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Excretion of putrescine by the putrescine–ornithine antiporter encoded by the *potE* gene of *Escherichia coli*

(polyamine transport/potE protein)

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ABSTRACT **Excretion of putrescine from *Escherichia coli* was assessed by measuring its uptake into inside-out membrane vesicles. The vesicles were prepared from wild-type *E. coli* or *E. coli* transformed with plasmids containing one of the three polyamine transport systems. The results indicate that excretion of putrescine is catalyzed by the putrescine transport protein, encoded by the *potE* gene located at 16 min on the *E. coli* chromosome. Loading of ornithine (or lysine) inside the vesicles was essential for the uptake of putrescine, indicating that the protein exchanges putrescine and ornithine (or lysine) by an antiport mechanism. The K_m and V_{max} values for the putrescine uptake by inside-out membrane vesicles were 73 μM and 0.82 nmol/min per mg of protein, respectively. The antiport protein (potE protein) also catalyzed putrescine–putrescine and ornithine–ornithine exchange. The transport activity was not disturbed by inhibitors of energy production such as KCN and carbonyl cyanide *m*-chlorophenylhydrazone. When intact *E. coli* was used instead of the inside-out membrane vesicles, excretion of putrescine was also catalyzed by the antiport protein in the presence of ornithine in the medium.**

Polyamines, aliphatic cations present in all living organisms, are known to be necessary for normal cell growth (1, 2). It has been suggested that the accumulation of excess polyamines may cause the inhibition of cell growth, since high levels inhibit macromolecule synthesis in a cell-free system (3, 4). Support for this suggestion—i.e., that optimal polyamine concentrations are maintained in cells—comes from the observation that cells excrete any excess amounts of polyamines (5, 6).

However, the mechanism of polyamine transport is still not well understood. We recently succeeded in producing three plasmids containing polyamine transport genes (pPT104, pPT79, and pPT71) (7–9). The transport system encoded by pPT104, mapped at 15 min on the *Escherichia coli* chromosome, catalyzes transport of both spermidine and putrescine. The transport systems encoded by pPT79, mapped at 19 min, and pPT71, mapped at 16 min, catalyze only putrescine transport. The putrescine transport system encoded by pPT71 showed a relatively low activity compared with that encoded by pPT104 or pPT79 (7). The transport system encoded by pPT104 or pPT79 was a periplasmic transport system consisting of four kinds of proteins: a periplasmic protein, a membrane-associated protein having the nucleotide-binding site, and two other membrane proteins consisting of 6 membrane-spanning segments (ref. 8 and unpublished results). In contrast, the putrescine transport system encoded by pPT71 consisted of one membrane protein having 12 transmembrane segments (9). Thus, we hypothesized that the putrescine transport system encoded by pPT71 possesses the ability to excrete putrescine. We tested this hypothesis by measuring the putrescine uptake into inside-out membrane vesicles. The results indicate that excretion of putrescine is catalyzed by the antiport protein (potE protein) encoded by the *potE* present on pPT71.

MATERIALS AND METHODS

Bacterial Strains, Plasmids, and Culture Conditions. *E. coli* DR112 (*speA speB thi*; ref. 10), kindly provided by D. R. Morris (University of Washington), was grown as described previously (5). When growth was sufficient to give an OD_{540} of 0.5, the cells were harvested and washed twice with 100 mM potassium phosphate buffer, pH 6.6/10 mM EDTA. *E. coli* MA261 (*speB speC thr leu thi*; ref. 11), kindly provided by W. K. Maas (New York University School of Medicine), and its polyamine transport-deficient mutant NH1596 (7) were grown in medium A in the absence of putrescine as described previously (12). Plasmids pPT104, pPT79, pPT71, and pPT71.30 containing polyamine transport genes were isolated as previously described (7, 9). Plasmid pODC, containing the *speC* gene for constitutive ornithine decarboxylase (13), was kindly supplied by S. M. Boyle (Virginia Polytechnic Institute and State University). Chloramphenicol (30 μg/ml) and/or tetracycline (15 μg/ml) was added during the growth of the strains containing plasmids.

