A periplasmic arginine transport system that is a member of the ATP-dependent transport superfamily was identified in Pasteurella haemolytica. The gene encoding the periplasmic binding protein (lapT) was cloned and the protein overexpressed in Escherichia coli. LapT was purified to homogeneity using a modified osmotic shock procedure and anion-exchange column chromatography. Filter-binding assays established that LapT is an L-arginine-binding protein. Various amino acids were tested for their ability to inhibit L-arginine binding to LapT. When present in 100-fold excess, only L-arginine, D-arginine and citrulline competed with L-arginine for binding. Arginine transport in P. haemolytica whole cells was competitively inhibited by the same amino acids, suggesting that the LapT permease specifically transports L-arginine. The dissociation constant for the L-arginine-LapT complex was 170 nM and the stoichiometry of binding was approximately 0.8 mol L-arginine (mol LapT)^{-1}. A polyclonal antibody raised against the purified protein permitted detection of LapT in P. haemolytica periplasmic fractions.

Keywords: Pasteurella haemolytica, arginine transport, periplasmic arginine-binding protein, permease, ABC transporters

INTRODUCTION

Gram-negative bacteria import nutrients through the outer membrane into the periplasmic space and across the cytoplasmic membrane. Many transport systems require a periplasmic ligand-binding protein, thus their transport is osmotic-shock-sensitive (Ames, 1986). Such systems are composed of a periplasmic ligand-binding protein, a dimeric membrane-bound component and a cytoplasmic nucleotide-binding protein (Higgins et al., 1986; Kerppola & Ames, 1992) and are members of the ATP-binding cassette (ABC) transport family. Binding-protein-dependent ABC transporters are also involved in translocation of polar and aliphatic amino acids, peptides, sugars and ions into the bacterial cell (Tam & Saier, 1993). Transport into the cytoplasm is initiated by the interaction of the ligand-bound periplasmic protein with the complex in the inner membrane. This interaction is thought to induce a conformational change that results in ATP hydrolysis and translocation of the ligand through the membrane to the cytoplasm (Davidson & Nikaido, 1990).

Binding-protein-dependent permeases that transport basic amino acids have been extensively characterized in Salmonella typhimurium and Escherichia coli. In S. typhimurium, histidine is transported by a system composed of a periplasmic histidine-binding protein (HisJ), a heterodimeric membrane complex composed of two hydrophobic proteins (HisQ and HisM), and an ATP-binding protein, HisP (Kerppola et al., 1991). The histidine membrane complex also functions to transport lysine, arginine and ornithine by interacting with an additional periplasmic binding protein called LAO (Kustu & Ames, 1973). In E. coli, arginine is transported using an LAO-dependent system (Rosen, 1971), and by one of two arginine-binding-protein-dependent systems: arginine-binding protein I (ArtI) (Wissenbach et al., 1995) or arginine-binding protein II (Rosen, 1973). Each of these systems includes the analogous ATP-binding and membrane-spanning components of the system (e.g. ArtQMP).

Pasteurella haemolytica is a bovine respiratory pathogen whose primary virulence factor is a secreted leucotoxin (Shewen & Wilkie, 1982). A four-gene cluster encoding proteins similar to those observed in other binding-protein-dependent transport systems maps immediately upstream of the P. haemolytica leucotoxin gene cluster (Highlander et al., 1993). This leucotoxin-associated permease (lap) gene cluster contains four open reading frames: lapA, lapB, lapC and lapT. The LapA protein
sequence contains two regions of sequence that match the consensus Walker motifs A and B for ATP-binding proteins (Walker et al., 1982) suggesting that it is a nucleotide-binding protein. Hydrophobicity profiles indicate that LapB and LapC could be the membrane-associated components of the permease (Highlander et al., 1993). The LapT sequence is most similar to the predicted periplasmic arginine-binding protein ArtI from the recently reported Haemophilus influenzae sequence (Fleischmann et al., 1995), to the E. coli ArtJ periplasmic binding protein (Wissenbach et al., 1993) and to the S. typhimurium periplasmic binding proteins HisJ and LAO (Higgins & Ames, 1981) (Fig. 1). The LapT sequence possesses a potential signal peptide sequence, suggesting that it is the periplasmic ligand-binding protein of the P. haemolytica permease. Here we report purification and characterization of the periplasmic binding component of the leucotoxin-associated permease and show that its ligand is l-arginine. This ABC transporter appears to be the primary system for arginine uptake in P. haemolytica.

