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Polyamine transport in *Escherichia coli*

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Summary. The polyamine content in cells is regulated by both polyamine biosynthesis and its transport. We recently obtained and characterized three clones of polyamine transport genes (pPT104, pPT79 and pPT71) in *Escherichia coli*. The system encoded by pPT104 was the spermidine-preferential uptake system and that encoded by pPT79 the putrescine-specific uptake system. Furthermore, these two systems were periplasmic transport systems consisting of four kinds of proteins: pPT104 clone encoded potA, -B, -C, and -D proteins and pPT79 clone encoded potF, -G, -H, and -I proteins, judging from the deduced amino acid sequences of the nucleotide sequences of these clones. PotD and -F proteins were periplasmic substrate binding proteins and potA and -G proteins membrane associated proteins having the nucleotide binding site. PotB and -C proteins, and potH and -I proteins were transmembrane proteins probably forming channels for spermidine and putrescine, respectively. Their amino acid sequences in the corresponding proteins were similar to each other. The functions of potA and -D proteins in the spermidine-preferential uptake system encoded by pPT104 clone were studied in detail through a combined biochemical and genetic approach. In contrast, the putrescine transport system encoded by pPT71 consisted of one membrane protein (potE protein) haveing twelve transmembrane segments, and was active in both the uptake and excretion of putrescine. The uptake was dependent on membrane potential, and the excretion was due to the exchange reaction between putrescine and ornithine.

Keywords: Amino acids – Polyamines – Putrescine – Spermidine – Spermine – Uptake – Excretion

Introduction

Polyamines (putrescine, spermidine and spermine) are known to be necessary for cell growth (Tabor and Tabor, 1984; Pegg, 1988). It is thus important to understand the mechanism by which the cellular polyamine is regulated. Polyamine transport is one of the important determining factors of polyamine

content in cells. In animal cells, polyamine uptake was shown to increase during hormonal stimulation and proliferation (Kano and Oka, 1976; Pohjanpelto, 1976) and to be energy-dependent (Reinhart Jr. and Chen, 1984; Kakinuma et al., 1988). In addition, it was shown that different polyamines appeared to share the same transport system (Porter et al., 1984; Reinhart Jr. and Chen, 1984; Kakinuma et al., 1988) and that multiple systems might exist for the uptake of polyamines (Byers et al., 1987). In *Escherichia coli*, polyamine uptake was energy-dependent, and the putrescine transport system was different from the spermidine and spermine transport system(s) (Tabor and Tabor, 1966; Kashiwagi et al., 1986). Furthermore, two transport systems for putrescine have been suggested in *E. coli* K12 grown in a low osmolarity medium (Munro et al., 1974). To clarify the properties of the polyamine transport systems in *E. coli* further, we tried to isolate clones of polyamine transport genes. We succeeded in obtaining three clones of polyamine transport genes and clarified some of the properties of polyamine transport.

Properties of polyamine transport and cloning of genes for polyamine transport proteins

Polyamine uptake activity is shown in Fig. 1. The activity was in the order putrescine > spermidine > spermine. The uptake activity was inhibited by KCN and CCCP (carbonylcyanide *m*-chlorophenylhydrazone), indicating that the uptake was energy-dependent. The addition of *N*-ethylmaleimide (NEM) and *p*-chloromercuribenzoic acid (PCMB) abolished polyamine uptake; the results suggest that a sulfhydryl group is involved in the polyamine transport system. The substrate specificity was then studied. Spermidine and

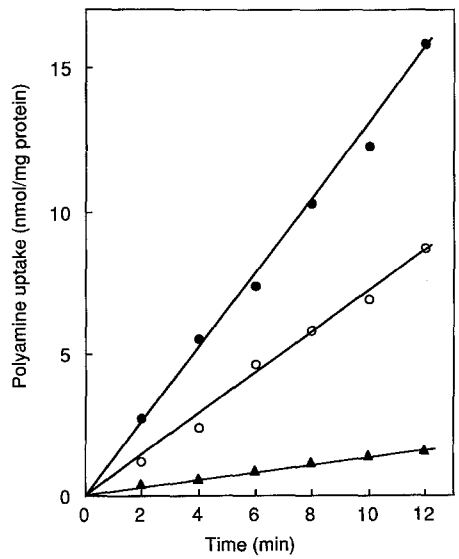

Fig. 1. Polyamine transport in *Escherichia coli* MA261. ●, putrescine uptake; ○, spermidine uptake; ▲, spermine uptake

spermine did not inhibit putrescine uptake significantly, and putrescine did not inhibit spermidine uptake. Spermidine uptake was inhibited by spermine. These results suggest that the putrescine uptake system differs from the spermidine and spermine uptake system and that spermine is transported by the same system as spermidine.

