

REVIEW ARTICLE

Polyamine transport in bacteria and yeast

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The polyamine content of cells is regulated by biosynthesis, degradation and transport. In *Escherichia coli*, the genes for three different polyamine transport systems have been cloned and characterized. Two uptake systems (putrescine-specific and spermidine-preferential) were ABC transporters, each consisting of a periplasmic substrate-binding protein, two transmembrane proteins and a membrane-associated ATPase. The crystal structures of the substrate-binding proteins (PotD and PotF) have been solved. They consist of two domains with an alternating β - α - β topology, similar to other periplasmic binding proteins. The polyamine-binding site is in a cleft between the two domains, as determined by crystallography and site-directed mutagenesis. Polyamines are mainly recognized by aspartic acid and glutamic acid residues, which interact with the NH_2 - (or NH -) groups, and by tryptophan and tyrosine residues that have hydrophobic

interactions with the methylene groups of polyamines. The precursor of one of the substrate binding proteins, PotD, negatively regulates transcription of the operon for the spermidine-preferential uptake system, thus providing another level of regulation of cellular polyamines. The third transport system, catalysed by PotE, mediates both uptake and excretion of putrescine. Uptake of putrescine is dependent on membrane potential, whereas excretion involves an exchange reaction between putrescine and ornithine. In *Saccharomyces cerevisiae*, the gene for a polyamine transport protein (TPO1) was identified. The properties of this protein are similar to those of PotE, and TPO1 is located on the vacuolar membrane.

Key words: ABC transporter, putrescine, putrescine/ornithine antiporter, spermidine, spermine.

INTRODUCTION

Polyamines (putrescine, spermidine and spermine) are necessary for cell growth [1–3]. They are among the major polycations in cells, together with Ca^{2+} and Mg^{2+} . Polyamines and Mg^{2+} , which are present in higher free concentrations than Ca^{2+} , can bind to intracellular polyanions such as nucleic acids and ATP to modulate their function. The polyamine content of cells is regulated by biosynthesis, degradation and transport. In animal cells, uptake of polyamines can increase during hormonal stimulation and cell proliferation [4–8]. Furthermore, although different polyamines appear to share the same transport system(s), multiple polyamine uptake systems exist [9]. In *Escherichia coli*, polyamine uptake is energy-dependent, and the putrescine transport system is different from the spermidine and spermine transport system [10,11]. Furthermore, two apparently distinct transport systems for putrescine have been described in *E. coli* grown in a low-osmolarity medium [12]. In recent years the genes encoding polyamine transporters in *E. coli* and yeast [13,14] have been identified, and some of the properties of these transporters have been studied at a biochemical, molecular and structural level.

GENES RELATED TO POLYAMINE METABOLISM IN *E. coli*

Polyamines stimulate growth of *E. coli*. There are many polyamine-related genes in *E. coli* (Table 1). Approximately half of them encode enzymes involved in the biosynthesis or degradation of polyamines, and the remainder are genes for polyamine-transport mechanisms. They occupy about 0.6% of the total number of genes and chromosomes in *E. coli*. This observation indirectly supports the idea that polyamines and polyamine regulation play important roles in *E. coli*. The genes involved in

polyamine metabolism are shown in Figure 1. Since cadaverine and aminopropylcadaverine can have functions similar to putrescine and spermidine [15], the genes involved in the synthesis of cadaverine are also shown in Figure 1. It is noted that two genes exist in the first step of biosynthesis of polyamines: those are *speC* and *speF* in the synthesis of putrescine from ornithine, *speA* and *adiA* in the synthesis of agmatine from arginine, and *ldcC* and *cadA* in the synthesis of cadaverine from lysine.

