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Cloning of the Gene for Na^+/Serine-Threonine Symporter (*sstT*) from *Haemophilus influenzae* Rd and Characteristics of the Transporter

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A protein, exhibiting a high similarity to the major serine transporter of *Escherichia coli*, *SstT*, was found in *Haemophilus influenzae* Rd. A Na^+-stimulated serine transport activity was also detected in the cells. The gene (*sstT*) for the Na^+/serine symporter from the chromosome of *H. influenzae* was cloned, and the properties of the transporter investigated. The serine transport activity was stimulated by Na^+. The uptake of Na^+ was elicited by the addition of serine or threonine into the cells, supporting the idea that these amino acids are transported by a mechanism of Na^+/substrate symport. No uptake of H^+ was elicited by the influx of serine. The serine transport via the *SstT* of *H. influenzae* was inhibited by excess threonine, which was used as another substrate. The K_m and the V_{max} values for the serine transport were 2.5 μM and 14 nmol/min/mg protein, respectively.

Key words: *Haemophilus influenzae* Rd, Na^+/serine-threonine symporter

Several transporters of serine have been reported in *Escherichia coli* K-12 (Anderson *et al.*, 1976; Hama *et al.*, 1987; Sumantran *et al.*, 1990; Shao *et al.*, 1994). It is also known that the major system for serine uptake is the constitutively produced Na^+/serine symporter, SstT (Hama *et al.*, 1987). The SstT of *E. coli* has exhibited a remarkable sequence homology to animal Na^+-dependent neutral and acidic amino acid transporters (Ogawa *et al.*, 1998). Domains responsible for the binding and translocation of substrates have been reported to be highly conserved in animal Na^+-dependent neutral and acidic amino acid transporter members (Kanai *et al.*, 1993; Utsunomiya *et al.*, 1996). This region was also conserved in the SstT of *E. coli*. For transporters, the comparison of family members that have different substrate selectivity, ion coupling stoichiometry or kinetic properties, provide important insights in understanding the structure-function relationships of a protein. Therefore, it is expected that studies of SstT should provide more information on animal Na^+-dependent neutral and acidic amino acid transporters.

Venter and coworkers reported the complete nucleotide sequence of the genome from *Haemophilus influenzae* Rd (Fleischmann *et al.*, 1995). A protein with a high similarity to SstT of *E. coli* was recently identified, and a Na^+-stimulated L-serine transport activity in this bacterium was also reported (Kim *et al.*, 2003). It is presumed that this protein in *H. influenzae* is homologous to the SstT of *E. coli*, due to their high similarity and similar hydropathy patterns (Kim *et al.*, 2003). However, no serine transporter gene(s) has previously been reported in *H. influenzae*, although the presence of a serine transporter was suspected from our recent work (Kim *et al.*, 2003).

In this paper, the cloning of a gene similar to *sstT* of *E. coli* from *H. influenzae* and the characteristics of the SstT protein are reported.

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Materials and Methods

Materials

The radioactive [^{14}C]serine was obtained from Amersham Life Science. All other reagents were of reagent grade and purchased from commercial sources.

Gene cloning by PCR and sequencing

The chromosomal DNA was prepared from cells of *H. influenzae* Rd, using the method described by Berns and Thomas (1965). Two oligonucleotides, based on *H. influenzae* Rd genomic sequences, were used to generate unique *Eco*RI and *Bam*HI: (sense) 5'-CCGGAATTCCG-GCATACAGGGTGTCAATAGGT-3' and (antisense) 5'-CGCGGATCCG CGTCCCGCTCTCTTATTTGCAT-3'. PCR was carried out using Ampli Taq Gold (Perkin Elmer, USA) as the polymerase. The PCR products were ligated into pSTV 29 (TaKaRa, Japan), which had been digested with *Eco*RI-*Bam*HI and dephosphorylated with bacterial alkaline phosphatase, using T4 DNA ligase. Competent cells of *E. coli* WAT9 (Ogawa *et al.*, 1997)

were transformed with the ligated hybrid plasmids and spread on the minimal medium consisting of 40 mM serine, 1 mM glycine, 1 mM idoleucine, 1 mM threonine, 15 μg/ml of chloramphenicol and 1.5% agar. The plates were incubated at 37°C for 3 day. Plasmids were prepared from the transformants, and the insert nucleotides sequenced by dideoxy-chain-termination (Sanger *et al.*, 1977) using a DNA sequencer (ALF Express; Pharmacia, Sweden).