To select a polyamine transport-deficient mutant, *E. coli* MA261 lacking polyamine biosynthesis was chosen as a parent strain. If the polyamine transport protein is deficient, cells will not grow well even in the presence of polyamines. Therefore, we picked up approximately 100 small colonies from 5000 colonies grown in the presence of either putrescine or spermidine after mutagenesis with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine, and the polyamine uptake activity of each colony was examined. One mutant named *E. coli* KK313, which was deficient in spermidine uptake and had a 30% decreased putrescine uptake activity, was isolated from 100 small colonies. The existence of two putrescine transport systems has been suggested in *E. coli* K12 grown in a low osmolarity medium (Munro et al., 1974). Thus, *E. coli* KK313 was treated again with *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine. *E. coli* NH1596, which was deficient in spermidine transport and had a 90% decreased putrescine uptake activity, was isolated from 100 small colonies. The polyamine content of the polyamine transport-deficient mutants was then measured. When putrescine was added to the medium, large amounts of putrescine were accumulated in *E. coli* KK313, whereas no significant amounts of spermidine were accumulated in *E. coli* KK313 by the addition of spermidine. In *E. coli* NH1596, as expected, small amounts of putrescine only were accumulated, not spermidine (Kashiwagi et al., 1990).

We tried to isolate the clones for the genes of the polyamine transport systems using *E. coli* NH1596 as a host strain and pACYC184 as a vector. Three independent clones were isolated from 5000 transformants and named pPT104, pPT79 and pPT71, according to the number of nucleotides of the inserts in 100 bases. These clones were mapped at 15, 19 and 16 min, respectively, on *E. coli* chromosome. Table 1 shows the kinetic parameters of polyamine uptake by *E. coli* NH1596 carrying these clones. The K_m values for

Table 1. The *Km* and *Vmax* values of polyamine transport

Cell	Polyamine	*Km* (μM)	*Vmax* (nmol/min/ mg protein)
NH1596[a]/ pPT104 (15 min)[b]	PUT	1.5	19.8
	SPD	0.10	14.9
NH1596/ pPT79 (19 min)	PUT	0.50	22.1
NH1596/ pPT71 (16 min)	PUT	1.8	3.4

[a] *E. coli* polyamine transport-deficient mutant.
[b] Position on *E. coli* chromosome.

putrescine uptake were in the order pPT71 > pPT104 > pPT79, and the V_{max} values were in the order pPT79 > pPT104 > pPT71. The putrescine uptake activity by the system encoded by pPT71 was considerably lower than the activities by the other two systems. Spermidine uptake activity was only observed in the transformant containing the pPT104 clone. The K_m value for the spermidine uptake (0.1 μM) was 1 order lower than that for putrescine (1.5 μM), and the V_{max} value was slightly smaller than that for putrescine (Kashiwagi et al., 1990).

Nucleotide sequence of the genes encoding polyamine transport proteins and characteristics of the deduced polyamine transport proteins

We determined the nucleotide sequence of three clones (Fig. 2). The spermidine-preferential uptake system encoded by pPT104 consisted of four proteins (potA, -B, -C and -D proteins), and the calculated molecular masses of the four proteins were 43 kDa, 31 kDa, 29 kDa and 39 kDa, respectively (Furuchi et al., 1991). Similarly, the putrescine-specific uptake system encoded by pPT79 consisted of four proteins (potF, -G, -H and -I proteins), and the calculated molecular masses of the four proteins were 41 kDa, 45 kDa, 35 kDa and 31 kDa, respectively (Pistocchi et al., 1993). In contrast, the putrescine transport system encoded by pPT71 consisted of one membrane protein (potE protein) having twelve transmembrane segments and had a calculated molecular mass of 46 kDa (Figs. 2 and 3) (Kashiwagi et al., 1991).

