Cloning and molecular analysis of the 
*Salmonella enterica ansP* gene, encoding an 
L-asparagine permease

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A gene (*ansP*), which encodes an L-asparagine permease, has been isolated from a cosmid library of *Salmonella enterica* during screening for recombinant clones which encode L-asparaginase. Nucleotide sequence analysis reveals that the gene product is a polypeptide of 497 amino acid residues, containing 12 putative transmembrane segments. The calculated molecular mass is 54 kDa, although maxicell analysis by SDS-PAGE gave an apparent molecular mass of 37 kDa. Comparison of the deduced amino acid sequence with sequence databases showed significant homology with a family of basic and aromatic amino acid permeases. Strains containing the cloned *ansP* gene demonstrated a many-fold increase in L-asparagine uptake in comparison with control strains.

**Keywords**: permease, L-asparagine transport, *Salmonella enterica*

**INTRODUCTION**

Specific systems for the uptake of L-asparagine in *Salmonella enterica* have not been described. A study of the closely related organism *Escherichia coli* has revealed that there are two distinct systems for L-asparagine uptake distinguishable on the basis of specificity and regulation (Willis & Woolfolk, 1975): a low-affinity system (*K*_m = 80 μM), and a high-affinity system (*K*_m = 3.5 μM) which is repressed by the presence of L-asparagine in the growth medium at concentrations greater than 1 mM. These systems have not been studied at the molecular level. In contrast, two enzymes for the subsequent degradation of L-asparagine have been described: a high-affinity, periplasmic L-asparaginase (asparaginase II; Jennings & Beacham, 1990) and a low-affinity cytoplasmic L-asparaginase (asparaginase I; Jerlstrom et al., 1889). The latter presumably functions to degrade L-asparagine when it has accumulated to an appropriate intracellular concentration. During early attempts to isolate the gene encoding the periplasmic L-asparaginase, a gene was identified (*ansP*) which encodes an L-asparagine permease.

Here we describe the molecular analysis of the *ansP* gene, whose product has a deduced amino acid sequence homologous to a family of amino acid transporters found in both bacteria and yeasts.

**METHODS**

**Bacterial strains and plasmids.** *Escherichia coli* strain TG1 [Δ(lac-pro) supE thi bsdΔF'-traD36 proAB lacI*G ZIP ΔM15; Sambrook et al., 1989] was used for the propagation of plasmids and genetic manipulations. *E. coli* CSR603 (recA aurA6 phr-1; Sancar et al., 1979) was the host strain used in maxicell analysis. Other *E. coli* strains used were GU8 (ansA rpsL fnr; Jerlstrom et al., 1989), HB94kan (ansB:kan; M. P. Jennings unpublished data) and DH1 [F' recA1 endA1 gyrA96 thi-1 bsdR17 (c**r** [m**P**] supE44 i**t**); Sambrook et al., 1989]. Cloning vectors used were pHP34 (Prentki & Krisch, 1982) and pBluescript SK (M13*) (Stratagene).

**Detection of plasmid-encoded proteins by maxicell analysis.** Proteins encoded by cloned DNA fragments were specifically labelled with [35S]methionine using maxicells and the radio-labelled proteins analysed by SDS-PAGE exactly as described by Calhoun & Gray (1981).

**Assay of high-affinity L-asparaginase activity by direct Nesslerization.** L-Asparaginase II activity was measured in whole cells or cell-free sonicated extracts by measuring the production of ammonia with Nessler’s reagent (Wriston & Yellin, 1973). L-Asparaginase was used at a concentration (0.1 mM) at which L-asparaginase I activity is not significant (Cedar & Schwartz, 1967; Chesney, 1983). Cultures were grown either anaerobically or aerobically to mid-exponential phase and harvested by centrifugation, washed twice in 50 mM Tris/HCl pH 7.4, then resuspended in 1 ml of the same buffer. The reaction was started by adding the substrate; the cells were incubated for 30 min at 37 °C, centrifuged briefly, and 600 μl of
the supernatant removed to a new tube. Then 60 µl Nessler's reagent (BDH) was added to each tube, and the Absorbance read immediately. The specific activity was expressed as nmol L-asparagine converted min⁻¹ (mg protein)⁻¹.