Preparation of Inside-Out and Right-Side-Out Membrane Vesicles. Inside-out membrane vesicles were prepared by French press treatment of *E. coli* DR112(pPT71) cells suspended in 100 mM potassium phosphate buffer, pH 6.6/10 mM EDTA containing each of 20 amino acids plus ornithine at 2.5 mM according to the method of Houng *et al.* (14). Right-side-out membrane vesicles of *E. coli* DR112(pPT71) were prepared by the procedure of Kaback (15), except that the concentration of lysozyme was decreased from 500 μg/ml to 50 μg/ml and the hypotonic medium contained 2 mM ornithine or putrescine.

Assay for Putrescine or Ornithine Uptake. The reaction mixture (0.1 ml) for the uptake by inside-out membrane vesicles contained 10 mM Tris·HCl at pH 8.0, 10 mM potassium phosphate buffer at pH 8.0, 0.14 M KCl, 50 μM [^{14}C]putrescine (1.48 GBq/mmol) or 100 μM [^{14}C]ornithine (1.48 GBq/mmol), and 50 μg of inside-out membrane vesicle protein. The reaction mixture (0.1 ml) for the uptake by right-side-out membrane vesicles contained 25 mM potassium phosphate buffer at pH 6.6, 50 mM Hepes–KOH at pH 7.6, 10 mM $MgSO_4$, 50 μM [^{14}C]putrescine (1.48 GBq/mmol) or 100 μM [^{14}C]ornithine (1.48 GBq/mmol), and 50 μg of right-side-out membrane vesicle protein. The reaction mixture was incubated at 22°C for 5 min without the substrate, and the reaction was started by the addition of the substrate. After incubation at 22°C for 10 sec to 2 min, membrane vesicles were collected on membrane filters (cellulose nitrate, 0.45 μm; Advantec Toyo, Tokyo) and washed, and

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Abbreviation: CCCP, carbonyl cyanide *m*-chlorophenylhydrazone.
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their radioactivities were measured with a liquid scintillation spectrophotometer. Polyamine uptake by intact cells was measured as described previously (7). The reaction mixture (0.5 ml) contained 0.48 ml of cell suspension (protein concentration 0.1 mg/ml) in buffer A [0.4% glucose/62 mM potassium phosphate, pH 7.0/1.7 mM sodium citrate/7.6 mM $(NH_4)_2SO_4$/0.41 mM $MgSO_4$] and 0.02 ml of 0.25 mM [^{14}C]putrescine (370 MBq/mmol).

Assay for Ornithine Excretion by Inside-Out Membrane Vesicles. Preloading of inside-out membranes with ornithine (the reaction mixture, 0.02 ml) was performed by incubating inside-out membrane vesicles (0.1 mg of protein) prepared in the absence of amino acids with 0.5 mM [^{14}C]ornithine (1.48 GBq/mmol) under the same ionic conditions as the reaction mixture of the uptake assay at 37°C for 1 hr. The excretion assay was started by 10-fold dilution of the above membrane vesicle solution with the solution of the uptake assay.

Assay for Putrescine Excretion by Intact Cells. The reaction mixture (1 ml) was the same as that of putrescine uptake by intact cells except that [^{14}C]putrescine was omitted and 2 mM ornithine was added. After incubation at 37°C for 5 or 10 min, the cells were harvested by centrifugation at 20,000 × *g* for 2 min. The putrescine in cells and medium was measured by HPLC as described previously (16).

Protein Assay. Protein content was determined by the method of Lowry *et al.* (17), using bovine serum albumin as the standard.