When the hydropathy of the proteins was evaluated, it became apparent that potB and -C proteins contained six putative transmembrane spanning segments linked by hydrophilic segments of variable length (Fig. 3). Models of the secondary structure of the potB and -C proteins are shown in Fig. 4. PotA and -D proteins do not contain significant amounts of hydrophobic segments (Fig. 3). When the amino acid sequence of potA protein was compared with that of the other proteins, consensus nucleotide-binding sequences were found in potA protein as observed in the sequences of α and β subunits of *E. coli* ATPase (Walker et al., 1982), hisP and malK proteins (Ames, 1986). Since both hisP and malK proteins are membrane-associated and constitute one of

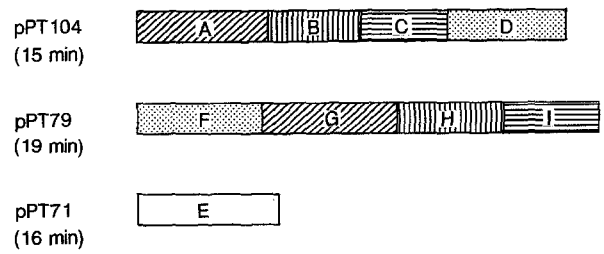

Fig. 2. Polyamine transport proteins encoded by pPT104, pPT79 and pPT71. The order and size of polyamine transport proteins on each clone was based on the nucleotide sequences of the genes encoding these proteins

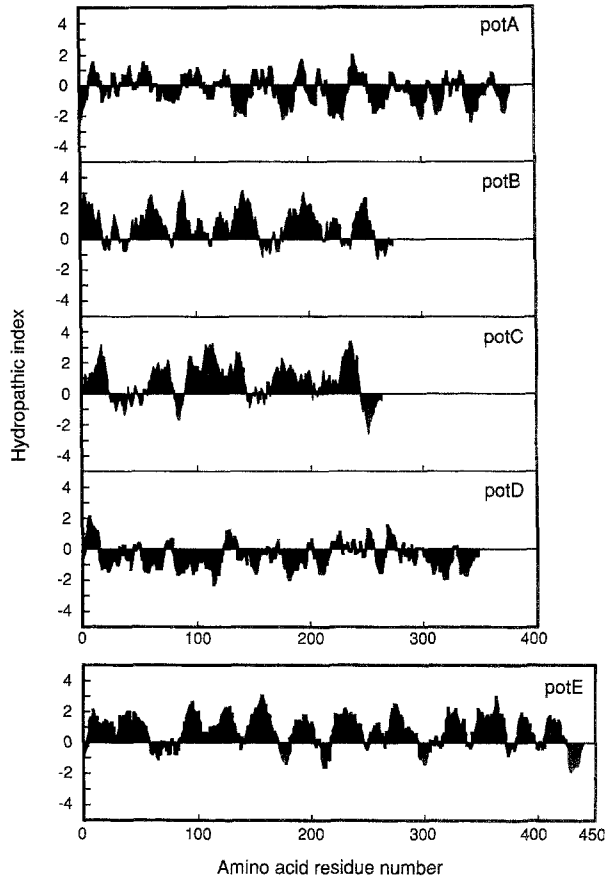

Fig. 3. Hydropathy profiles of potA, -B, -C and -D proteins encoded by pPT104, and potE protein encoded by pPT71

the components of the histidine and maltose transport systems, the intracellular localization of potA protein was determined by Western blot analysis. PotA protein existed mainly in the inner membrane fraction. The protein was much more strongly expressed in *E. coli* NH1596 carrying pPT104 than in *E. coli* NH1596.

Transport systems possessing a membrane-associated ATP binding protein also have a periplasmic substrate-binding protein (Ames, 1986). Therefore, we purified potD protein to homogeneity, and the amino-terminal amino acid sequence was determined by Edman degradation. The amino-terminal 30-amino acid sequence was exactly the same as the deduced 24th to 53rd amino acid sequence from the nucleotide sequence of *potD* gene. The results show that the processing site of potD protein by signal peptidase for destination to the periplasm is between Ala-23 and Asp-24. The existence of potD protein in a periplasmic fraction was confirmed by Western blot analysis. The amount of potD protein in *E. coli* NH1596/pPT104 was greater than that in *E. coli* NH1596 (Furuchi et al., 1991).

When the hydropathy of the proteins encoded by pPT79 was evaluated, it became apparent that potH and -I proteins contained six putative