Transport assay

For the serine transport assay, cells were grown under aerobic conditions at 37°C, in a minimal medium (Ogawa *et al.*, 1998) supplemented with 40 mM potassium lactate. The cells were harvested at the late logarithmic phase of growth, and washed twice with buffer A (0.1 M MOPS-Tris and 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 μg/ml chloramphenicol and 10 mM lactate. The assay mixture consisted of buffer A containing 50 μg/ml chloramphenicol and 10 mM lactate. 100 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 the addition of 50 μl of various concentrations of [^{14}C]serine (to a final concentration of 0.2 μCi/ml). When necessary, other amino acids were added to the assay mixture. 100 μl of the mixture was taken at the times indicated, 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 the radioactivity measured with a liquid scintillation counter.

Measurement of Na^+ or H^+ movement

To measure the entry of Na^+ or H^+ into cells elicited by the serine influx, the cells were grown in minimal medium (Ogawa *et al.*, 1998), supplemented with 40 mM glycerol, under aerobic conditions at 37°C. The ion movement was measured as described previously for a Na^+ electrode (Hama *et al.*, 1987) and the H^+ uptake elicited by the serine influx by the method described previously for a H^+ electrode (Hama *et al.*, 1987).

Protein Assay

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

Results and Discussion

Cloning of the sstT gene from H. influenzae

To clone a gene similar to *sstT* of *E. coli*, the direct PCR method, using chromosomal DNA of *H. influenaze* Rd, was carried out as described in Materials and Methods. The *E. coli* WAT9 strain was unable to grow on serine as a major carbon source, as it lacked the principal serine uptake system, the Na^+/serine symporter (SstT) (Ogawa *et al.*, 1997). Therefore, this strain has been used as a host cell for the cloning of the serine transport gene(s) of *H. influenaze* (Ogawa *et al.*, 1997). Candidate recombinant plasmids were able to grow the WAT9 cells on serine as a carbon source. Finally, a candidate recombinant plasmid was obtained, and designated as pSHS2.

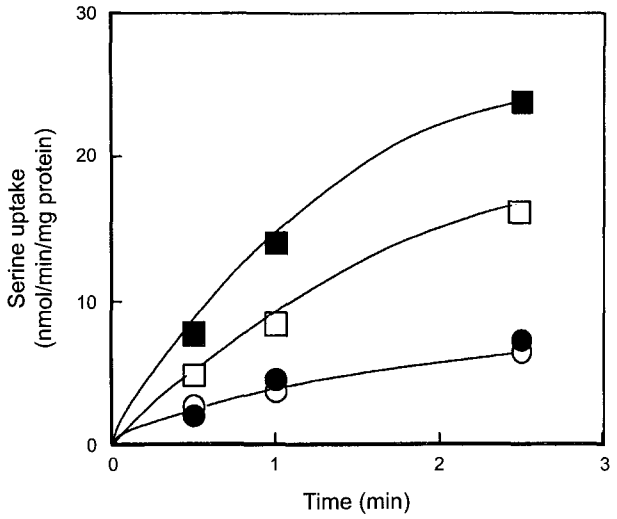

Fig. 1. Serine transport on WAT9/pSTV29 and WAT9/pSHS2 cells. The transport assay was performed as described in Materials and Methods. The transport assay was initiated by the addition of [^{14}C]serine (final 0.1 mM, 0.2 μCi/ml). Each experimental point is the average of three determinations. ○, WAT9/pSTV29 in the absence of NaCl; ●, WAT9/pSTV29 in the presence of 10 mM NaCl; □,WAT9/pSHS2 in the absence of NaCl; ■, WAT9/pSHS2 in the presence of 10 mM NaCl.

