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Characteristics of Na^+-dependent Serine Transport in *Haemophilus Influenzae* Rd

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We identified two proteins in *Haemophilus influenzae* Rd that exhibited high similarity to two major serine transporters of *Escherichia coli* (SstT and SdaC). Then, we investigated serine transport in *H. influenzae* Rd and detected Na^+-stimulated L-serine transport activity. The optimum NaCl concentration for this stimulation was about 20 mM. The uptake of Na^+ by *H. influenzae* Rd was found to be elicited by L-serine influx, which supports the idea that L-serine is transported by a mechanism of Na^+/serine symport. No uptake of H^+ elicited by L-serine influx was detected. Na^+/serine symport activity was not inhibited by other amino acids such as L-threonine or D-serine. Two distinct *Km* values were obtained from the kinetic analysis of serine transport. Thus, two serine transport pathways may exist in *H. influenzae* Rd, and it appears that both systems are stimulated by Na^+.

Key words: *Haemophilus influenzae* Rd, serine transport

Several transporters for serine in *Escherichia coli* K-12 have been reported. The major system for serine uptake is the Na^+/serine symporter, SstT, which is produced constitutively (Hama *et al.*, 1987). The second system is SdaC, which is induced by L-leucine, and the L-serine specific symporter is stimulated by H^+ (Hama et al., 1988; Shao *et al.*, 1994). Other systems are the TdcC and the leucine-isoleucine-valine-1 (LIV-1) system. TdcC, which is induced in *E. coli* cells incubated in an amino acid-rich medium under anaerobic conditions, mediates H^+/threonine and H^+/L-serine transport (Sumantran *et al.*, 1990). The LIV-1 system mediates the transport of leucine, isoleucine, valine, and serine, and does not require ion-coupling but ATP as an energy source (Robbins and Oxender, 1973; Anderson *et al.*, 1976). The presence of multiple transport systems for serine in *E. coli* makes it difficult to isolate a serine-transport defective mutant, which would be very useful for cloning gene(s) encoding the serine transporter(s). We have succeeded in isolating a mutant that lacks the principal serine transporter, SstT, which makes it easier to clone the serine transporter genes (Ogawa *et al.*, 1997). In fact, we cloned the *sstT* gene and *tdcC* gene using the mutant as the cloning host (Ogawa *et al.*, 1998). Recently we reported upon the overproduction of His-tagged SstT protein, and upon the purification, reconstitution into liposomes, and functional properties of the SstT, Na^+/serine transport protein (Kim *et al.*, 2002).

Venter and coworkers (Fleischmann *et al.*, 1995) reported the complete nucleotide sequence of the genome from *Haemophilus influenzae* Rd. In the present study, we identified proteins with a high similarity to two major serine transporters of *E. coli*, SstT and SdaC, in this bacterium. No serine transporter has been previously reported in *H. influenzae*. However, the presence of a serine transporter was suspected from homology research in this bacterium (Fleischmann *et al.*, 1995; Ogawa *et al.*, 1998). In this paper, we report upon the characteristics of serine transport in *H. influenzae* Rd.

Materials and Methods

Materials

Hemin was obtained from Sigma, USA, NAD from Wako, Japan; and radioactive [^{14}C] serine from Amersham Life Science, USA. All other reagents were of reagent grade and purchased from commercial sources.

Transport assay

For the serine transport assay, *H. influenzae* Rd was grown under aerobic conditions at 37°C in a minimal medium (Ogawa *et al.*, 1998) supplemented with 40 mM of 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,

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Table 1. A comparison of the amino acid sequences of the putative membrane proteins of *Haemophilus influenzae* and of the major serine transporters of *Escherichia coli*

Protein	Lengths	Identity	Similarity	Property
To SstT of *E. coli*				
YgjU in *H. influenzae*	414	246/406 (61%)	357/406 (88%)	Unknown
To SstC of *E. coli*				
ScaC in H. influenzae	412	246/413 (60%)	363/413 (88%)	Unknown

Homology search was conducted using standard protein-protein BLAST.

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 μg/ml chloramphenicol and 10 mM lactate. The assay mixture was buffer A containing 50 μg/ml chloramphenicol and 10 mM lactate. One hundred μl of cell suspension was added to 850 μl of the assay mixture and incubated for 150 sec at 25°C. The transport assay was initiated by adding 50 μl of various concentrations of [^{14}C] serine (final 0.2 μCi/ml). When necessary, other amino acids were added to the assay mixture. One hundred μl of the mixture was sampled at the indicated times, filtered on a membrane filter (0.45 μm, ADVANTEC Toyo, Japan), and washed with 2 ml of buffer A. The 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 (Ogawa *et al.*, 1998) supplemented with 40 mM glycerol under aerobic conditions at 37°C. Ion movement was measured as described previously for a Na^+ electrode (Hama *et al.*, 1987) and the uptake of H^+ elicited by serine influx was measured as described previously for a H^+ electrode (Hama *et al.*, 1987).