RESULTS

Putrescine and Ornithine Uptake by Inside-Out Membrane Vesicles. Excretion of putrescine from cells was demonstrated by measuring its uptake into inside-out membrane vesicles. As shown in Table 1, putrescine uptake was clearly observed only with the membrane vesicles prepared from *E. coli* transformed with pPT71 (*potE*) and not with the membrane vesicles prepared from *E. coli* or *E. coli* transformed with either pPT104 or pPT79. The uptake activity was not disturbed by inhibitors of energy production such as KCN and CCCP. The addition of D-lactate did not influence the putrescine uptake significantly (data not shown). The uptake was not inhibited by spermine, spermidine, or acetylated polyamines. These results indicate that there is an energy-independent putrescine excretion system in *E. coli*, catalyzed by the product of a gene on pPT71; we have characterized the sequence of this putrescine transport protein and designated the gene *potE* (9).

Since energy was not necessary for the excretion of putrescine from *E. coli*, we asked whether or not the putrescine transport protein has an antiport activity. Under standard conditions, inside-out membrane vesicles were prepared in the presence of 20 amino acids plus ornithine. When membrane vesicles were prepared in the absence of amino acids, putrescine uptake was greatly diminished (Table 2). Similarly, when the membrane vesicles were prepared in the presence of 18 amino acids (without ornithine, lysine, and arginine) or glutamic acid only, only 10% putrescine uptake activity was observed. As shown in Table 2, only ornithine-loaded or lysine-loaded membrane vesicles showed high putrescine uptake activity. Significant putrescine uptake activity (32% of control) was also observed with arginine-loaded vesicles.

When 2.5 mM ornithine or lysine was added together with 50 μM [^{14}C]putrescine, the uptake activity was strongly inhibited. Arginine inhibited the uptake significantly, but glutamic acid did not (Table 2). Agmatine (decarboxylated arginine and a precursor of putrescine) also did not influence putrescine uptake (data not shown). The K_m and V_{max} values for putrescine uptake by the inside-out membrane vesicles were 73 μM and 0.82 nmol/min per mg of protein, respectively. The K_i values for ornithine and lysine were 208 μM and 1.26 mM, respectively (Fig. 1). These results show that the putrescine transport protein encoded by the *potE* gene on pPT71 has an antiport activity between putrescine and ornithine (or lysine).

We next examined whether the excretion of ornithine was accompanied by the uptake of putrescine. For this purpose, the inside-out membrane vesicles were prepared in the absence of amino acids and then preloaded with 0.5 mM ornithine. The reaction was started by diluting the membrane vesicles 10-fold and by adding putrescine. As shown in Fig. 2, the amount of ornithine retained in the membrane vesicles decreased with putrescine uptake. The exchange ratio between putrescine and ornithine was 1 to 1. Furthermore, a putrescine–putrescine or ornithine–ornithine exchange activity was also observed with the inside-out membrane vesicles (Fig. 3). Similar exchange activities of energy-independent ornithine–putrescine, ornithine–ornithine, and putrescine–putrescine were observed with right-side-out membrane vesicles prepared from *E. coli* transformed with pPT71 (data not shown).

Table 1. Effect of polyamine transport proteins, inhibitors of energy production, and polyamine analogs on putrescine uptake by inside-out membrane vesicles

Exp.	Plasmid	Gene(s)	Addition	Putrescine uptake: pmol/min per mg of protein	Putrescine uptake: %
1	pPT71	*potE*	—	252	100
	pPT79	*potF, -G, -H, -I**	—	14	6
	pPT104	*potA, -B, -C, -D*	—	13	5
	None		—	17	7
2	pPT71	*potE*	—	274	100
	pPT71	*potE*	CCCP, 40 μM	283	103
	pPT71	*potE*	KCN, 10 mM	247	90
	pPT71	*potE*	Spermine, 0.2 mM	285	104
	pPT71	*potE*	Spermidine, 0.2 mM	288	105
	pPT71	*potE*	N^1-Acetylspermine, 0.2 mM	260	95
	pPT71	*potE*	N^1-Acetylspermidine, 0.2 mM	252	92
	pPT71	*potE*	N^1-Acetylputrescine, 0.2 mM	241	88

Inside-out membrane vesicles from *E. coli* DR112 containing various plasmids were prepared as described in *Materials and Methods*. The assays were performed under standard conditions except that the reaction mixture contained inhibitors of energy production or polyamine analogs as indicated. CCCP, carbonyl cyanide *m*-chlorophenylhydrazone. Each value is the average of duplicate determinations.