When assaying a large number of samples during the screening of the cosmids library, a single colony was inoculated into 2 ml LB containing ampicillin (50 µg ml⁻¹), and grown at 37 °C with vigorous shaking until mid-exponential phase was reached. Then 1.5 ml of the culture was transferred to a microfuge tube and the cells collected by centrifugation for 5 min. The cells were washed in 0.5 ml 50 mM Tris/HCl pH 7.4, spun for further 5 min, and resuspended in 1 ml of the same buffer. To begin the assay, 10 µl 10 mM L-asparagine was added and incubated at 37 °C for 1 h. At the end of the incubation period, the assay mixture was centrifuged for 5 min and 100 µl Nessler's reagent was added. The colour of the supernatant was compared to negative (aerobically grown E. coli) and positive (anaerobically grown E. coli) controls. Any positive clones were re-assayed by the quantitative method (see above).

**Cloning of the ansP gene of S. enterica.** A cosmids library of S. enterica strain SA2656 (Garrett et al., 1989) was screened by assaying 500 individuals for high-affinity, aerobic L-asparaginase activity by direct Nesslerization (see above), resulting in a single positive cosmid, pMJ5. Restriction mapping revealed an insert size of 37 kb. Selected fragments were subcloned into pHP34, and the resulting strains assayed for high-affinity, aerobic L-asparaginase activity (results not shown). One of these subclones, pMJ5, which retained the activity of pMJ5 and contained a 6 kb Sall/HindIII fragment (see Fig. 1), was chosen for further analysis. A further subclone, pMJ6, contained a 3.3 kb Sall/PstI fragment from pMJ5 cloned into EcoRV/PstI-digested pBluescript SK (M13⁺). Unidirectional deletions were made by digesting pMJ6 with BamHI and SacI, followed by digestion for various times with exonuclease III as described previously (McKay et al., 1992). The resulting plasmids were assayed for high-affinity, aerobic L-asparaginase activity, to define the ansP coding region, and were also used as sequencing templates (see Fig. 1).

L-[¹⁴C]Asparagine uptake assay. The method used was a modification of that described by Wallace et al. (1990). Strain HB94kan (an ansB mutant strain) containing pMJ5 or pBluescript was grown in Luria Broth (20 ml) to an OD₆₀₀ of 0.5, harvested by centrifugation, and washed three times in 3 ml Tris/HCl buffer (50 mM, pH 8.0). The pellet was finally resuspended in 1.8 ml of the same buffer to which 20 µl 250 mM glucose was added. Each 1 ml assay mixture was pre-incubated at 37 °C for 30 min. The assay was started by the addition of 14C-labelled L-asparagine (25 mCi mmol⁻¹, 0.925 MBq mmol⁻¹; Amersham) to a final concentration of 100 µM. Samples (250 µl) were removed at various times and filtered through a 0.45 µm cellulose nitrate filter (Millipore) on an Amicon vacuum filtration apparatus, and then washed immediately with 10 ml ice-cold Tris/HCl buffer (50 mM, pH 8.0). The filters were dried and placed in 5 ml ACS liquid scintillant and the radioactivity determined using a Beckman scintillation counter.

**Recombinant DNA techniques and nucleotide sequence analysis.** Unless otherwise stated, recombinant DNA techniques were as described in Sambrook et al. (1989). Nucleotide sequence was determined on both strands by the Sanger dideoxy method using Sequenase (United States Biochemicals) and double-stranded templates (Lim & Penc, 1988). Nucleotide sequence analysis and database searching were performed using the GCG sequence analysis package (Devereux et al., 1984).

**RESULTS**

**Cloning and identification of ansP**

The ansP gene was isolated during attempts to clone the S. enterica analogue of the ansB gene of E. coli, which encodes a high-affinity L-asparaginase (Jennings & Beacham, 1990). An S. enterica cosmids library in the E. coli host DH1 (Garret et al., 1989) was screened by growing each individual in the library under aerobic conditions such that the endogenous high-affinity L-asparaginase II ac-
anP gene of S. enterica

Fig. 2. Maxicell analysis of cells containing pMJS5. [35S]methionine-labelled maxicell samples from aerobic cultures were run on a 12% SDS-polyacrylamide gel, and the gel was fixed, dried and autoradiographed. The samples are: 1, 125I-labelled low-molecular-mass standards (bovine serum albumin (66.2 kDa), ovalbumin (44 kDa), carbonic anhydrase (31 kDa) and trypsin inhibitor (21 kDa)); 2, CSR603(pMJS5); 3, CSR603(pHP34). The bands which correspond to β-lactamase (Bla) and the fragment-specific gene product (X) are indicated with arrows.

