text{ mM NaCl}$ . To determine the initial velocity, samples were taken  $1 \text{ min}$  after adding [ $^{14}\text{C}$ ]serine. Each experimental point is the average value of three determinations. To calculate the kinetic parameters of serine transport, data were expressed as a double-reciprocal plot of the initial rate of [ $^{14}\text{C}$ ]serine uptake and the serine concentration (refer to inset).

high affinity transport system with a  $K_m$  value of  $1.7 \mu\text{M}$ , and the other is a low affinity transport system with a  $K_m$  value of  $22 \mu\text{M}$ . The high affinity transporter, the  $\text{Na}^+$ /serine-threonine symporter (SstT), with the  $K_m$  value of  $2.5 \mu\text{M}$  was observed (unpublished material). Therefore, the SdaC would be the serine transporter with low affinity in *H. influenzae* Rd.

### Effects of Other Amino Acids on L-Serine Transport

It has been known that the SdaC of *E. coli* is the L-serine specific transport system and is not inhibited by other amino acids such as L-threonine or D-serine [8]. Therefore, the same phenomena would be observed in the SdaC of *H. influenzae*, if both proteins are homologous as was expected. To investigate this possibility, the effect of L-threonine or D-serine on the L-serine uptake was tested. As shown in Fig. 5, the serine transport was not inhibited by excess L-threonine or D-serine (50 fold). These transport properties are similar to those of the SdaC of *E. coli* and are consistent with the results as shown in Fig. 3. Other amino acids (homoserine, L-glycine, and L-valine) were also examined. None of them were found to have an inhibitory effect at 50-fold concentration of that of L-serine (data not shown). Thus, it is clear that the SdaC of *H. influenzae* is a  $\text{Na}^+$ -dependent L-serine specific symporter while its homologue, the SdaC of *E. coli*, is an  $\text{H}^+$ -dependent symporter.

The results presented in this paper indicate that the L-serine specific symporter of *H. influenzae*, SdaC, is a  $\text{Na}^+$ -coupled symport system, though the homologous SdaC of *E. coli* is an  $\text{H}^+$ -coupled symporter. In fact, much is known about important amino acids or domains for ion-coupled transporters due to analysis such as amino acid substitutions [10, 11] and chimeric protein [9, 16]. However,



**Fig. 5.** Effects of other amino acids on serine transport.

Each experimental point is the average value of three determinations.  $\circ$ , control ( $8 \mu\text{M}$  L-serine);  $\bullet$ ,  $0.4 \text{ mM}$  D-serine;  $\blacksquare$ ,  $0.4 \text{ mM}$  L-threonine.

in spite of the knowledge accumulated on functional properties, little is known about the structure responsible for the binding and translocation of ions, and the mechanisms of the drive of the transporter by ions. It is expected that studies of SdaC should provide more information about ion-coupling stoichiometry and the translocation of ion-coupled transporters.