METHODS

Bacterial strains and growth conditions. P. haemolytica strain PHL101 (Highlander et al., 1989) was grown with aeration in Brain Heart Infusion broth (Difco) at 37°C; E. coli K-12 strains MG1655 (Bachmann, 1987) and JM109 were grown at 37°C in Luria broth (Miller, 1972). When needed, 100 μg ampicillin ml⁻¹ and 50 μg spectinomycin ml⁻¹ were added to E. coli cultures. For protein overexpression, recombinant E. coli strains were grown to OD₆₀₀ 0.2, then expression of the plasmid-encoded lapT gene was induced by adding isopropyl β-D-thiogalactopyranoside (IPTG) to a final concentration of 0.5 mM. Incubation was continued at 37°C and cells were harvested 2 h after the addition of IPTG.

Construction of a plasmid for overexpression of LapT. Basic DNA manipulations were as previously described (Highlander et al., 1989). The expression vector pKK223-3 (Pharmacia), which contains a trp/lac fusion promoter (P₉₀) and expresses ampicillin resistance, was used to clone and express lapT. The lapT gene was excised from plasmid pSH224 (Highlander et al., 1989) by cleavage with NolI and SphI. The resulting 1.0 kb fragment was purified, the NolI end was filled by using the Klenow fragment of DNA polymerase I and the fragment was ligated using T4 ligase into the blunt-ended Smal site of pKK223-3 then transformed into E. coli strain JM109. The resulting plasmid, pLC2027, carrying the lapT fragment in the A orientation with respect to lacZ, was transformed into E. coli strain MG1655 carrying the spectinomycin resistant, lacI¹ plasmid, pMS421 (Grafia et al., 1988), to permit controlled expression of lapT.

Cell fractionation and LapT purification. Periplasmic proteins were isolated from a 4 l culture by osmotic shock by the method of Kondo et al. (1988). LapT protein was purified by anion exchange column chromatography using a NaCl gradient (0–150 mM) in 20 mM ethanolamine, pH 9.5. Fractions were collected and analysed on silver-stained SDS-polyacrylamide gels (Heukeshoven & Dernick, 1988; Laemmli, 1970). L-Arginine-binding activity was measured by filter-binding as described below. Protein concentrations were determined using the amidoschwartz method (Schaffner & Weissman, 1973), modified by increasing the final concentration of SDS in the assay to 1% (w/v) and filtering the precipitated proteins through a 47 mm diameter nitrocellulose filter with a pore size of 0.45 μm (Newman et al., 1981). Bovine serum albumin was used as a standard.

Nitrocefin assays. β-Lactamase activity was measured by cleavage of nitrocefin monitored spectrophotometrically (O’Callaghan et al., 1972). A 10 μl sample from each column fraction was incubated with 50 mM sodium phosphate buffer, pH 7.0, 0.1 mg nitrocefin ml⁻¹ for 1 min, then the absorbance read at 525 nm. The specific activity of β-lactamase is reported in pmol min⁻¹ (mg protein)⁻¹.