Table 1. Assays of high-affinity L-asparaginase activity in strains grown aerobically or anaerobically

<table>
<thead>
<tr>
<th>Strain</th>
<th>L-Asparaginase activity*</th>
</tr>
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<tbody>
<tr>
<td></td>
<td>Whole cells</td>
</tr>
<tr>
<td>DH1</td>
<td>&lt; 0.5 (22)</td>
</tr>
<tr>
<td>DH1(pMJS5)</td>
<td>18</td>
</tr>
<tr>
<td>GU8</td>
<td>&lt; 0.5</td>
</tr>
<tr>
<td>GU8(pMJS5)</td>
<td>&lt; 0.5</td>
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</tbody>
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* Specific activity [nmol ammonia liberated min⁻¹ (mg protein)⁻¹].
† Figures in parenthesis are values determined from strains grown anaerobically. All other cultures were grown aerobically.

activity, which is oxygen-regulated, was absent. The cells were then incubated with L-asparaginase at a concentration below the \( K_m \) of L-asparaginase I (cytoplasmic, low-affinity L-asparaginase; see Methods) in order to detect cells expressing L-asparaginase II activity. Of the 500 individual cosmid clones screened, one positive colony was obtained.

The positive cosmid clone, pMJS1, was isolated and subcloned, resulting in pMJS5, which retained the aerobic high-affinity L-asparaginase activity and contained a 6 kb SalI/HindIII fragment from the original clone (see Methods and Fig. 1). Maxicell analysis of this clone revealed a single detectable fragment-specific gene product, with an estimated molecular mass of 37 kDa (Fig. 2). This size is consistent with the molecular mass of L-asparaginase II from E. coli (the product of the \( antP \) gene); however, when extracts of aerobically grown cells containing pMJS5 were analysed by immunoblotting, there was no cross-reactivity with polyclonal antisera raised against E. coli L-asparaginase II (results not shown).

The possibility was then examined that the cloned gene may encode an L-asparagine transport protein. This would be consistent with the positive result in the whole-cell assay, as the intracellular L-asparagine concentration may have been increased to the point where the endogenous, low-affinity, L-asparaginase I is able to hydrolyse the accumulated L-asparagine efficiently. To test this hypothesis, sonicated extracts were prepared from cells containing pMJS5 and assayed for L-asparaginase activity (see Table 1). Sonicated extracts of cells containing pMJS5 did not exhibit the high-affinity, L-asparaginase II activity. To confirm that the activity observed in the whole-cell assay was due to the activity of L-asparaginase I, pMJS5 was transformed into an L-asparaginase I deficient strain, GU8 (Jerlstrom et al., 1989). This strain showed no L-asparaginase activity in the whole-cell assay (see Table 1). Both results are consistent with the cloned gene encoding an L-asparagine transport protein.

In order to confirm that the cloned gene did encode an L-asparagine transport system, L-[^14C]asparagine uptake assays were performed (Fig. 3). The cells harbouring pMJS5 exhibited an approximately fivefold increase in the uptake of L-asparagine compared to cells containing a control plasmid. Uptake was not observed in experiments in which either lower substrate concentrations or much shorter time courses were used (results not shown).
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Fig. 4. Nucleotide sequence analysis of the orientation of the insert (see Fig. 1). The nucleotide and sequence which may constitute a ribosome-binding site which may act as a terminator in the mRNA, are underlined.

| PstI | CTCGAGGTTACCCATGATTAATCCTTCCGACGAGACACATCTTGTTGTCGC |
| GCTTCATTCACTGACGAGGACACCCATCAGGCAACACCTCGTTGC |
| GCTGGTACTGATGCCGAGGACCCATCAGGCAACACCTCGTTGC |
| CTGGCGGACTTCCACCGAGGACCCATCAGGCAACACCTCGTTGC |
| Nuclease site is indicated above the sequence to show activity (results not shown), was subcloned to create PMJS6 (see Methods and Fig. 1). Unidirectional deletions were made in PMJS6, and the resulting nested deletions were then assayed for L-asparagine transport activity, and also used as templates for nucleotide sequence analysis. The nucleotide sequence of the region which encoded the L-asparagine transport activity, as determined by the assay of deletions, was determined on both strands and is shown in Fig. 4.