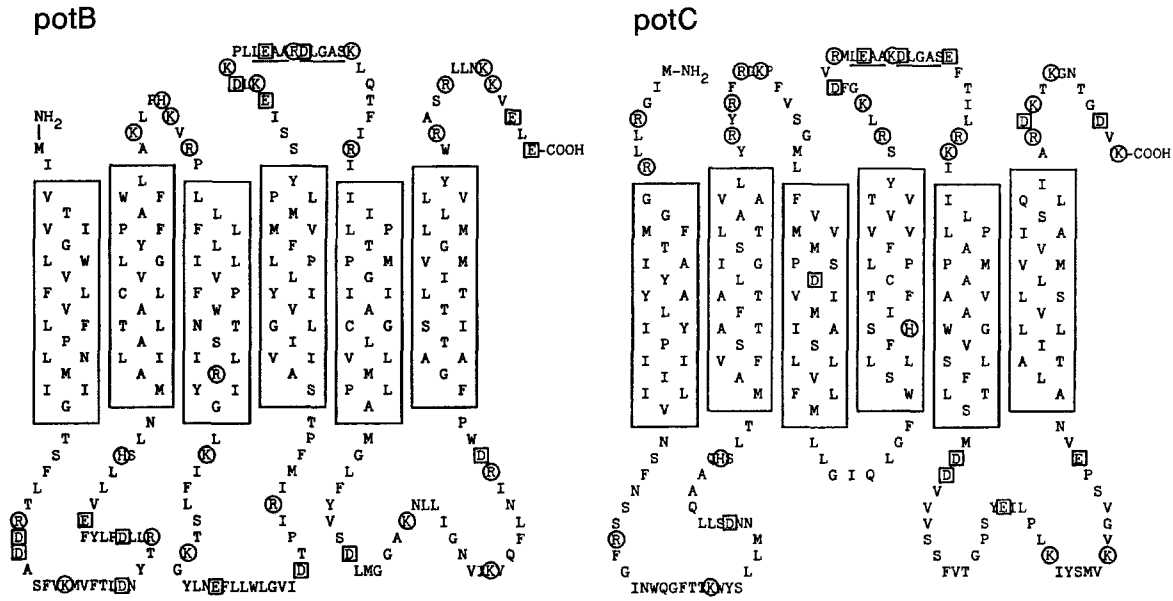

Fig. 4. Models of secondary structure of potB and -C proteins. Putative transmembrane segments are shown in large boxes. Homologous amino acid sequences between potB and -C proteins are underlined. Basic amino acids are shown in *circles*, and acidic amino acids are shown in *squares*

transmembrane spanning segments linked by hydrophilic segments of variable length. When the sequence homology of the proteins was analyzed, the percent homologies between potH and -B proteins, and between potI and -C proteins, were 37% and 36%, respectively, indicating that the transmembrane proteins in the two transport systems are very similar to each other. PotF and -G proteins do not contain significant amounts of hydrophobic regions. When the amino acid sequence of potG protein was compared with that of other proteins, consensus nucleotide-binding sequences were found in the potG protein. The percent homology between potG and -A proteins was 42%, indicating that potG protein is a membrane-associated protein like the potA protein in the spermidine-preferential uptake system. PotF protein was a periplasmic substrate binding protein like the potD protein. The processing site of potF protein was between Ala-26 and Ala-27, judging from the amino-terminal amino acid sequence of potF protein and the nucleotide sequence of the open reading frame of the *potF* gene. The percent homology between potF and -D proteins was 35% (Pistocchi et al., 1993).

Further characteristics of spermidine-preferential uptake system

In order to clarify whether the four proteins are absolutely necessary for spermidine uptake, the genes for the four proteins were disrupted by inserting the gene for kanamycin resistance. Spermidine uptake was not observed in *E. coli* MA261 *potA*::Km, MA261 *potB*::Km, MA261 *potC*::Km or MA261 *potD*::Km, in which one of the genes for the four proteins was disrupted. The

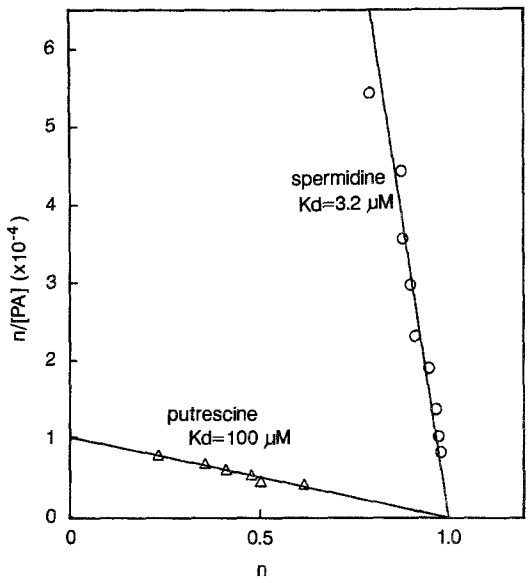

Fig. 5. Scatchard plot of polyamine binding to potD protein. n, polyamine bound to potD protein; [*PA*], concentration of polyamine

transformation of plasmid containing a gene corresponding to the disrupted one into these cells recovered spermidine uptake activity. The results indicate that all proteins are necessary for spermidine uptake.