Ligand-binding assays. l-Arginine-binding activity was measured both by filtration assay and by equilibrium dialysis (Lever, 1972). For binding assays, crude or purified protein samples were combined with 6 μM l-¹⁴C]arginine in 62.5 mM sodium phosphate buffer, pH 7.0. Crude protein concentrations ranged from 1 μM to 25 μM, and purified LapT was used at 1 μM. The protein/arginine mixture was rapidly filtered through a pre-wetted 25 mm diameter nitrocellulose filter with a pore size of 0.45 μm, and washed once with 1.2 ml 10 mM sodium phosphate buffer, pH 7.0, at room temperature. The filter was dried for 1 h, then the radioactivity was measured in 10 ml Aquasol scintillant (New England Nuclear). Background counts were obtained using samples lacking protein. For equilibrium dialysis measurements, 0.3 ml 5 μM LapT protein was dialysed in a 12000–14000 Da Spectramembrane (Spectrum) versus 50 ml 10 mM sodium phosphate buffer, pH 7.0 containing l-¹⁴C]arginine ranging in concentration from 50 nM to 400 nM, with gentle rocking at 4°C overnight. The radioactivity of aliquots from inside the dialysis bag and from the surrounding buffer was measured in 10 ml Aquasol scintillant. Competition experiments were performed by dialysis using l-¹⁴C]arginine at 8 nM in competition with competitors at 0.8 μM (100-fold molar excess) or 1 μM (1.25 × 10⁵-fold molar excess). Kᵣ values were obtained by dialysis using l-¹⁴C]arginine at 8 nM and competitors ranging in concentration from 40 μM to 400 μM.

Transport assays. Arginine transport in P. haemolytica was measured in washed-cell uptake assays (Speiser & Ames, 1991). Cells were grown to exponential phase, harvested by centrifugation at 2000 g for 10 min, washed twice with an arginine-free defined medium (S. K. Highlander, unpublished), and resuspended at a concentration of 10⁷ cells (5 mg dry wt) ml⁻¹ in the same medium. To initiate transport, l-¹⁴C]arginine was added to a concentration of 1 μM. Samples (100 μl) were withdrawn at various time intervals, filtered through a pre-wetted 25 mm diameter nitrocellulose filter with a pore-size of 0.45 μm, washed with 50 ml arginine-free defined medium, dried and the radioactivity measured as described above. Competition experiments were performed by simultaneous addition of l-¹⁴C]arginine and competitors to concentrations of 1 μM and 125 mM, respectively, to the cell suspension.

Arginine transport was also measured in sphaeroplasts isolated from 100 ml culture by the method of Witholt et al. (1976). To prepare sphaeroplasts, cells were recovered by centrifugation and washed once in 2.5 ml 0.2 M Tris/HCl buffer, pH 8.0. An equal volume of 1 M sucrose, 0.2 M Tris/HCl buffer (pH 8.0) was added, followed by lysozyme and EDTA at 60 μg ml⁻¹ and 0.5 mM, respectively. The suspension was diluted rapidly with an equal volume of distilled water, and after 2 min MgCl₂ and pancreatic DNase were added to 3 mM and 20 μg ml⁻¹, respectively. Sphaeroplasts, recovered by centrifugation at 4500 g for 10 min, were resuspended in 250 μl 25 mM sucrose, 50 mM potassium phosphate buffer (pH 6.6), 5 mM MgCl₂ (Witholt et al., 1976). Transport assays were performed as described above using 1 μM l-¹⁴C]arginine or 1 μM l-³H]proline (Witholt et al., 1976).
Antiserum production. Antiserum to LapT was produced using ostensibly germ-free New Zealand rabbits. Animals were injected subcutaneously with 100 μg pure LapT mixed with an equal volume of Freund's complete adjuvant. At 14 and 28 d following the primary injection, booster injections were given using 50 μg of pure LapT and Freund's incomplete adjuvant. Blood was collected at 35 d. Serum was separated from whole citrated blood by low speed centrifugation (2000g) and was analysed for the presence of LapT antibodies by Western blotting.

Immunoblot analysis. LapT protein was detected in whole cell lysates and periplasmic fractions by Western blot analysis (Towbin et al., 1979), using polyclonal rabbit antiserum (1/500 dilution), biotin-conjugated mouse anti-rabbit IgG (1/500) and hors-radish-peroxidase-conjugated streptavidin (1/1000), obtained from Kirkegaard & Perry, as previously described (Highlander et al., 1989).