*Characteristics of the genes will be described elsewhere.

Table 2. Effect of amino acids on putrescine uptake by inside-out membrane vesicles

Exp.	Addition: Inside	Addition: Outside	Putrescine uptake: pmol/min per mg of protein	Putrescine uptake: %
1	20 AAs + ornithine	—	278	100
	18 AAs*	—	31	11
	Ornithine	—	230	83
	Lysine	—	241	87
	Arginine	—	89	32
	Glutamate	—	28	10
	—	—	39	14
2	Ornithine	—	233	100
	Ornithine	Ornithine	23	10
	Ornithine	Lysine	37	16
	Ornithine	Arginine	175	75
	Ornithine	Glutamate	217	93

The membrane vesicles were prepared from *E. coli* in the presence of various amino acids (AAs) shown in the column under "Inside" at 2.5 mM each. The assays were performed under standard conditions except that the reaction mixture contained amino acids shown in the column under "Outside." Each value is the average of duplicate determinations.
*Ornithine, lysine, and arginine were not added.

Excretion of Putrescine from Intact Cells. In *E. coli* having normal putrescine uptake activity, putrescine transport is mainly catalyzed by pPT104- and pPT79-encoded systems (7). The addition of ornithine or putrescine together with CCCP did not cause significant excretion of accumulated putrescine from cells (Fig. 4*C*). In *E. coli* NH1596 (pPT71), in which putrescine transport is catalyzed by potE protein (7), the accumulated [^{14}C]putrescine was excreted when nonlabeled putrescine was added at either 5 or 10 min of incubation. Whereas ornithine did not inhibit putrescine uptake at the onset of the reaction, *E. coli* started to excrete putrescine as its accumulated amount increased (Fig. 4*A*). When putrescine uptake was inhibited by the addition of CCCP, accumulated putrescine was rapidly excreted by the addition of putrescine or ornithine (Fig. 4*B*). Essentially the same results were obtained with *E. coli* NH1596 (pPT71.30), in which only potE protein, not speF protein, was expressed (9). These findings indicate that the excretion of putrescine from intact cells was catalyzed by potE protein.

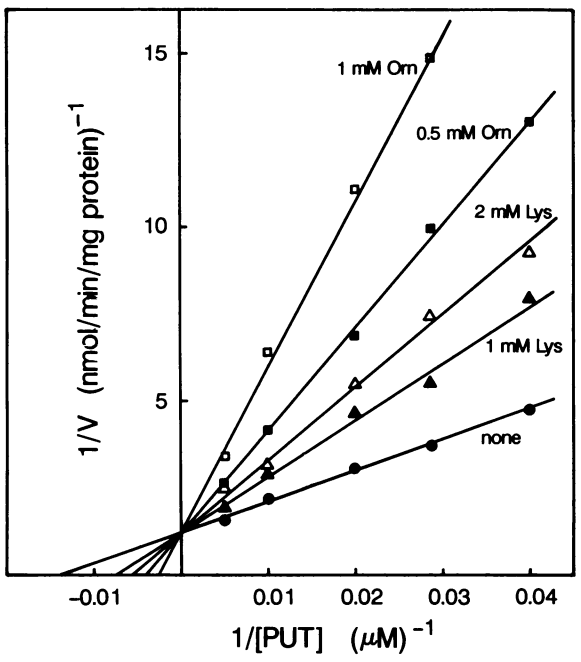

FIG. 1. Double-reciprocal plots of putrescine (PUT) uptake by inside-out membrane vesicles. The assays were carried out by changing the concentration of substrate and amino acids. ●, No addition; ▲, 1 mM lysine; △, 2 mM lysine; ■, 0.5 mM ornithine; □, 1 mM ornithine.