GENERAL CHARACTERISTICS OF THE POLYAMINE UPTAKE SYSTEMS

The rate of polyamine uptake in *E. coli* is in the order: putrescine > spermidine > spermine [11]. Uptake is mainly catalysed by two polyamine-uptake systems: one is a putrescine-specific system and the other is spermidine-preferential system (Figure 2). Both systems are ABC (ATP binding cassette) transporters [16] consisting of a substrate-binding protein in the periplasm, two channel-forming proteins and a membrane-associated ATPase that is involved in energy supply. The operon encoding the putrescine-specific uptake system was mapped to 19 min on the *E. coli* chromosome and encodes four proteins: PotF (the substrate-binding protein), PotG (an ATPase), and PotH and PotI (channel-forming proteins). The operon for the spermidine-preferential uptake system was mapped to 26 min on the *E. coli* W3110 chromosome (or 15 min of *E. coli* DR112 chromosome) and encodes PotA (an ATPase), PotB and PotC (channel-forming proteins) and PotD (a substrate-binding protein) [13,17,18]. The K_m value for putrescine in the putrescine-specific uptake system was $0.5 \mu\text{M}$, and the K_m values for spermidine and putrescine in the spermidine-preferential uptake system were 0.1 and $1.5 \mu\text{M}$ respectively [13].

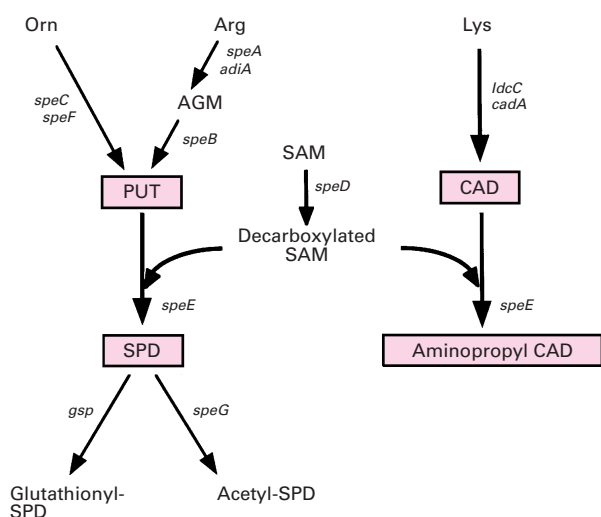
Abbreviation used: ABC, ATP binding cassette.

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Table 1 Polyamine-related genes in *E. coli*

Gene	Length (kbp)	Enzyme	Direction*	Location (min)	Ref.
<i>IdcC</i>	2.1	Constitutive lysine decarboxylase	+	0.5	[55]
<i>speE</i>	1.7	Spermidine synthase	-	2.9	[84]
<i>speD</i>		S-Adenosylmethionine decarboxylase			
<i>speF</i>	3.5	Inducible ornithine decarboxylase	-	15.5	[47]
<i>potE</i>		Putrescine/ornithine antiporter			
<i>potFGHI</i>	4.2	ATP-binding cassette putrescine-specific uptake system	+	19.3	[18]
<i>potABCD</i>	3.8	ATP-binding cassette spermidine-preferential uptake system	-	25.5	[17]
<i>speG</i>	0.6	Spermidine acetyltransferase	+	35.7	[85]
<i>speA</i>	3.1	Biosynthetic arginine decarboxylase	-	66.4	[86]
<i>speB</i>		Agmatinase (agmatine ureohydrolase)			
<i>speC</i>	2.2	Constitutive ornithine decarboxylase	-	66.9	[87]
<i>gsp</i>	1.9	Glutathionylspermidine synthetase/amidase	-	67.6	[88]
<i>yjdE</i>	1.4	Putative arginine/agmatine transporter	-	93.4	[55]
<i>adiA</i>	3.4	Inducible arginine decarboxylase	-	93.5	[89]
<i>adiY</i>		Putative AraC-type regulatory protein			[90]
<i>cadB</i>	3.6	Lysine/cadaverine antiporter	-	93.9	[54]
<i>cadA</i>		Inducible lysine decarboxylase			
<i>cadC</i>	1.6	Transcriptional activator of cad operon	-	94.0	[91]

* +, Clockwise; -, counterclockwise.