Chemicals. L-[14C]Arginine (0.1 mCi ml⁻¹; specific activity 339.3 mCi mmol⁻¹ (1.3 x 10⁴ MBq mmol⁻¹)) and L-[1H]proline (1 mCi ml⁻¹; specific activity 38.1 Ci mmol⁻¹ (1.4 x 10⁴ MBq mmol⁻¹)) were obtained from DuPont-New England Nuclear. Amino acids were purchased from Sigma.

RESULTS

L-Arginine binding to LapT in crude cell fractions

The predicted amino acid sequence of the LapT protein suggested that it is similar to the periplasmic binding proteins ArtJ (Wissenbach et al., 1993), HisJ and LAO (Higgins & Ames, 1981) and to the predicted arginine-binding protein ArtI from the recently reported sequence from *H. influenzae* (Fleischmann et al., 1995). Fig. 1 illustrates the deduced amino acid sequences and alignment of LapT, ArtJ, ArtI, HisJ and LAO amino acid sequences. LapT is most similar to the sequence predicted for the *H. influenzae* ArtI gene product, showing 59% identity and 75% similarity, when conservative amino acid substitutions are allowed, and to the *E. coli* ArtJ gene product, showing 50% identity and 67% similarity. The *S. typhimurium* proteins HisJ and LAO both show 35% identity and 57% similarity to LapT. Thus, it seemed likely that LapT could bind and transport arginine and/or histidine. To test if histidine was a ligand for LapT, we used a plasmid (pSH224) that expressed LapT (Highlander et al., 1989) in an effort to provide a periplasmic binding protein to complement a *hisJ* *S. typhimurium* histidine auxotroph (*hisJ* hisJAF645) (Speiser & Ames, 1991) for growth on minimal medium containing histidine. Though the protein was expressed in *S. typhimurium*, LapT was unable to permit histidine transport in these strains; this could be due to failure of LapT to bind histidine, or to the inability of LapT to interact with the HisQMP transport complex. Nevertheless, we then sought to determine if the LapT protein could bind the basic amino acid arginine.

We examined whether crude periplasmic fractions containing LapT could bind l-arginine by filter-binding. The *lapT* gene was cloned onto the Pₜₐₑ expression vector.

![Alignment of the deduced amino sequence of LapT with the related periplasmic amino-acid-binding-proteins ArtJ (from *E. coli*), ArtI (from *H. influenzae*), HisJ and LAO (both from *S. typhimurium*). The alignment was generated using the PILEUP algorithm (Feng & Doolittle, 1987) and the output manipulated using the BOXSHADE server at the Swiss Institute for Experimental Cancer Research, Lausanne. Identical residues are indicated by black boxes and functionally similar residues by shaded boxes. Dots mark the positions of gaps introduced to maximize alignment.](image-url)
plasmid, pKK223-3, to create plasmid pLC2027. Periplasmic shockates prepared from cells carrying pLC2027 (lapT) or pKK223-3 (vector) were mixed with \[^{14}\text{C}\text{]arginine and filtered through nitrocellulose filters to trap protein-associated L-arginine. Crude periplasmic fractions from cells expressing LapT bound}
507 ± 113 pmol L-[14C]arginine (mg protein)^{-1}, whereas fractions from cells carrying pKK223-3 bound only 44 ± 11 pmol L-[14C]arginine (mg protein)^{-1}. Previous studies using this filter-binding method indicated that the amount of bound ligand retained by the filter is influenced by the wash and filtration steps and that approximately 40% of the protein-bound ligand is retained by the filter (Lever, 1972). As a result, these results demonstrated that LapT bound L-arginine, but were not quantitative.