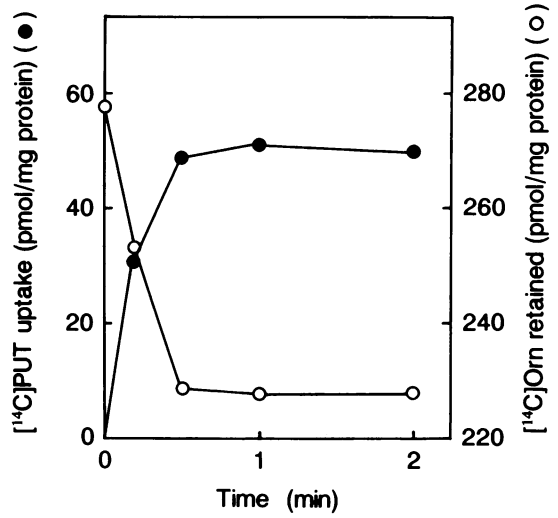

FIG. 2. Exchange of putrescine (PUT) and ornithine by inside-out membrane vesicles. To measure the excretion of ornithine, inside-out membrane vesicles preloaded with [^{14}C]ornithine were prepared. Similarly, inside-out membrane vesicles preloaded with unlabeled ornithine were prepared and [^{14}C]putrescine uptake was measured.

To confirm that potE protein is involved in excreting excess putrescine, putrescine excretion rates were measured in *E. coli* transformed with pODC (*speC*), which contains a constitutive ornithine decarboxylase gene (13). In *E. coli* NH1596, in which genomic potE protein is expressed, slow but significant excretion of putrescine was observed (Fig. 5*B*). The rates of excretion of putrescine from *E. coli* NH1596 and the parent strain, *E. coli* MA261, were nearly equal. This suggested that polyamine transport proteins encoded by pPT104 (*potA, potB, potC, potD*) and pPT79 (*potF, potG, potH, potI*) are not involved in the excretion; the excretion was not inhibited by CCCP. When *E. coli* NH1596 was transformed with pPT71 (*potE*), very rapid excretion of putrescine was observed and CCCP even slightly stimulated this rate (Fig. 5*A*). *E. coli* transformed with pPT104 or pPT79

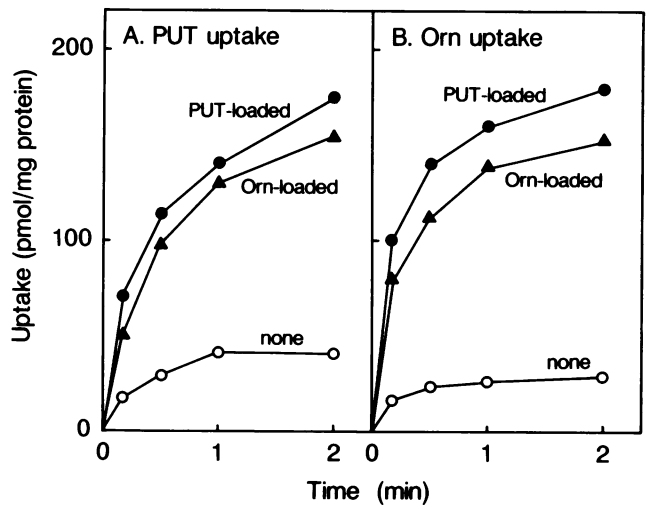

FIG. 3. Putrescine (*A*) and ornithine (*B*) uptake by inside-out membrane vesicles. The inside-out membrane vesicles were prepared in the presence of 2.5 mM putrescine (●) or 2.5 mM ornithine (▲) or in the absence of amino acids (○).