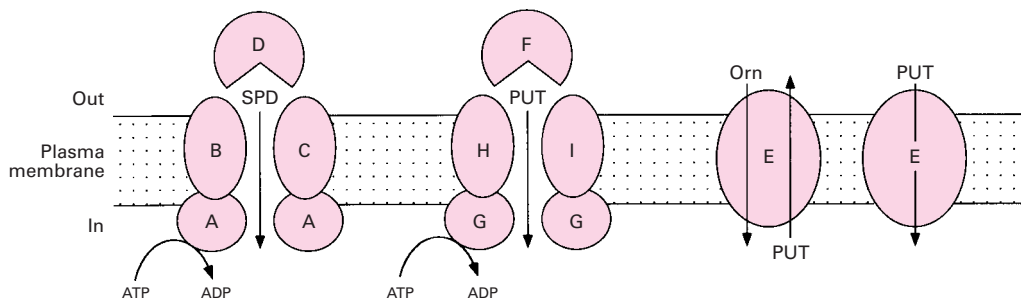
**Figure 1 Polyamine metabolism and related genes in *E. coli***

PUT, putrescine; SPD, spermidine; CAD, cadaverine; SAM, S-adenosylmethionine.

SPERMIDINE-PREFERENTIAL UPTAKE SYSTEM

To determine whether all four proteins are necessary for spermidine uptake in the PotA/B/C/D system, the genes for each protein were individually disrupted by inserting the gene for kanamycin resistance in place of the Pot protein. Spermidine uptake was not observed in *E. coli* in which any one of the four proteins was disrupted. Transformation with a plasmid containing an intact gene corresponding to the disrupted one restored spermidine uptake activity. Thus all four proteins (PotA, PotB, PotC, and PotD) are necessary for spermidine uptake.

The calculated molecular masses of PotA, PotB, PotC, and PotD were 43, 31, 29 and 39 kDa respectively [17]. On the basis of hydrophobicity analysis, PotB and PotC contain six putative transmembrane segments linked by hydrophilic segments of variable length. PotA and PotD do not contain notable hydrophobic segments. When the amino acid sequence of PotA was compared with that of other proteins, a consensus nucleotide-binding sequence was found in PotA similar to that seen in the α and β subunits of *E. coli* ATPase [19], HisP and MalK proteins [20]. Both HisP and MalK are membrane-associated and constitute one component of the histidine and maltose transport systems. We therefore speculated that PotA may also be mem-