**Purification of LapT**

LapT was purified to further characterize its interaction with L-arginine and other amino acids. An IPTG-induced culture of a strain carrying pLC2027 was osmotically shocked to release the periplasmic proteins. As shown in Fig. 2(a), lane 1, the majority of the protein in the crude osmotic periplasmic fraction was LapT. The periplasmic fraction (1.5 mg ml^{-1}, approx. 35 mg total protein) was chromatographed on a FPLC MonoQ column and fractions were monitored for the presence of LapT by SDS-PAGE and silver staining. Representative fractions are shown in Fig. 2(a) and an FPLC chromatography profile is shown in Fig. 2(b). The LapT protein, with an apparent molecular mass of 29 kDa, eluted as a single species in fractions 5–11, from 55 to 60 mM NaCl [Fig. 2(a), lanes 4–7]. The apparent molecular mass is slightly larger than the 25 kDa molecular mass predicted from the sequence for the signal-cleaved protein (Highlander et al., 1993). Fractions 13–15 [Fig. 2(a), lanes 8 and 9] contained additional proteins, including the β-lactamase protein (approx. 30 kDa). Fractions that contained LapT (5–11), as observed by SDS-PAGE [Fig. 2(a), lanes 4–7], correlated with the major protein peak (1.8 mg ml^{-1}) and with a high level of L-arginine-binding activity (6000 pmol mg^{-1}), as determined by filter-binding assay, Fig. 2(b).

Plasmid pLC2027 also encodes the 30 kDa periplasmic protein β-lactamase. Since this protein is similar in size to LapT, fractions containing LapT were assayed for the presence of β-lactamase activity by nitrocefin hydrolysis. Fractions containing LapT (5–11) did not show detectable β-lactamase activity. β-Lactamase activity was observed in fractions 13–21; this correlates with the second major protein peak in the elution profile.

**Dissociation constant and stoichiometry of the LapT-arginine complex**

Equilibrium dialysis was used to determine the dissociation constant (K_D) of the LapT-L-arginine complex. Experiments were performed using LapT at a constant concentration of 5 μM and L-[14C]arginine at variable concentrations from 50 nM to 400 nM. The averaged results of equilibrium dialysis, performed in triplicate, are shown in Fig. 3. The plot of r/L versus r yields a straight line whose slope is −1/K_D. Thus, the K_D of LapT for L-arginine is 170 nM. The number of L-arginine-binding sites per molecule of LapT was also determined by equilibrium dialysis using LapT at a constant concentration of 5 μM and L-[14C]arginine at variable concentrations from 50 nM to 400 nM. The stoichiometry of binding, from averaged triplicate experiments, was obtained by extrapolation of the Scatchard plot to the x-intercept equals n. The continuous line was generated by linear regression.

**Fig. 3. Dissociation constant of the LapT-arginine complex and stoichiometry of L-arginine binding by LapT obtained by dialysis assay.** Purified LapT was used at 5 μM. Arginine concentrations ranged from 50 nM to 400 nM. The dissociation constant (K_D) was calculated using the equation r/L = 1/K_D + r/K_B, where r is the concentration of bound L-[14C]arginine (in nM) divided by the concentration of LapT protein (in nM), and L is the total concentration of L-[14C]arginine (in nM) (Freifelder, 1982). The number of binding sites was obtained by extrapolation to the point where the x-intercept equals n. The continuous line was generated by linear regression.

**LapT binding specificity**

To determine if L-arginine analogues and other amino acids could bind to LapT, binding affinity was analysed by measuring the ability of an unlabelled amino acid to compete for L-[14C]arginine binding by equilibrium dialysis (Table 1). Potential competitors were chosen that contained a positive charge or were structurally similar to arginine. Alanine was included as a negative control. The competitors were present at 100-fold or 1.25 × 10^5-fold molar excess to L-[14C]arginine and were dialysed versus 1 μM LapT within the dialysis membrane. Values reported in Table 1 are expressed as a percentage of total L-arginine binding in the presence of competitor. Additional concentrations were also tested (results not shown) and gave results consistent with those reported. At 100-fold molar excess, only L-arginine, D-arginine and citrulline exhibited any competition with L-arginine for binding. At 1.25 × 10^5-fold excess some competition by canavanine, l-lysine and homoarginine was also observed, but this is not likely to be physiologically relevant.