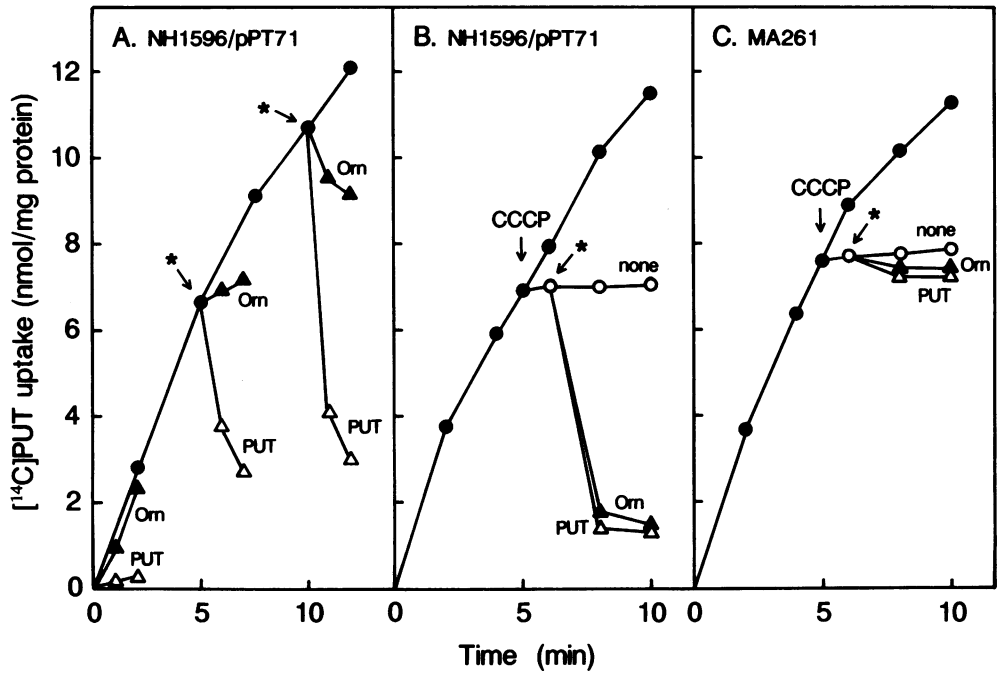

FIG. 4. Excretion of putrescine from *E. coli* transformed with pPT71 (*potE*). (*A* and *B*) *E. coli* NH1596 transformed with pPT71. (*C*) The parent strain, *E. coli* MA261. (*A*) At 0, 5, or 10 min after the onset of the reaction, either 1 mM ornithine (▲) or 1 mM putrescine (△) was added to the reaction mixture. ●, No addition. (*B* and *C*) At 5 min, 40 μM CCCP (○) was added to the reaction mixture. At 6 min, 1 mM ornithine (▲) or putrescine (△) was added to the reaction mixture containing 40 μM CCCP. *, Time of addition of either ornithine or putrescine.

did not show the rapid excretion of putrescine. When ornithine was removed from the medium, putrescine excretion was greatly diminished (data not shown).

DISCUSSION

Because polyamine excretion is usually slow, it has been difficult to investigate. In this study, we prepared membrane vesicles that overproduced one of the three polyamine transport systems. This enabled us to easily determine if one of the transport systems was involved in the excretion. Our data show that potE protein (encoded by pPT71) can excrete putrescine as the result of its antiport activity between putrescine and ornithine (or lysine).