## REFERENCES

- Anderson, J. J., S. C. Quay, and D. L. Oxender. 1976. Mapping of two loci affecting the regulation of branched chain amino acid transport in *Escherichia coli* K12. *J. Bacteriol.* **126**: 80–90.
- Berns, K. I. and C. A. Thomas. 1965. Isolation of the high molecular DNA from *Haemophilus influenzae*. *J. Mol. Biol.* **11**: 476–490.
- Chen, C. C., T. Tsuchiya, T. Tamane, J. M. Wood, and T. H. Wilson. 1985. Na<sup>+</sup> (Li<sup>+</sup>)-proline cotransport in *Escherichia coli*. *J. Mem. Biol.* **84**: 157–164.
- Choi, K. M., J. K. Park, and S. Y. Hwang. 2003. Pathogenetic impact of vacuolar degeneration by accelerated transport of *Helicobacter pylori* VacA. *J. Microbiol. Biotechnol.* **13**: 666–672.
- Eisenberg, D., E. Schwarz, M. Komaromy, and R. Wall. 1984. Analysis of membrane and surface protein sequences with hydrophobic moments plot. *J. Mol. Biol.* **179**: 125–142.
- Fleischmann, R. D., M. D. Adams, O. White, R. A. Clayton, E. F. Kirkness, C. J. Butt, J. F. Tomb, B. A. Dougherty, J. M. Merrick, K. McKenney, G. Sutton, W. FitzHugh, C. Fields, J. D. Gocayne, J. Scott, R. Shirley, L. Liu, A. Glodek, J. M. Kelly, J. F. Weidman, C. A. Phillips, T. Spriggs, E. Hedblom, M. D. Cotton, T. R. Utterback, M. C. Hanna, D. T. Nguyen, D. M. Saudek, R. C. Brandon, L. D. Fine, J. L. Fritchman, J. L. Fuhrmann, N. S. M. Geoghagen, C. L. Gnehm, L. A. McDonald, K. V. Small, C. M. Fraser, H. O. Smith, and J. C. Venter. 1995. Whole-genome random sequencing and assembly of *Haemophilus influenzae* Rd. *Science* **269**: 496–512.
- Hama, H., T. Shimamoto, M. Tsuda, and T. Tsuchiya. 1987. Properties of a Na<sup>+</sup>-coupled serine-threonine transport system in *Escherichia coli*. *Biochim. Biophys. Acta* **905**: 231–239.
- Hama, H., T. Shimamoto, M. Tsuda, and T. Tsuchiya. 1988. Characterization of a novel L-serine transport system in *Escherichia coli*. *J. Bacteriol.* **170**: 2236–2239.
- Hama, H. and T. H. Wilson. 1993. Cation-coupling in chimeric melibiose carriers derived from *Escherichia coli* and *Klebsiella pneumoniae*. *J. Biol. Chem.* **268**: 10060–10065.
- Hama, H. and T. H. Wilson. 1994. Replacement of alanine 58 by asparagine enables the melibiose carrier of *Klebsiella pneumoniae* to couple sugar transport to Na<sup>+</sup>. *J. Biol. Chem.* **269**: 1063–1067.
- Kaback, H. R. 1987. Use of site-directed mutagenesis to study the mechanism of a membrane transport protein. *Biochemistry* **26**: 2071–2076.
- Kim, H. J., J. H. Lee, J. E. Rhee, H. S. Jeong, H. K. Choi, H. J. Chung, S. Ryu, and S. H. Choi. 2002. Identification and functional analysis of the *putAP* genes encoding *Vibrio vulnificus* proline dehydrogenase and proline permease. *J. Microbiol. Biotechnol.* **12**: 318–326.
- Kim, Y. M., I. K. Rhee, M. Y. Park, D. S. Chang, and T. Tsuchiya. 2003. Characteristics of Na<sup>+</sup>-dependent serine transport in *Haemophilus influenzae* Rd. *J. Microbiol.* **41**: 78–82.
- Kim, Y. M., W. Ogawa, E. Tamai, T. Kurota, T. Mizushima, and T. Tsuchiya. 2002. Purification, reconstruction, and characterization of Na(+)/serine symporter, SstT, of *Escherichia coli*. *J. Biochem.* **132**: 71–76.
- Lowry, O. H., N. J. Rosebrough, A. L. Farr, and R. J. Randall. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* **193**: 265–275.
- Nishizawa, K., E. Shimoda, and M. Kasahara. 1995. Substrate recognition domain of the Gal2 galactose transporter in yeast *Saccharomyces cerevisiae* as revealed at chimeric galactose-glucose transporter. *J. Biol. Chem.* **270**: 2426–2433.
- Ogawa, W., T. Kayahara, M. Tsuda, T. Mizushima, and T. Tsuchiya. 1997. Isolation and characterization of *Escherichia coli* mutant lacking the major serine transporter, and cloning of a serine transporter gene. *J. Biochem.* **122**: 1241–1245.
- Ogawa, W., Y. M. Kim, T. Mizushima, and T. Tsuchiya. 1998. Cloning and expression of gene for the Na<sup>+</sup>-coupled serine transporter from *Escherichia coli* and characteristics of the transporter. *J. Bacteriol.* **180**: 6749–6752.
- Sanger, F., S. Nicklen, and A. R. Coulson. 1977. DNA sequencing with chain-terminating inhibitors. *Proc. Natl. Acad. Sci. USA* **74**: 5463–5467.
- Shao, Z. Q., R. T. Lin, and E. B. Newman. 1994. Sequencing and characterization of the *sdaC* gene and identification of the *sdaCB* operon in *Escherichia coli* K12. *Eur. J. Biochem.* **222**: 901–907.
- Stephens, R. S., S. Kalman, C. J. Lammel, J. Fan, R. Marathe, L. Aravind, W. P. Mitchell, L. Olinger, R. V. Tatusov, Q. Zhao, E. V. Koonin, and R. W. Davis. 1998. Genome sequence of an obligate intracellular pathogen of humans: *Chlamydia trachomatis*. *Science* **282**: 754–759.
- Sumantran, V. N., H. P. Schweizer, and P. Datta. 1990. A novel membrane-associated threonine permease encoded by the *tdcC* gene of *Escherichia coli*. *J. Bacteriol.* **172**: 4288–4294.
- Tsuchiya, T., J. Raven, and T. H. Wilson. 1977. Co-transport of Na<sup>+</sup> and methyl-beta-D-thiogalactopyranoside mediated by the melibiose transport system of *Escherichia coli*. *Biochem. Biophys. Res. Commun.* **76**: 26–31.