Properties of the transporter derived from pSHS2

The serine transport activity of the WAT9/pSHS2 cells was measured as described in Materials and Methods. As shown in Fig. 1, high levels of serine transport activity were observed with the WAT9/ pSHS2 cells, but not with the control cells, WAT9/pSTV29. To determine whether the Na^+-stimulated system is also used for serine transport, the serine transport assay was performed in assay mixtures in the presence or absence of NaCl. If a Na^+/serine symport system exists, the stimulation of serine transport by the addition of NaCl would be detected. Actually, a 2-3 fold stimulation in the presence of 10 mM NaCl (Fig. 1) was observed. Considerable serine uptake was also detected in the absence of NaCl. This was presumed to be due to Na^+ contaminating the assay mixture (Chen *et al.*, 1985; Hama *et al.*, 1987), as the control cells, WAT9/pSTV29, were unaffected by the addition of NaCl. Therefore, it was supposed that the transporter derived from the gene carried on pSHS2 was a Na^+-stimulated serine symporter. In addition, the relationship between serine transport and the NaCl concentration was investigated. Although a large stimulation was obtained at a considerably low NaCl concentration (a few mM), the optimum

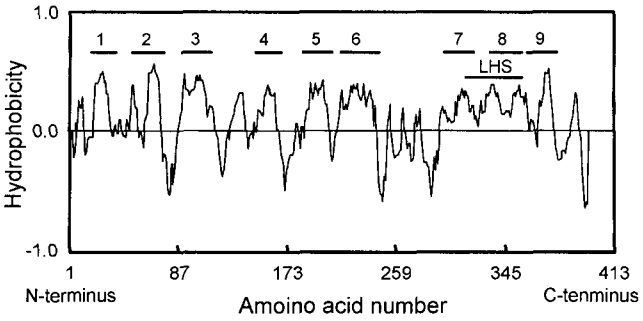

Fig. 2. Hydropathy patterns of YgjU (SstT) from *H. influenzae* Rd. The hydropathy values were calculated by the method of Eisenberg *et al.* (1984) along the deduced amino acid sequence of the YgjU. The portions above and below the midpoint line indicate the hydrophobic and hydrophilic regions, respectively. The nine hydrophobic domains of YgjU are indicated.

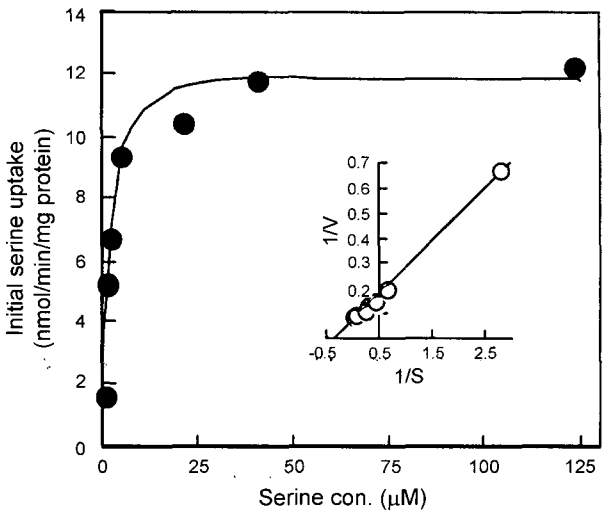

Fig. 3. Effect of the L-serine concentration on the serine transport. The transport assay was performed as described in Materials and Methods. Various concentrations of [^{14}C]serine (final 0.2 μCi/ml) were added to the assay mixture containing 10 mM NaCl. To determine the initial velocity, samples were taken at 1 min after the addition of [^{14}C]serine. Each experimental point is the average value of three determinations. To calculate the kinetic parameters of serine transport, the data were expressed as a double-reciprocal plot of the initial rate of [^{14}C]serine uptake and the serine concentration (refer to inset).

NaCl concentration for the stimulation was 10 mM (data not shown).