Protein assay

Protein contents were determined by the Lowry method with bovine serum albumin as the standard (Lowry *et al.*, 1951).

Results and Discussion

Serine transport activity

In *H. influenzae*, we found two proteins that exhibited very high similarity (88%, respectively) to SdaC and SstT of *E. coli*, two major serine transporters. Thus, we presume that these proteins in *H. influenzae* are homologous to SdaC and SstT of *E. coli*, because of this similarity (Table 1) and their similar hydropathy patterns (data not shown).

Serine transport activity in *H. influenaze* cells was determined as described in Materials and Methods. As shown in Fig. 1, we observed high levels of serine transport activity. Additionally, using Na^+- or H^+-stimulated systems, serine

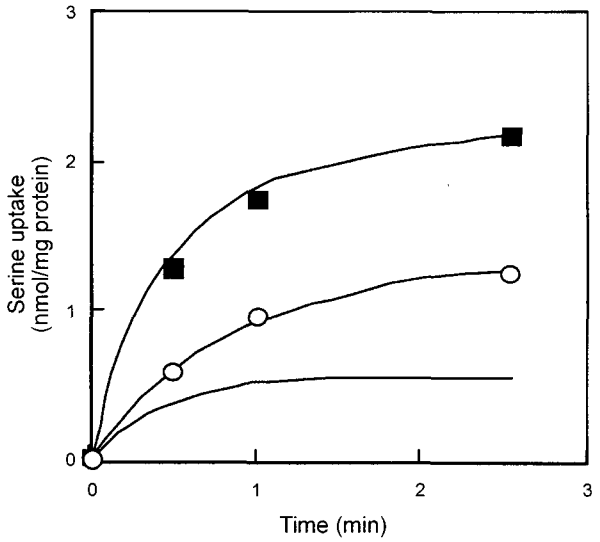

Fig. 1. Serine transport on *Haemophilus influenzae* Rd cells. The transport assay was performed as described in Materials and Methods. The transport assay was initiated by addition of [^{14}C]serine (final 5 μM). Each experimental point is the average of three determinations. ○, control; ■, 20 mM NaCl; ◆, 50 μM CCCP.

transport assays were performed in assay mixtures containing either NaCl or CCCP, the latter of which inhibits the electrochemical potential of H^+. If a Na^+/serine symporter exists, then serine transport should be stimulated by NaCl. Actually, we observed a 2-3 fold stimulation on NaCl addition, supporting the idea that *H. influenzae* Rd contains a Na^+/serine symporter. On the other hand, serine transport was strongly inhibited by CCCP, a potent H^+ conductor, which suggests that the electrochemical potential of H^+ affects this system, and that other serine transport systems may exists in *H. influenzae* Rd. In addition, we investigated the relationship between NaCl concentration and serine transport. Although a large stimulation was observed at a low NaCl concentration (a few mM), this was presumed to be due to contaminating Na^+ in the assay mixture. The optimum NaCl concentration for the stimulation was about 20 mM (Fig. 2).

Kinetics of L-serine transport

We investigated the relationship between serine concentration and serine transport upon the initial rates of [^{14}C]serine

uptake (Fig. 3). Serine uptake is a saturable process displaying a hyperbolic curve. We also investigated the kinetics of L-serine transport by preparing a double-reciprocal plot of serine concentration versus initial serine uptakes (Fig. 3, inset). The data obtained suggested two distinct systems. The K_m value and the V_{max} at serine concentration of 0 to 10 μM were 1.7 μM and 2.3 nmol/min/mg protein, respectively. On the other hand, the K_m value and the V_{max} at serine concentration of 30 to 300 μM serine were 22 μM and 5.9 nmol/min/mg protein, respectively. The kinetics of serine transport suggested that the Na^+-dependent serine transporter system seems to operate in a duplex manner in *H. influenzae* Rd. One is a high affinity transport system with a K_m value of 1.7 μM at low concentrations of serine (up to 10 μM), and the other is a low affinity transport system with a K_m value of 22 μM at high serine concentration (over 30 μM).