Since only D-arginine and citrulline could compete appreciably for L-arginine binding by LapT, we determined their K_I values (Hammes, 1982). Those for
### Table 1. LapT binding specificity

Binding assays were performed using \( l-[^{14}C] \)arginine at 8 nM in the presence of the competitors at 100-fold or \( 1.25 \times 10^5 \)-fold molar excess. Measurements were made in triplicate and the values reported are means.

<table>
<thead>
<tr>
<th>Competitor</th>
<th>R-Group</th>
<th>Inhibition (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>100-fold molar excess</td>
</tr>
<tr>
<td>L-Arginine</td>
<td>(-\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}(\text{NH})\text{CNH}_2)</td>
<td>100(\dagger)</td>
</tr>
<tr>
<td>D-Arginine</td>
<td>(-\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}(\text{NH})\text{CNH}_2)</td>
<td>20</td>
</tr>
<tr>
<td>Citrulline</td>
<td>(-\text{CH}_2\text{CH}_2\text{OH}(\text{O})\text{CNH}_2)</td>
<td>12</td>
</tr>
<tr>
<td>Canavanine</td>
<td>(-\text{CH}_2\text{CH}_2\text{ON}(\text{NH})\text{CNH}_2)</td>
<td>0(\dagger)</td>
</tr>
<tr>
<td>L-Lysine</td>
<td>(-\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2)</td>
<td>0(\dagger)</td>
</tr>
<tr>
<td>Homoarginine</td>
<td>(-\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}(\text{NH})\text{CNH}_2)</td>
<td>0(\dagger)</td>
</tr>
<tr>
<td>L-Histidine</td>
<td>(-\text{CH}_2\text{imidazole})</td>
<td>0(\dagger)</td>
</tr>
<tr>
<td>Ornithine</td>
<td>(-\text{CH}_2\text{CH}_2\text{CH}_2\text{NH}_2)</td>
<td>0(\dagger)</td>
</tr>
<tr>
<td>L-Alanine</td>
<td>(-\text{CH}_3)</td>
<td>0(\dagger)</td>
</tr>
<tr>
<td>None</td>
<td>(-)</td>
<td>0(\dagger)</td>
</tr>
</tbody>
</table>

\(\dagger\) 100% inhibition represents < 0.2 pmol \( l-[^{14}C] \)arginine bound.

\(\dagger\) 0% inhibition represents \( \geq 40 \) pmol \( l-[^{14}C] \)arginine bound.

L-arginine and citrulline were 340 \( \mu \)M and 560 \( \mu \)M, respectively. From this analysis, we conclude that the primary ligand for LapT is L-arginine. L-Lysine and L-ornithine did not compete for L-arginine binding to a significant extent, indicating that LapT is not an LAO-binding protein.