The function of potD protein was studied in detail (Kashiwagi et al., 1993). First, the polyamine dissociation constants of potD protein were measured by gel filtration under the condition of 1 mM Mg^{2+} and 100 mM K^+ at pH 7.5. As shown in Fig. 5, the dissociation constants of spermidine and putrescine for potD protein were 3.2 and 100 μM, respectively. These values reflect the uptake affinity of spermidine and putrescine through the spermidine-preferential uptake system in intact cells (Kashiwagi et al., 1990). There was a single binding site for polyamines on potD under these conditions.

PotD protein dependency on spermidine uptake was examined using right-side-out membrane vesicles. When membrane vesicles were prepared from the cells containing the plasmid for *potABCD* genes, they had significant uptake activity, and the addition of potD protein did not stimulate spermidine uptake strongly (data not shown), probably due to the association of potD protein with membrane vesicles. When membrane vesicles were prepared from the cells containing the plasmid for *potABC* genes, spermidine uptake with membrane vesicles became potD protein dependent (Fig. 6). The optimal concentration of potD protein was 5 μM when the assay was carried out using 10 μM spermidine as substrate.

The function of potA protein was then studied in detail (Kashiwagi et al., 1993). ATP dependency for spermidine uptake was first examined using the proton-translocating ATPase mutant *E. coli* DK8. ATP content in the cells was normal (about 1 mM) only when the cells were energized with glucose. As shown in Fig. 7A, spermidine uptake was only observed with cells energized by glucose but not by succinate. When membrane potential was extinguished

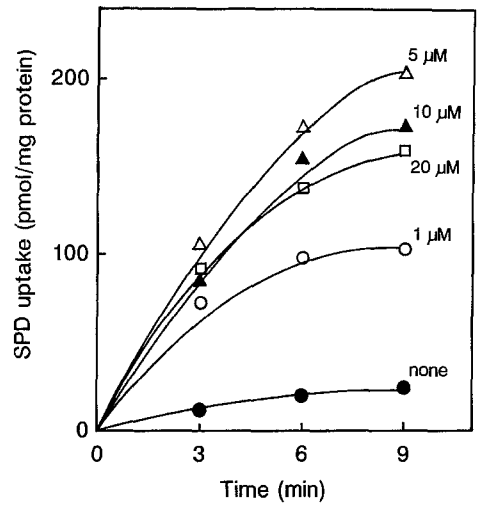

Fig. 6. Effect of potD protein on spermidine uptake by rightside-out membrane vesicles. Various concentrations (1 to 20μM) of potD protein was added to the reaction mixture

by CCCP, the uptake activity was decreased by 60% even if ATP existed and the change in pH was small. The results indicate that ATP is essential for spermidine uptake and that membrane potential is also involved in the uptake. Proline uptake, which is known to be membrane potential dependent, was measured as a control (Fig. 7B). Proline uptake was observed in the ATP-depleted cells and in the cells energized by glucose and succinate. However, the uptake was completely inhibited by CCCP.

Next, we examined whether potA protein could react with the photoaffinity labeling reagent 8-azido-ATP. After the overexpression of

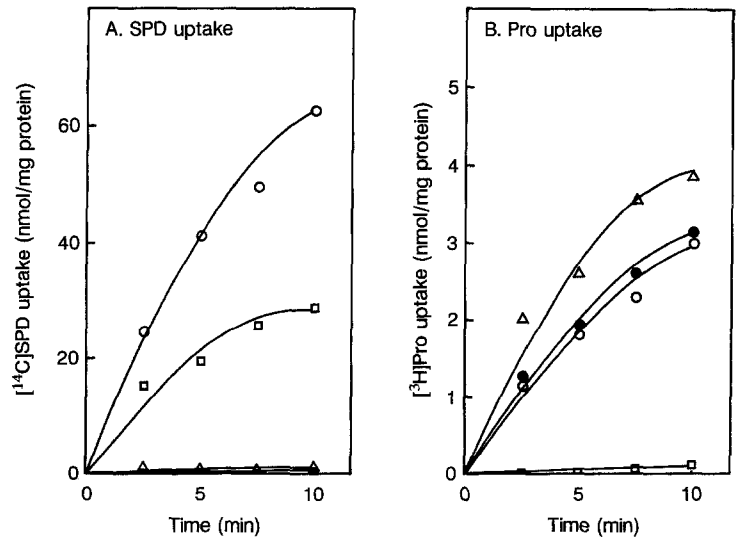

Fig. 7. Energy dependent spermidine (**A**) and proline (**B**) uptake by the *atp* mutant cells. ○, 0.4% glucose; △, 0.5% succinate; □, 0.4% glucose and 40μM CCCP; ●, non-treated cells