An open reading frame (ORF) is present which presumably initiates with the ATG at nucleotide (nt) 311. Located seven base pairs (bp) upstream is a sequence which may serve as a ribosome-binding site (AGG; see Fig. 4). The deduced amino acid sequence consists of 497 residues, with a calculated molecular mass of 54 kDa. This calculated mass is not consistent with the observed molecular mass of the putative ORF-encoded product, as determined experimentally from the maxicell studies; however, aberrant migration on SDS-PAGE is a common feature of integral membrane proteins, particularly those homologous with the ansP gene product (Honore & Cole, 1990; Chye et al., 1986; Steffes et al., 1992; Wallace et al., 1990). A further possibility is that the difference in molecular mass may be due to processing.

The plasmid derivatives of PMJS6 generated by exonuclease III deletion, PMJS6.1 and PMJS6.2, which retain the ORF described above, retained L-asparagine transport activity (see Figs 1 and 4). In contrast, PMJS6.3 and PMJS6.4, from which large sections of the ORF are deleted, have lost the L-asparagine transport activity: this result is consistent with the ORF described above encoding an L-asparagine permease. This ORF is designated ansP.

Nucleotide sequence analysis

Further subcloning and nucleotide sequence analysis were carried out in order to identify the gene encoding the putative L-asparagine transport system. A 3.3 kb fragment from PMJS5, which retained the L-asparaginase uptake activity (results not shown), was subcloned to create PMJS6 (see Methods and Fig. 1). Unidirectional deletions were made in PMJS6, and the resulting nested deletions were then assayed for L-asparagine transport activity, and also used as templates for nucleotide sequence analysis. The nucleotide sequence of the region which encoded the L-asparagine transport activity, as determined by the assay of deletions, was determined on both strands and is shown in Fig. 4.

Sequence homology with amino acid permeases

Comparison of the deduced amino acid sequence with the protein databases revealed a high level of similarity with a family of basic and aromatic amino acid permeases found in both prokaryotes and eukaryotes (Steffes et al., 1992). The highest sequence identity was with the phenylalanine-specific (PheP 36.9%; Pi et al., 1991), lysine-specific (LysP 31.6%; Steffes et al., 1992) and aromatic amino acid (AroP 36.9%; Honore & Cole, 1990) permeases of E. coli, and the arginine (ArgP 28.2%; Jauniaux et al., 1992) and serine (SerP 29.3%; Janniau & Grenson, 1990) permeases of Saccharomyces cerevisiae.
HYDROPATHIC INDEX PLOT OF ANSP

A plot of average hydrophatic index was performed using the method of Kyte & Doolittle (1982), using a window of 19 amino acids (Fig. 5). This analysis reveals 12 hydrophobic segments which may constitute transmembrane domains. This feature is common to the other members of this class of permease (Pi et al., 1991).

DISCUSSION

The ansP gene was initially isolated on the basis that it conferred the capacity for L-asparagine hydrolysis upon whole cells of L-asparaginase I proficient E. coli, at low substrate concentration. This L-asparagine hydrolysis is lost when the cells are sonicated, or when the plasmid containing ansP is present in an L-asparaginase I deficient strain. These observations suggest that the observed L-asparagine hydrolysis is due to an increased uptake of L-asparagine when ansP is in multicopy, in conjunction with hydrolysis of the accumulated L-asparagine by L-asparaginase I. The identity of the putative L-asparaginase permease was supported by the increased L-asparagine uptake in cells containing the ansP gene in multicopy. In addition, homology was found between the deduced amino acid sequence of AnsP and a family of amino acid permeases.

The 12 hydrophobic segments revealed by the hydrophatic index plot of AnsP probably correspond to 12 transmembrane domains. This structural feature is characteristic of a large number of transporters and has been suggested to be essential for one-component transport systems (Gött & Boos, 1988). It seems likely that the family of amino acid transporters which share significant sequence homology with ansP (see Results) share a common evolutionary origin and possibly have similar tertiary structures. The substrate specificity of the members of this family described to date has been for basic and aromatic amino acids. The addition of AnsP to this group indicates that the substrate range of this group is not limited to basic and aromatic amino acids.

The specificity and kinetics of AnsP function have not been addressed in this study. However, evidence for two specific L-asparagine uptake systems in E. coli has been reported (Willis & Woolfolk, 1975). As S. enterica and E. coli are closely related, it is possible that ansP may represent a homologue of one of these L-asparagine uptake systems. The observation that increased L-asparagine uptake by cells harbouring the ansP gene in multicopy is not detected at substrate concentrations below 100 μM is consistent with the hypothesis that ansP encodes the S. enterica homologue of the low-affinity uptake system (Km = 80 μM) reported in E. coli.

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