**Figure 2 Polyamine transport systems in *E. coli***

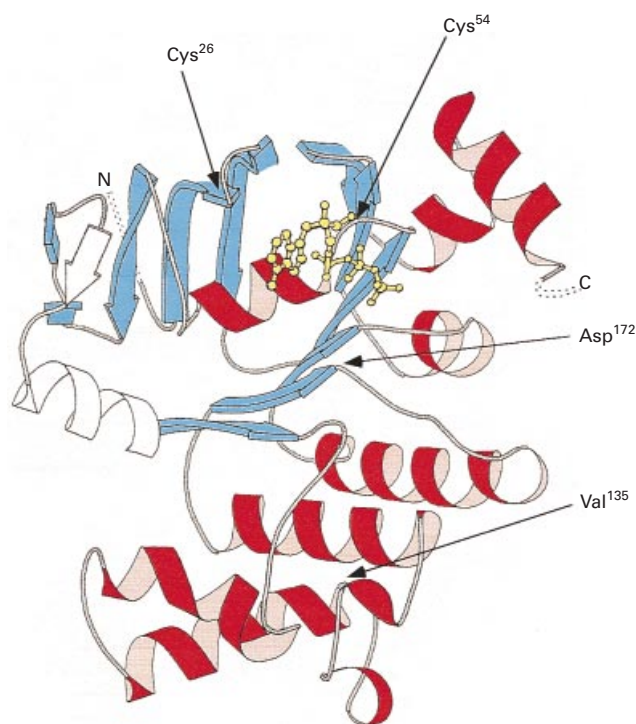


Figure 3 A model of the ATP-binding domain of PotA

The Figure shows a projected structure of HisP based on the known crystal structure of HisP (modified from reference [23] and reproduced with the permission of the authors and publishers). The predicted positions of α -helices and β -sheets in PotA were determined by computer modelling using the LIBRA 1 program [92]. White α -helix and β -sheet in the left exist in HisP but not PotA. Other α -helices and β -sheets exist in both HisP and PotA. ATP is shown in yellow. Reprinted with permission from Nature [23] © 1998 Macmillan Magazines Limited.

brane-associated, and, indeed, it was shown that PotA exists mainly in the inner-membrane fraction [17]. PotA was associated with membranes through the interaction with PotB and PotC [21].

As PotA was suggested to be involved in the energy-coupling step, like HisP and MalK [20], the ATP-dependency of spermidine uptake was examined. ATP was found to be essential for spermidine uptake [22]. To confirm that PotA is indeed an ATP-binding protein, we examined whether PotA could react with the photoaffinity labelling reagent 8-azido-ATP. Using membrane vesicles overexpressing PotA, PotB and PotC, membrane proteins were allowed to react with 8-azido- $[\alpha\text{-}^{32}\text{P}]\text{ATP}$. Only the PotA protein was clearly photolabelled with ATP. The nucleotide specificity of binding to PotA was in the order: $\text{ATP} > \text{GTP} = \text{ADP} > \text{CTP} = \text{UTP}$ [22]. These results indicate that PotA is involved in the energy-coupling step in the spermidine-preferential uptake system.

The ATPase activity of PotA was studied using purified PotA [21]. The specific activity was approx. 400 nmol/min per mg of protein, and the K_m value for ATP was 385 μM . The ATP-binding site on PotA was determined by photoaffinity-labelling with 8-azido-ATP, and it was found that Cys²⁶ was photoaffinity-labelled with 8-azido-ATP. Amino acid residues involved in the ATPase activity were then identified: these were Cys⁵⁴, Val¹³⁵ and Asp¹⁷². The results indicate that the amino acid residues necessary for ATP hydrolysis of PotA are located both within and between the two consensus amino acid sequences for

nucleotide binding (GPSGC⁵⁴GKT and LLLLD¹⁷²E). Recently, the crystal structure of HisP has been reported [23]. Since the similarity in amino acid sequence between HisP and PotA is high, the important amino acid residues in PotA are tentatively shown on the HisP structure (Figure 3). Although Cys⁵⁴ and Asp¹⁷² are located at the ATP-binding domain, the position of Val¹³⁵ is distant from the ATP-binding domain, suggesting that Val¹³⁵ may be important for the structure of the active site of ATP hydrolysis.

PotD was purified to homogeneity, and the N-terminal sequence was determined by Edman degradation. The processing site of PotD by signal peptidase was between Ala²³ and Asp²⁴. Dissociation constants of spermidine and putrescine for purified PotD under the condition of 1 mM Mg²⁺ and 100 mM K⁺ at pH 7.5 were 3.2 and 100 μM respectively. These values reflect the uptake system in intact cells. There was a single binding site for spermidine or putrescine on PotD, and spermidine uptake was shown to be PotD-dependent using right-side-out membrane vesicles.