potA, -B, and -C proteins on membrane vesicles prepared from *E. coli* cells containing pKK*potABC* was confirmed, the membrane proteins were photolabeled with 8-azido-[α-^{32}P]ATP. PotA protein was clearly photolabeled, but not potB and -C proteins. However, the addition of 1 mM ATP together with 8-azido-[α-^{32}P]ATP inhibited the photolabeling of potA protein. When membrane vesicles were prepared from cells transformed with pKK*potA*::Km*BC* instead of pKKpotABC, the photolabeling of potA protein disappeared. These results strongly suggest that potA is involved in the energy-coupling step in the spermidine-preferential uptake system. The nucleotide specificity of binding to potA protein was examined by adding competing nucleotides during the photoaffinity reaction. The degree of inhibition of 8-azido-[α-^{32}P]ATP labeling of potA protein was in the order ATP > GTP = ADP > CTP = UTP.

ATPase activity of potA protein was examined using purified potA protein (Kashiwagi et al., 1995). The activity was dependent on Mg^{2+}, and 10 mM Mg^{2+} was necessary to obtain the maximal activity (Fig. 8A). The ATPase activity was strongly inhibited by spermidine and the function of Mg^{2+} could not be replaced by spermidine (Fig. 8B). Since spermidine uptake was inhibited by the already accumulated spermidine, the inhibition may be in operation during this process. The specific activity was approximately 400 nmol/min/mg protein and the K_m value for ATP was 385 μM. Since spermidine uptake was inhibited by NEM and PCMB, it was suggested that a sulfhydryl group is involved in the uptake (Kashiwagi et al., 1986). The ATPase activity of potA protein was also inhibited by NEM and PCMB, and the inhibition was restored by the addition of dithiothreitol (DTT). The results suggested that one of the three cysteines in the potA protein may be involved in the spermidine uptake.

PotA protein contains three molecules of cysteines (C26, C54 and C276). To identify which cysteine is involved in the ATPase activity of potA protein, cysteines 26, 54 and 276 were converted to alanine, threonine and alanine, respectively, using site-directed mutagenesis on *potA* gene. ATPase activity was measured using inside-out membrane vesicles prepared from *E. coli*

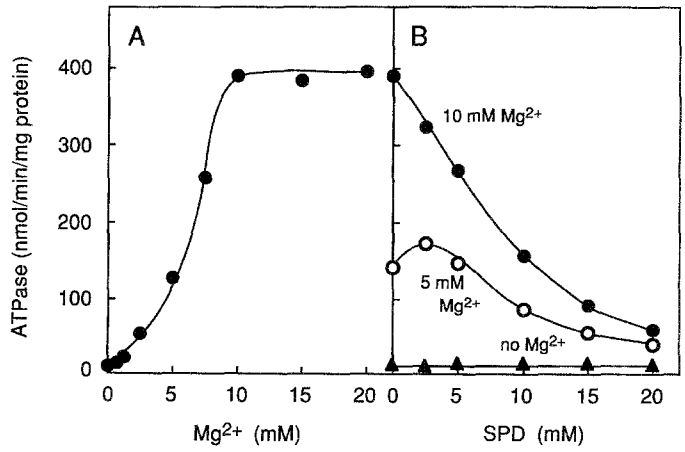

Fig. 8. Effect of Mg^{2+} (**A**) and spermidine (**B**) on ATPase activity of potA protein

DK8*atp*$^-$/pKK*potABC*, in which *potA* gene was modified by site-directed mutagenesis. Only potA3 (C54T) protein did not have ATPase activity. The other proteins A2 (C26A) and A4 (C276A), showed the ATPase activity. When another potA3* (C54A) protein was made, the vesicles containing the protein did not show any ATPase activity, in spite of its small amount (rapidly degraded).

The ATP binding site on potA protein was determined by photoaffinity-labeling with 8-azido-ATP. The photoaffinity-labeled potA protein was digested with trypsin, and the sequence of peptide labeled with 8-azido-ATP was determined by Edman degradation. It was found that cysteine 26 was photoaffinity-labeled with 8-azido-ATP. With regard to potA2 protein (C26A), threonine 39 may be photoaffinity-labeled with 8-azido-ATP instead of cysteine 26.