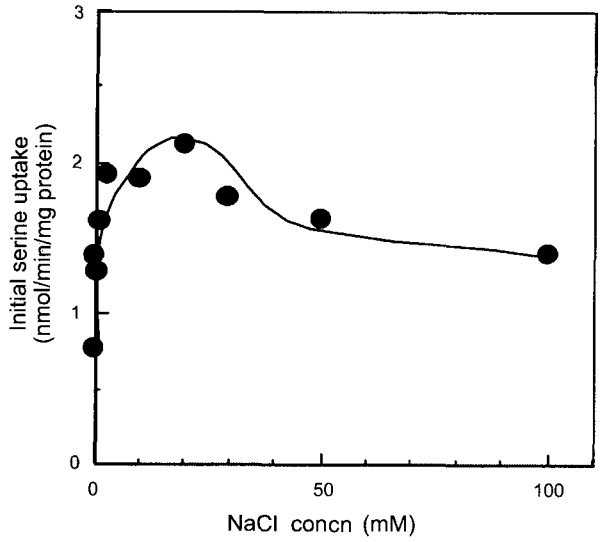

Fig. 2. Effect of NaCl concentration on serine transport in *Haemophilus influenzae* Rd cells. The transport assay was performed as described in Materials and Methods. To determine the initial velocity, samples were taken 1 min after the addition of [^{14}C]serine. Each experimental point is the average of three determinations.

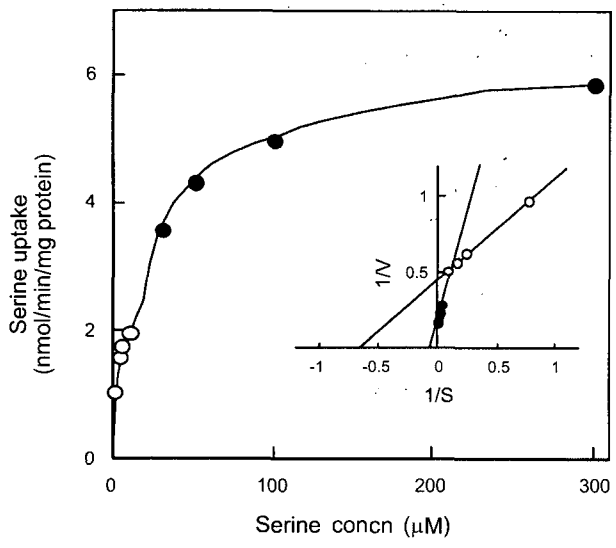

Fig. 3. Effect of L-serine concentration on serine transport. The transport assay was performed as described in Materials and Methods. Various concentrations of [^{14}C]serine were added to the assay mixture containing 20 mM NaCl. To determine the initial velocity, samples were taken 1 min after adding [^{14}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}C]serine uptake and the serine concentration (refer to inset).

Effect of other amino acids on L-serine transport

We tested the effect of D-serine and L-threonine on L-serine uptake (Fig. 4), neither were found to have an inhibitory effect at concentrations 100-fold that of L-serine. The same result was obtained with L-valine and L-glycine (data not shown). Therefore, it is evident that this system is very specific for L-serine transport. This property is very similar to SdaC of *E. coli*, an L-serine specific transport system, which is not inhibited by other amino acids like L-threonine and D-serine, and uses H^+ as a coupling ion (Hama et al., 1988). However, it is believed that the system in *H. influenzae* Rd uses Na^+ as a coupling ion rather than H^+, as shown in Fig. 1.

Na^+ or H^+ uptake elicited by L-serine influx

From the above results, it is supposed that the serine transport system in *H. influenaze* Rd cells uses Na^+ as a coupling cation. The most convincing evidence of this is the finding of Na^+ uptake into *H. influenzae* Rd cell elicited by L-serine influx. We confirmed that the addition of serine to a cell suspension elicited Na^+ uptake in *H. influenzae* Rd (Fig. 5A), which suggests that L-serine is trans-

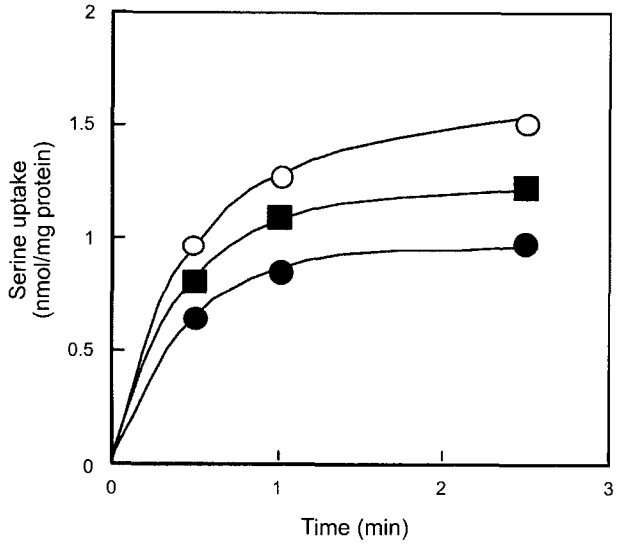

Fig. 4. Effect of other amino acids on serine transport. Each experimental point is the average value of three determinations. ○, control (2 μM L-serine); ■, 0.2 mM L-threonine; ●, 0.2 mM D-serine.