Sequence and characteristics of the gene product

The sequence data of the insert nucleotides to the pSHS2 revealed them to be identical to the sequences registered in Genbank database as *ygjU*, HI1545. According to the deduced amino acid sequences of *ygjU*, the protein consisted of 414 amino acid residues with a calculated molecular mass of 43,341 Da.

A search for sequence similarity between YgjU and other proteins was conducted using the standard protein-protein BLAST. The SstT of *E. coli* showed only high sequence similarity: 61% identity and 78% similarity. Human SATT (Shafqat *et al.*, 1993), an amino acid transporter, showed high sequence similarity throughout the entire sequence: 20% identity and 43% similarity. Some other amino acid transporters from animal cells, ASCT1, ASCT2, and GLAST, (Arriza *et al.*, 1993; Utsunomiya *et al.*, 1996) also showed similar levels of identity and similarity to the SstT, similarly to the SATT (data not shown). No other transporters showed such high similarity.

Hydropathy values were calculated by the method of Eisenberg *et al.* (1984) and plotted along the deduced amino acid sequence of *ygjU* (Fig. 2). There were nine hydrophobic domains of sufficient length to span the membrane. The hydropathy patterns were similar to those of SstT (Ogawa *et al.*, 1998), SATT (Shafqat *et al.*,1993), ASCT1 (Arriza *et al.*, 1993) and ASCT2 (Utsunomiya *et al.*, 1996), which showed nine hydrophobic domains. It has been reported that a long hydrophobic stretch is present at the C-terminal portion of SstT, GLAST, and ASCT2 (Utsunomiya *et al.*, 1996; Ogawa *et al.*, 1998). The hydropathy pattern of YgjU also revealed the presence of a similarly long hydrophobic stretch at the C-terminal portion, including the hydrophobic regions 7, 8 and 9 (Fig. 2).

It was presumed that the protein encoded by *ygjU* would be a homologous protein of SstT, due to both their high similarity and similar hydropathy patterns. Indeed, a Na^+-stimulated serine transport activity was detected in the WAT9 cells harboring the plasmid carrying the *ygjU* gene (Fig. 1). Therefore, the gene encoding the Na^+-stimulated serine transporter was designated as sstT of *H. influenaze*.

Kinetics of L-serine transport

The relationship between the serine concentration and the serine transport upon the initial rates of [^{14}C] serine uptake were investigated (Fig. 3). Serine uptake is a saturable process that displays a hyperbolic curve. The kinetics of L-serine transport was also investigated by preparing a double-reciprocal plot of the serine concentration versus the initial serine uptakes (Fig. 3, inset). The K_m value and the V_{max} values were 2.5 μM and 14 nmol/min/mg protein, respectively.

Two distinct pathways for serine transport in *H. influenzae* Rd cells have been reported (Kim *et al.*, 2003). One is a high affinity transport system with a K_m value of 1.7 μM, and the other is a low affinity transport system with a K_m value of 22 μM. Considering the above results, it was presumed that the SstT (YgjU) would be the serine transporter with high affinity in *H. influenzae* Rd.

Effects of other amino acids on L-serine transport

The serine transport is known to be inhibited by L-threonine in the SstT of *E. coli*, since the SstT mediates the transport of L-serine and L-threonine (Hama *et al.*, 1987;

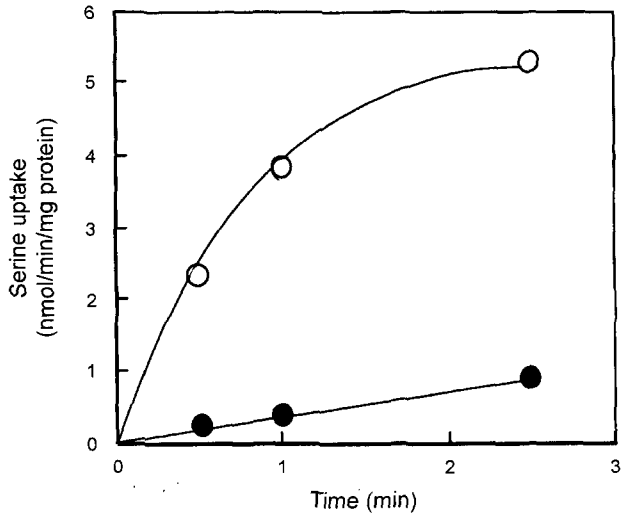

Fig. 4. Effect of other amino acids on serine transport. Each experimental point is the average value of three determinations. ○, control (2.5 μM L-serine); ●, 0.1 mM L-threonine.