STRUCTURE OF PotD

The polyamine-binding site on PotD was localized and characterized by X-ray crystallography of a PotD-spermidine complex

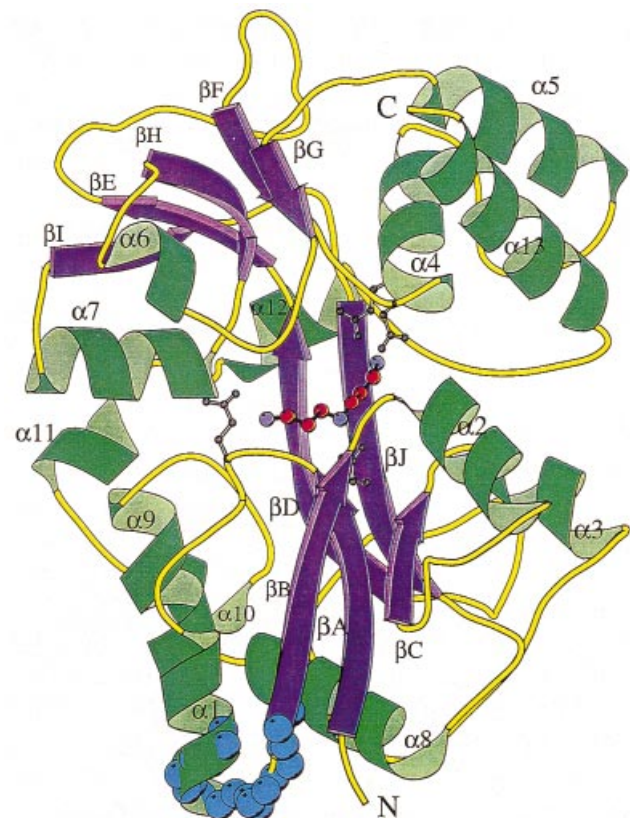


Figure 4 Structure of PotD.

The N domain lies at the bottom, and the C domain is at the top. The α -helices are green, the β -strands are purple, the coils and loops are yellow, and the conserved sequence motif observed in maltodextrin-binding protein and PotD is mid-blue. Spermidine is bound to the central cleft between the two domains. The binding site for the spermidine molecule is marked by the side chains of the four acidic residues.

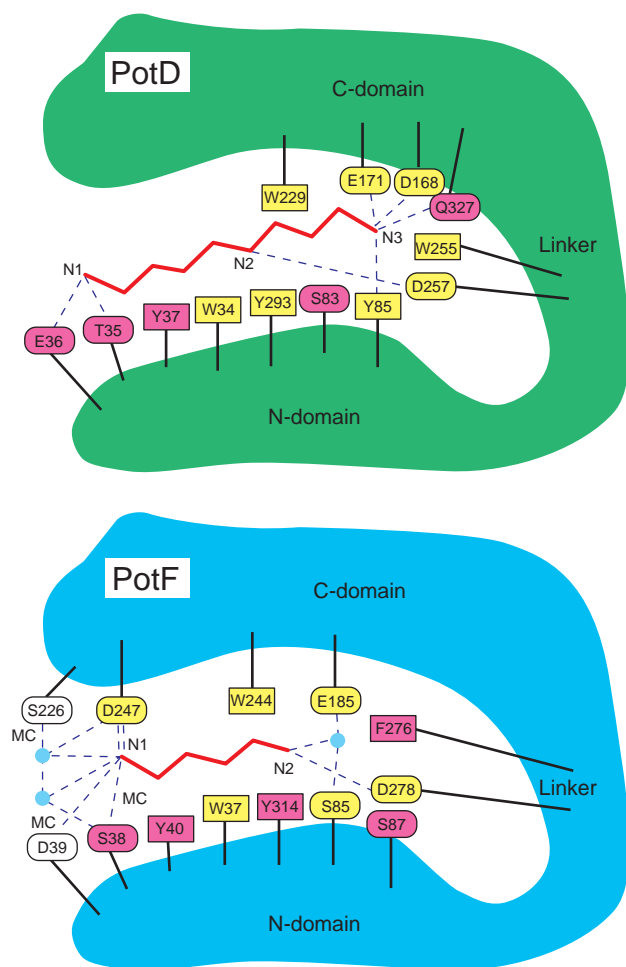


Figure 5 Schematic drawing of PotD (green) and PotF (mid-blue)

The polar and hydrophobic residues are drawn as ellipses and rectangles respectively. The most crucial residues from the structural and mutational analyses are coloured yellow, and the other residues, which affect substrate binding, are coloured pink. Substrates are shown as red zig-zag lines. The polar interactions between proteins and substrates are shown as broken blue lines. Putrescine binding to PotF Ser²²⁶ (S226) and Asp³⁹ (D39) is shown as white ellipses, as their mutations did not strongly affect putrescine binding to PotF. The water molecules are shown as pale-blue circles. MC, main chain.