### Arginine transport in *P. haemolytica*

*P. haemolytica* whole cells were analysed for their ability to accumulate \( l-[^{14}C] \)arginine by washed-cell uptake assay (Fig. 4). The quantity of \( l-[^{14}C] \)arginine retained by the cells increased nearly linearly over the time examined. To establish that the primary mechanism of L-arginine uptake in *P. haemolytica* occurs via a periplasmic binding-protein-dependent system, transport assays were also performed using sphaeroplasts (Fig. 4). During the formation of sphaeroplasts, the periplasmic binding protein component needed for transport should be lost. As predicted, \( l-[^{14}C] \)arginine accumulation in sphaeroplasts was minimal. When sphaeroplasts were lysed by dilution in water, \( l-[^{14}C] \)arginine accumulation was nearly abolished indicating that few whole cells were present following sphaeroplasting. To verify transport competence of the sphaeroplasts, control assays were performed using \( l-[^{3}H] \)proline (Fig. 4, right axis). In *E. coli*, proline is transported via the proton motive force and does not require a periplasmic binding protein (Chen et al., 1985). In *P. haemolytica*, \( l-[^{3}H] \)proline transport occurred both in whole cells and in sphaeroplasts, but was lost upon lysis of sphaeroplasts in water. Sphaeroplasting decreased \( l-[^{3}H] \)proline transport by 30% suggesting that the manipulation caused lysis of about 30% of the cells. Considering the reduction of viability during sphaeroplasting, it appears that periplasmic binding-protein-dependent arginine transport represents at least 80% of the total arginine transport in whole cells. This suggests that periplasmic binding-protein-independent transport systems, if any, play a minor role in the transport of \( l-[^{14}C] \)arginine.

Amino acid analogues, analysed in the LapT-binding experiments, were also tested for their ability to inhibit \( l-[^{14}C] \)arginine transport in *P. haemolytica*. A whole-cell or sphaeroplast suspension containing \( 10^7 \) cells ml\(^{-1}\) was incubated with \( 1 \mu M l-[^{14}C] \)arginine (filled symbols) or \( l-[^{3}H] \)proline (open symbols) and transport assays were performed by filter-binding assay. 

\(\bullet\), Whole cells plus arginine; 
\(\bigcirc\), whole cells plus proline; 
\(\blacklozenge\), sphaeroplasts plus arginine; 
\(\square\), sphaeroplasts plus proline; 
\(\triangle\), sphaeroplasts diluted in water plus arginine; 
\(\Delta\), sphaeroplasts diluted in water plus proline.

Fig. 4. \( l-[^{14}C] \)arginine and \( l[^{3}H] \)proline transport in *P. haemolytica*. A whole-cell or sphaeroplast suspension containing \( 10^7 \) cells ml\(^{-1}\) was incubated with \( 1 \mu M l-[^{14}C] \)arginine (filled symbols) or \( l-[^{3}H] \)proline (open symbols) and transport assays were performed by filter-binding assay. 

\(\bullet\), Whole cells plus arginine; 
\(\bigcirc\), whole cells plus proline; 
\(\blacklozenge\), sphaeroplasts plus arginine; 
\(\square\), sphaeroplasts plus proline; 
\(\triangle\), sphaeroplasts diluted in water plus arginine; 
\(\Delta\), sphaeroplasts diluted in water plus proline.
arginine transport in *P. haemolytica* whole cells. When L-[^14]C]arginine transport was assayed in the presence of a 1:25×10^5-fold molar excess of each competitor, D-arginine and citrulline inhibited transport by approximately 75% and 70%, respectively. These values are similar to those reported for binding inhibition presented in Table 1. None of the other analogues examined (listed in Table 1) were able to compete for transport.

**Identification of LapT in *P. haemolytica***

LapT in whole cells and periplasmic fractions from *E. coli* cells carrying pLC2027 and from *P. haemolytica* cells was compared by Western blotting (Fig. 5). LapT was found in the periplasm (lane 7) and in whole cells (lane 8) of *E. coli* cells carrying pLC2027, but was not observed in cells carrying the vector alone (lanes 5 and 6). LapT was present in the periplasmic fraction of *P. haemolytica* (lane 3), but could not be detected in whole cell samples (lane 4). Note that the *P. haemolytica* samples shown do not contain equivalent quantities of cells since a greater concentration of whole cell sample could not be effectively resolved by SDS-PAGE. Pre-immune serum was not reactive with LapT (lanes 1 and 2). LapT was observed only when samples were probed with anti-LapT antiserum (lanes 3–8).