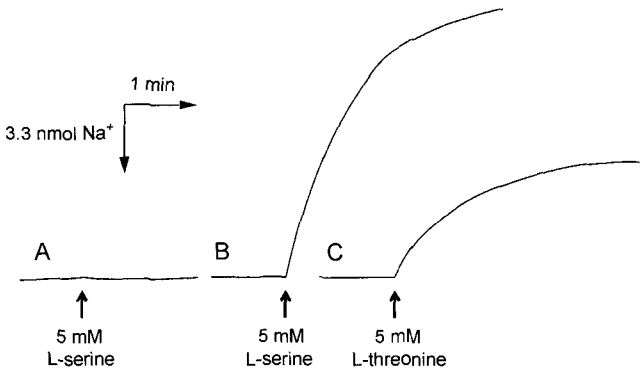

Fig. 5. Uptake of Na^+ elicited by the influx of amino acids on WAT9/pSTV29 (A) and WAT9/pSHS2 (B and C). The Na^+ movement was measured as described in Materials and Methods. The indicated amino acids were added to the assay mixture. The upward deflection in the chart represents the entry of the Na^+ into the cells.

Kim *et al.*, 2002). Therefore, the same phenomena should be observed in the SstT of *H. influenaze*, as both proteins are homologous. To investigate this possibility, the effect of L-threonine on the L-serine uptake was tested. As shown in Fig. 4, the serine transport was inhibited by excess L-threonine (50 fold). Thus, L-serine and L-threonine compete with each other, suggesting that the L-serine and L-threonine are transported via the same Na^+/serine-threonine symporter (SstT) system. Other amino acids (D-serine, homoserine, L-glycine, L-valine) examined were found not to have an inhibitory effect at concentrations 50-fold that of L-serine (data not shown). Considering the above results, it was concluded that the SstT of *H. influenaze* also mediates the transport of L-serine and L-threonine, similarly to that of *E. coli*.

Na^+ uptake elicited by L-serine influx

From the above results it was also supposed that the SstT of *H. influenaze* uses Na^+ as a coupling cation, similarly to the SstT of *E. coli* (Hama *et al.*, 1987; Kim *et al.*, 2002). The most convincing evidence of this was that the Na^+ uptake into cells was elicited by the substrate influx. It was confirmed that the addition of serine or threonine to a cell suspension of WAT9/ pSHS2 elicited the Na^+ uptake (Fig. 5B and C) but not in the control cells, WAT9/ pSTV29 (Fig. 5A). These observations suggest that the SstT of *H. influenzae* transports L-serine and L-threonine by a mechanism of Na^+/serine-threonine symport. Thus, the SstT of *H. influenzae* also uses Na^+ as a coupling cation. The possibility of H^+/L-serine symport activity in the SstT of *H. influenzae* was also investigated. However, no uptake of H^+ elicited by L-serine or L-threonine influx was detected (data not shown).

The results presented in this paper indicate that the SstT of *H. influenzae* Rd is a Na^+-coupled serine-threonine symporter, with similar transport properties to those of the SstT of *E. coli*. The SstT of *H. influenzae* Rd also exhibited a remarkable sequence homology to the animal Na^+-dependent neutral and acidic amino acid transporters, similarly to its homologue, the SstT of *E. coli*. Despite the knowledge accumulated on the functional properties of the animal Na^+-dependent neutral and acidic amino acid transporters, little information is available on the structure of the ion binding domains and on the mechanisms of transport. Therefore, studies on SstT as a model system should provide important insights in understanding the structure-function relationships of the animal Na^+-dependent neutral and acidic amino acid transporters.

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