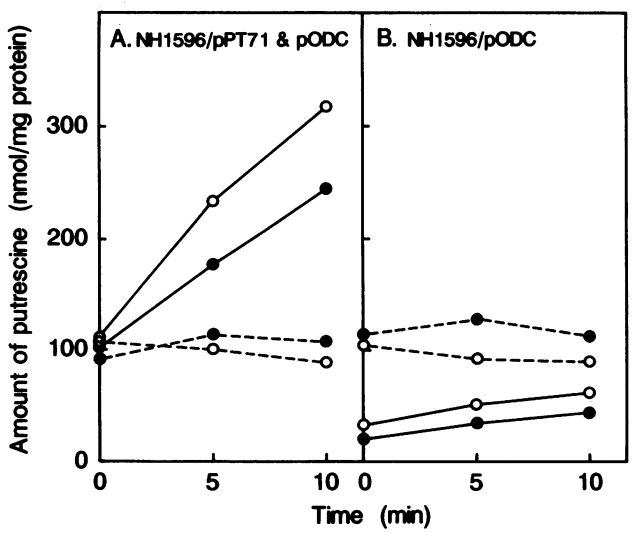

FIG. 5. Putrescine excretion into the medium by intact cells. *E. coli* NH1596 was transformed with pPT71 (*potE*) and pODC (*speC*) (*A*) or by pODC only (*B*). ——, Putrescine in the medium; – – –, putrescine in cells. The assays were performed in the presence (○) or absence (●) of 40 μM CCCP.

Exchange systems in which ions do not participate are commonly found in mitochondria (18) but rarely in bacteria (19–21). The arginine–ornithine and the agmatine–putrescine antiporters in *Enterococcus faecalis* play important roles in energy production through the degradation of guanidine compounds (20, 21). The antiport protein (*potE* product) in *E. coli* is expressed together with an inducible ornithine decarboxylase (*speF* product) (9). When excess amounts of putrescine accumulate in cells, they inhibit both constitutive and inducible ornithine decarboxylase activities (5, 22, 23) and are excreted into medium by the potE protein, to keep the cellular polyamine content optimal. Furthermore, in acidic culture medium, potE protein and inducible ornithine decarboxylase may be involved in neutralization of the medium or protection of cells at acidic pH through the synthesis and excretion of putrescine. It still remains to be clarified how the genes for the two proteins (i.e., *potE* and *speF*) are expressed and regulated under specific circumstances.

The *potE*-encoded transport system also catalyzes putrescine uptake, although the activity is low compared with the pPT104 and pPT79 systems (7). The uptake was clearly inhibited by CCCP and not inhibited by ornithine unless putrescine was accumulated in cells. The K_m value for putrescine uptake with intact cells was 1.8 μM (7). In contrast, putrescine uptake by the inside-out membrane vesicles was not inhibited by CCCP and KCN, and the K_m value for the uptake was 73 μM. In addition, putrescine uptake was greatly inhibited in the absence of ornithine. These results suggest that the excretion of ornithine as a counterpart for the uptake of putrescine by potE protein in intact cells may not always be necessary. Energy may be required when putrescine is taken up into cells without excretion of ornithine. In any event, the involvement of the potE protein in putrescine uptake is probably very small.

The potE protein consists of 12 putative membrane-spanning segments linked by hydrophilic segments of variable length (9). In the eighth hydrophilic segment from the

NH_2 terminus of potE protein, amino acid sequences in common with ornithine decarboxylase (Pro-Phe-Xaa-Leu-Ala; ref. 9 and personal communication from S. M. Boyle†) and with potD protein (Pro-Xaa-Met-Glu; ref. 8) were found. The potD protein is a polyamine-binding protein existing in the periplasm and is part of the spermidine and putrescine transport system (the pPT104-encoded system). Thus, the recognition sites for putrescine and ornithine of potE protein may exist in this region. The ATP/ADP antiporter of *Rickettsia prowazekki* (24) also consisted of 12 putative membrane-spanning segments linked by hydrophilic segments of variable length, but it showed no significant homology with potE protein. Furthermore, we have not been able to find potE homologies among the DNA and protein data bases.

Excess polyamines are excreted in the form of putrescine, acetylspermidine, or acetylspermine in both prokaryotes and eukaryotes (unpublished results). Since potE protein can excrete only putrescine, another protein or proteins must be involved in the excretion of acetylspermidine and acetylspermine. It is now clear that we are beginning to understand the mechanism by which the polyamine content in *E. coli* is variously and elaborately regulated by biosynthesis, uptake, and excretion.

†GenBank data base accession no. M33766.

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