[24,25] and by site-directed mutagenesis of the *potD* gene [26]. In this section we review the results of those studies and compare the structure of PotD with other bacterial periplasmic binding proteins.

Crystal structures of several periplasmic binding proteins have been solved by X-ray analysis. The proteins are specific for various substrates, such as amino acids (lysine/arginine/ornithine [27,28], leucine/isoleucine/valine [29], leucine [30] and glutamine [31]), di- and oligo-peptides [32,33], tetrahedral oxy-anions (sulphate [34] and phosphate [35]), and saccharides (arabinose [36], galactose/glucose [37], ribose [38], maltodextrin [39,40], and D-allose [41]). These binding proteins, which have a remarkable range of substrate specificities, show very little sequence similarity at the amino acid or nucleotide level, but they do share a similar main-chain fold. Furthermore, all of these proteins have a similar tertiary structure, consisting of two domains that show an 'opening-closing' movement, likened to a Venus flytrap, which is depending upon substrate binding [40].

The PotD protein also consists of two domains with alternating β - α - β topology [24] with the polyamine binding site located in a central cleft between these domains (Figure 4). Four acidic residues recognize the three positively charged nitrogen atoms of spermidine, and five aromatic side chains anchor the methylene backbone by van der Waals interactions (Figure 5). The overall fold of PotD is similar to that of other periplasmic binding proteins, in particular to the maltodextrin-binding protein from *E. coli* [39], even though the sequence identity is low. The crystal structure of PotD in the absence of spermidine is not known. However, a comparison of the PotD-spermidine structure with that of the maltodextrin-binding protein, determined in the presence and absence of its substrate, suggests that binding of spermidine rearranges the relative orientation of the PotD domains to create a more compact structure.

We also analysed the polyamine-binding site on PotD using mutated PotD proteins [26]. It was found that 13 amino acid residues were involved in binding spermidine (Figure 5). Among these residues, Glu¹⁷¹, Trp²⁵⁵, and Asp²⁵⁷ were the most important for binding spermidine, Trp³⁴, Tyr⁸⁵, Asp¹⁶⁸, Trp²²⁹ and Tyr²⁹³ had moderate contributions and the other five amino acids made weak contributions to binding of spermidine (Figure 5). The dissociation constants of spermidine for PotD mutated at Glu¹⁷¹, Trp²⁵⁵ and Asp²⁵⁷ increased greatly compared with the other mutants. Since these three residues interact with the diamino-propane moiety of spermidine, the results are in accordance with the finding that PotD has a higher affinity for spermidine than for putrescine. Similarly, using PotD mutants, putrescine was found to bind at the position of the diamino-butane moiety of spermidine.

PUTRESCINE-UPTAKE SYSTEM

The putrescine-uptake system, similar to the spermidine-preferential uptake system, consisted of PotF (a 38 kDa putrescine-binding protein), PotG (a 45 kDa membrane-associated ATPase) and PotH and PotI (31–35 kDa channel-forming proteins). The corresponding proteins of the two polyamine uptake systems, F and D, G and A, H and B, and I and C, had showed 35–42% sequence homology. By making several subclones and a mutant lacking the *potF* gene, we showed that the expression of all four proteins (PotF, PotG, PotH and PotI) was necessary for maximal putrescine transport activity.