We also analyzed the properties of our mutated potA (potA1) protein expressed in *E. coli* NH1596, a spermidine uptake-deficient mutant (Kashiwagi et al., 1993). In potA1 protein, valine 135, which is located between two consensus amino acid sequences for nucleotide binding, was replaced by methionine. Among the nucleotide binding proteins in periplasmic transport systems, there are four other conserved sequences (sites C to F) in addition to two consensus sequences for nucleotide binding (sites A and B) according to the analysis by Ames (1986). The mutated position in potA protein corresponds to site C. Although purified potA1 protein was photolabeled with 8-azido-ATP, it did not show ATPase activity, indicating that potA1 protein is a mutant of ATPase activity similar to potA3 protein. The results taken together suggest that the active sites of ATP hydrolysis of potA protein are located both within and between the two consensus amino acid sequences for nucleotide binding (Fig. 9).

It was then tested whether membrane association of potA protein is influenced by the channel forming potB and -C proteins. Inside-out membrane

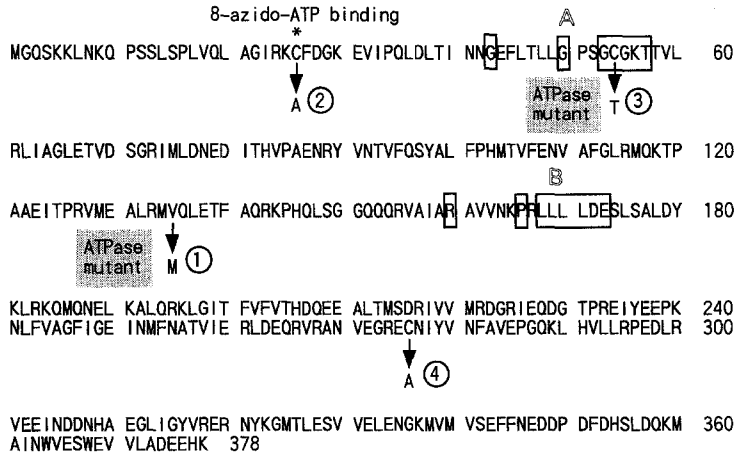

Fig. 9. Structure and function of potA protein. Consensus amino acid sequences for nucleotide binding (sites A and B), photoaffinity-labeled amino acid with 8-azido-ATP (C26) and ATPase-deficient mutants (C54T and V135M) are shown in the figure

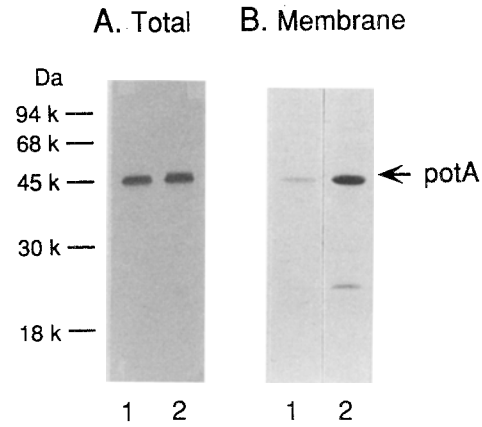

Fig. 10. PotA protein in total and inner membrane proteins. PotA protein was analyzed by Western blotting. *1, E. coli* JM105/pKK*potA*; *2, E. coli* JM105/pKK*potABC*

vesicles were prepared from *E. coli* JM105/pKK*potA* and JM105/pKK*potABC* cultured in the presence of IPTG. Although the total amount of potA protein was almost the same in both cells (Fig. 10A), the association of potA protein with membranes was greatly stimulated by potB and -C proteins (Fig. 10B). The results indicate that potA protein is associated with membranes through the interaction with potB and -C proteins.

Functions of potE protein

The putrescine transport system encoded by pPT71 consisted of one membrane protein (potE protein) having 12 transmembrane segments. The putrescine uptake was dependent on membrane potential rather than ATP, and the K_m value for putrescine was 1.8μM (Table 1). However, expression of th gene was strongly repressed at neutral pH.

We next examined whether potE protein possesses the ability to excrete putrescine by measuring the putrescine uptake into inside-out membrane vesicles (Kashiwagi et al., 1992). As shown in Table 2, putrescine uptake was clearly observed only with the membrane vesicles prepared from *E. coli* transformed with the 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 the inhibitors of energy production such as KCN and CCCP. The addition of D-lactate did not influence the putrescine uptake significantly. The uptake was not inhibited by spermine, spermidine or acetylated polyamines. These results indicate that there is an energy-independent putrescine excretion system catalyzed by potE protein.