**DISCUSSION**

We have characterized the periplasmic binding protein, LapT, of the leucotoxin-associated permease of *P. haemolytica* (Highlander *et al.*, 1993). The purification procedure yielded pure LapT as observed by gel electrophoresis. Since the majority of the protein in crude periplasmic fractions consisted of LapT, we were able to obtain it in a one-step purification. We utilized the anion-exchange column used previously to purify other binding proteins (Lever, 1972; Nikaido & Ames, 1992). The fact that the predicted amino acid sequence of LapT is similar to arginine- and histidine-binding proteins from *E. coli* (Wissenbach *et al.*, 1993) and *S. typhimurium* (Higgins & Ames, 1981) helped us identify L-arginine as the primary ligand for LapT. From the binding competition experiments, we concluded that LapT does not bind histidine nor is it an LAO-like periplasmic binding protein.

Analysis of binding properties provided additional information about LapT. The LapT dissociation constant (170 nM) is intermediate when compared to the *K*ₐ values of other arginine-binding proteins for L-arginine: that of the *S. typhimurium* LAO is 14 nM (Nikaido & Ames, 1992); of the *E. coli* LAO, 1500 nM (Rosen, 1971); of the *E. coli* ArtJ binding protein, 400 nM (Wissenbach *et al.*, 1995); and of the *E. coli* arginine-binding protein II, 30 nM (Rosen, 1973). Our studies indicate that the primary ligand for LapT is L-arginine, suggesting that the binding pocket of LapT is very specific. LapT is most similar in terms of primary sequence, ligand specificity and ligand affinity to the recently described ArtJ protein (Wissenbach *et al.*, 1995), previously recognized by Rosen (1973) as arginine binding protein I. Alignment of the predicted amino acid sequences of LapT and LAO determined that all but three residues (92, 94 and 139), located in the ligand-binding pocket of LAO (residues 33, 36, 52, 74, 91, 92, 94, 99, 139, 143 and 183) (Oh *et al.*, 1994), as determined by the crystal structure (Kang *et al.*, 1991), corresponded to identical or functionally similar residues in LapT.

Since only D-arginine and citrulline could compete for binding to LapT, it appears that ligand side chain length is critical. The larger analogue, homoarginine, which possesses an additional carbon was a poor competitor. Citrulline, a molecule similar in chain length and charge to L-arginine but with a carbonyl oxygen in place of the guanido group in arginine, was a modest inhibitor of L-arginine binding to LapT. Stereospecificity is significant since the D-enantiomer of arginine did not bind as well as the L-enantiomer. The *K*ₐ values for D-arginine and citrulline were 1000-fold greater than the L-arginine *K*ₐ, again illustrating the specificity of the interaction.

Whole-cell transport assays demonstrated that *P. haemolytica* transports L-arginine very efficiently. Transport assays performed with sphaeroplasts indicated that a periplasmic binding-protein-dependent transport system...
is the major mechanism of L-arginine transport by *P. haemobtica*. The ability of amino acids to inhibit L-arginine transport in *P. haemobtica* was compared to the data obtained in the LapT binding specificity experiments. D-Arginine and citrulline were the best inhibitors of both L-arginine binding to LapT and to L-arginine transport in *P. haemobtica*. Other charged amino acids such as L-histidine and ornithine had little effect on binding and transport activity. Thus, our results indicate that the leucotoxin-associated permease is very specific for the transport of L-arginine, but it is also possible that other arginine transport systems are present in *P. haemobtica*.

Despite the close proximity of the arginine transport genes to the leucotoxin gene cluster, no role for arginine transport in leucotoxin expression has been identified. However, it has been shown that a component of the arginine transport locus, LapA, negatively regulates leucotoxin expression (Highlander et al., 1993). It is also hypothesized that leucotoxin and lapT transcription are coordinately regulated (Highlander & Weinstock, 1994). Studies using defined growth medium have determined that L-arginine is not required nor does it enhance the growth of *P. haemobtica* (S. K. Highlander, unpublished data). We are hopeful that a more complete characterization of the arginine transport system in *P. haemobtica* may yield information about its possible relationship to the regulation of leucotoxin expression.

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### REFERENCES


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