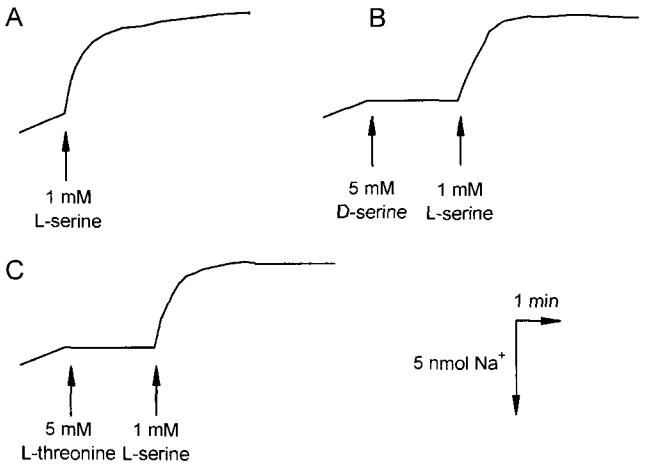

Fig. 5. Uptake of Na^+ elicited by amino acids influx into *Haemophilus influenzae* Rd. Na^+ movement was measured as described in Materials and Methods. The indicated amino acids were added to the assay mixture. The upward deflection in the chart represents the entry of Na^+ into cells.

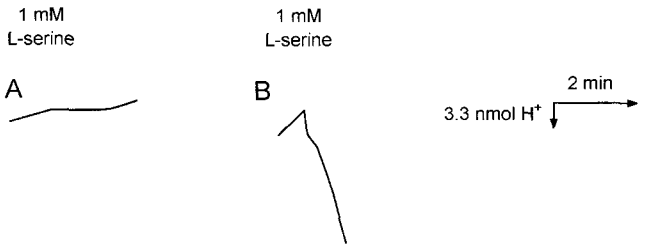

Fig. 6. Uptake of H^+ elicited by serine influx into *Haemophilus influenzae* Rd. H^+ movement was measured as described in Materials and Methods. The indicated amino acid was added to assay mixtures in the absence (A) or presence of 20 mM NaCl (B). The upward deflection in the chart represents H^+ entry into cells.

ported by a mechanism of Na^+/serine symport. Thus, the serine transport system of *H. influenzae* Rd uses Na^+ as a coupling cation.

Additionally, we tested the effects of L-threonine or D-serine on Na^+ fluxes into cells as elicited by L-serine (Fig. 5B and C). The Na^+ uptake elicited by L-serine was not affected in the presence of excess L-threonine or D-serine - consistent with the results shown in Fig. 4. These observations suggest that the serine transport system of *H. influenzae* transports L-serine by a mechanism of Na^+/serine symport, which is not inhibited by other amino acids like L-threonine or D-serine. Thus, this system is a Na^+-dependent L-serine specific symporter.

We also investigated the possibility of H^+/L-serine symport in *H. influenzae*. However, no uptake of H^+ elicited by L-serine influx was detected (Fig. 6A). On the other hand, we observed a large H^+ exclusion in the presence of NaCl (Fig. 6B). This observation is consistent with the view that a membrane potential is established by an electrogenic Na^+/serine symporter mechanism, which produces secondary electrophoretic H^+ extrusion. A similar phenomenon has been reported for Na^+/substrate symport via the serine transporter (Hama *et al.*, 1987) in *E. coli*.

The results presented in this paper indicate that the L-serine transport system in *H. influenzae* Rd is a Na^+-coupled symport system, and that it involves a Na^+-dependent L-serine specific transporter. These transport properties are similar to those of SdaC of *E. coli*, though it uses Na^+ as a coupling ion rather than H^+. Considering the results outlined in this paper, we conclude that the Na^+-dependent L-serine specific transport system in *H. influenzae* Rd is homologous to SdaC of *E. coli*. However, the L-serine transport system uses Na^+ as a coupling ion while the SdaC of *E. coli* uses H^+ (Hama *et al.*, 1988). Therefore, it is possible that an unusual natural mutant type exists. In order to study this issue in more detail, the serine transport gene of *H. influenzae* will be cloned and characterized.

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