Since energy was not necessary for the excretion of putrescine from *E. coli*, the question arose 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 mem-

Table 2. Effect of polyamine transport proteins, inhibitors of energy production, and polyamine analogs on putrescine uptake by inside-out membrane vesicles

Exp.	Plasmid in cells	Addition	Putrescine uptake	
			pmol/min/ mg protein	%
1.	pPT71	none	252	100
	pPT79	none	14	6
	pPT104	none	13	5
	none	none	17	7
2.	pPT71	none	274	100
	pPT71	CCCP (40μM)	283	103
	pPT71	KCN (10mM)	247	90
	pPT71	Spermine (0.2mM)	285	104
	pPT71	Spermidine (0.2mM)	288	105
	pPT71	N^1-Acetylspermine (0.2mM)	260	95
	pPT71	N^1-Acetylspermidine (0.2mM)	252	92
	pPT71	N^1-Acetylputrescine (0.2mM)	241	88

brane vesicles were prepared in the absence of amino acids, putrescine uptake was greatly diminished (Table 3). 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. 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 2.5mM

Table 3. Effect of amino acids on putrescine uptake by inside-out membrane vesicles

Exp.	Addition		Putrescine uptake	
	Inside	Outside	pmol/min/ mg protein	%
1.	20 amino acids + Orm	–	278	100
	18 amino acids (−Orn, Lys, Arg)	–	31	11
	Orn	none	230	83
	Lys	none	241	87
	Arg	none	89	32
	Glu	none	28	10
	none	none	39	14
2.	Orn	none	233	100
	Orn	Orn	23	10
	Orn	Lys	37	16
	Orn	Arg	175	75
	Orn	Glu	217	93

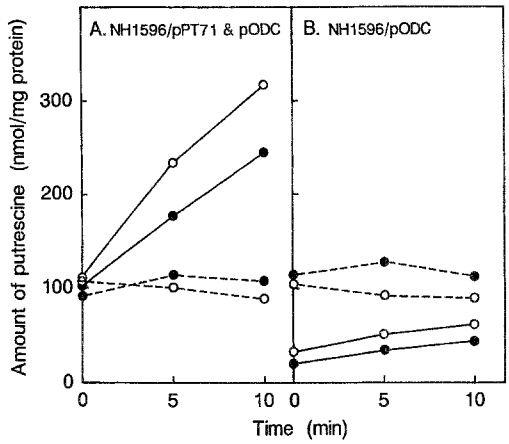

Fig. 11. Putrescine excretion into the medium by intact cells. *E. coli* NH1596 was transformed with pPT71 (*potE*) and pODC (*speC*) (**A**) or by pODC only (**B**). —, Putrescine in the medium; ----, putrescine in cells. The assays were performed in the presence (○) or absence (●) of 40 μM CCCP

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. Agmatine (decarboxylated arginine and a precursor of putrescine) also did not influence putrescine uptake. The K_m and V_{max} values for putrescine uptake by the inside-out membrane vesicles were 73 μM and 0.82 nmol/min/mg protein, respectively. The K_i values for ornithine and lysine were 208 μM and 1.26 mM, respectively. These results show that potE protein has an antiport activity between putrescine and ornithine (or lysine). The exchange ratio between putrescine and ornithine was 1 to 1.

To confirm that the potE protein is involved in excreting excess putrescine, putrescine excretion rates were measured in *E. coli* transformed with pODC (*speC*) containing a constitutive ornithine decarboxylase gene. In *E. coli* NH1596, in which genomic potE protein is expressed, slow but significant excretion of putrescine was observed (Fig. 11B). The excretion was not inhibited by CCCP. When *E. coli* NH1596 was transformed with pODC (*speC*) and pPT71 (*potE*), very rapid excretion of putrescine was observed and CCCP even slightly stimulated this rate (Fig. 11A). *E. coli* transformed with pPT104 or pPT79 did not show the rapid excretion of putrescine. When ornithine was removed from the medium, putrescine excretion was greatly diminished.

Further perspective

We have thus far clarified some properties of polyamine transport in *E. coli*. However, many characteristics still remain to be clarified. Detailed analysis of the functional domains of each protein (potA, -B, -C, D and -E proteins) are now in progress using the mutated proteins prepared by site-directed mutagenesis and X-ray analysis of crystal proteins. Regulation of the gene

expression of polyamine transport genes, especially negative regulation by polyamines, should also be clarified.

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