The crystal structure of a PotF-putrescine complex has been solved. The crystal structure, together with studies of mutated PotF proteins, has provided detailed information about the putrescine-binding site of PotF [42]. The structure of PotF was reminiscent of other periplasmic substrate-binding proteins, with the highest structural similarity to that of PotD (Figure 6). Putrescine was tightly bound in a deep cleft between the two domains of PotF. The PotF structure, in combination with the mutational analysis, revealed the residues crucial for putrescine binding (Trp³⁷, Ser⁸⁵, Glu¹⁸⁵, Trp²⁴⁴, Asp²⁴⁷ and Asp²⁷⁸) and the importance of water molecules for putrescine recognition (Figure 5).

POLYAMINE BINDING TO PotD AND PotF

PotD can recognize not only spermidine, but also putrescine, with lower affinity. However, PotF recognizes putrescine specifically. Among the 13 amino acid residues that were shown to be important for spermidine binding to PotD, seven residues are absolutely conserved in PotF. They are Trp³⁷, Tyr⁴⁰, Ser⁸⁵, Glu¹⁸⁵, Trp²⁴⁴, Asp²⁷⁸ and Tyr³¹⁴ in PotF. All of these residues are crucial for the activity of PotF. In addition, Asp²⁴⁷ in the C-

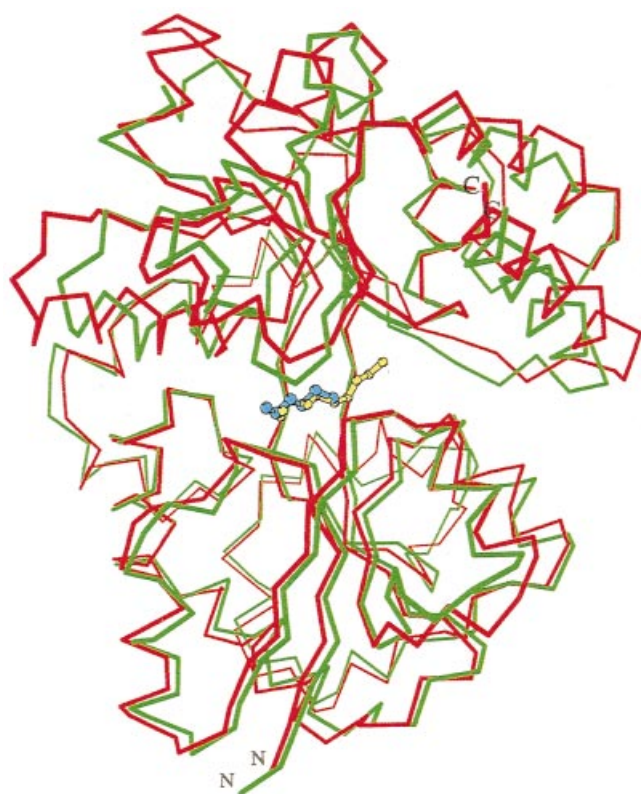


Figure 6 Comparison of PotD and PotF

The PotD molecule (green) is superimposed on the PotF molecule (red). Spermidine bound to PotD and putrescine bound to PotF are shown as yellow and blue ball-and-stick models respectively.

domain of PotF was strongly involved in the recognition of one of the amino groups (N1) of putrescine. Surprisingly, mutations at Ser⁸⁵ and Glu¹⁸⁵, which do not directly contact putrescine, substantially reduced the activity of PotF. They probably interact with the amino group (N2) of putrescine through a water molecule (Figure 5). The presence of this water molecule may be important, as it makes a barrier for putrescine in the binding cleft. Displacement of the water molecule would facilitate penetration of putrescine deeper into the cleft and would disturb the interactions of the substrate with PotF. The carboxyl oxygen atoms of Glu¹⁸⁵ form a strong hydrogen bond with Trp²⁴⁴, which stacks on the substrate. Therefore, a second possible role of Glu¹⁸⁵ may be to maintain the favourable orientation of the Trp²⁴⁴ side chain.

Two residues in PotD, Thr³⁵ and Glu³⁶, the side chains of which make hydrogen bonds with the N1 nitrogen of spermidine, are replaced by Ser³⁸ and Asp³⁹ in PotF. In contrast with PotD, putrescine makes hydrogen bonds with the main-chain carbonyl oxygens of these two residues (Figure 5). The side chain of Ser³⁸ interacts with the N1 nitrogen of putrescine through the water molecule. Moreover, in PotF, the carboxy oxygen atoms of Asp²⁴⁷ make both direct and water-mediated hydrogen bonds with the N1 atom of putrescine. Thus binding at the N1 site of putrescine in PotF is much stronger than that observed in PotD. This observation may explain the lower affinity of PotD than PotF for putrescine because the other interactions of the proteins with putrescine (i.e. the diamino-butane portion of spermidine) are very similar.

In PotF, the position of the N1 atom of putrescine is strictly fixed, whereas in PotD, the N1 atom of spermidine is more flexible. To understand whether this difference may prevent binding of spermidine to PotF, we made a docking model of the spermidine molecule with a fixed position of its N1 amino group. There was no conformation that lacked steric hindrance with some amino acid residues in the PotF-binding sites. Thus we presume that the substrate selectivity of PotF is dominated by the unique hydrogen-bond network with the N1 amino group, such that polyamines larger than putrescine cannot fit into the PotF-binding cavity.

In addition to PotD and PotF of *E. coli*, seven other candidate polyamine-binding proteins have been reported in bacteria [43–46]. In general, the amino acid sequences show very high similarity among these nine proteins (Figure 7). The 13 amino acid residues involved in the recognition of spermidine are shown above the sequences (#) in Figure 7. On the basis of amino acid similarity and the known structures of PotD and PotF, the first five proteins (nos. 1–5, including PotD) probably recognize spermidine, but the other four proteins (nos. 6–9, including PotF) possibly may not recognize spermidine.

In summary, the crystal structures of PotD and PotF provide the first insight into the molecular mechanisms by which proteins bind and discriminate different polyamines. The structural results, in combination with the mutational analyses, revealed that polyamine recognition could be achieved by the cooperation of multiple polar and hydrophobic interactions.

POLYAMINE TRANSPORT INVOLVING THE PotE PROTEIN

In *E. coli* there is a third polyamine transport system. This system involves the PotE protein. PotE is a 46 kDa protein containing 12 transmembrane segments linked by hydrophilic segments of variable length with the N- and C-termini located in the cytoplasm [47,48]. The gene for PotE (*potE*) together with the gene for inducible ornithine decarboxylase (*speF*) constitutes an operon [47].

PotE can catalyse both the uptake and excretion of putrescine (see Figure 2) [48,49]. Uptake of putrescine by PotE was dependent on the membrane potential, and the K_m value for putrescine was 1.8 μM . Substrate specificity of putrescine uptake by PotE was strict. Ornithine, cadaverine, spermidine, spermine and acetyl polyamines did not inhibit the uptake activity of PotE. In contrast, the excretion of putrescine was catalysed by a putrescine/ornithine antiporter activity of PotE. The K_m values for antiporter activities of putrescine and ornithine were 73 and 108 μM respectively. The exchange ratio between putrescine and ornithine was 1:1. Furthermore, a putrescine/putrescine or ornithine/ornithine exchange activity was also observed. The excretion of putrescine was increased by carbonyl cyanide *m*-chlorophenylhydrazone, which inhibits the membrane potential-dependent reuptake of putrescine and extinguishes the hyperpolarization caused by putrescine excretion.

To identify the amino acids involved in the transport activity of PotE, we studied PotE using site-directed mutagenesis. Uptake and excretion of putrescine decreased greatly with PotE mutants at Glu⁷⁷, Glu²⁰⁷, and Glu⁴³³. All three residues are located in the hydrophilic regions on the cytoplasmic side of PotE (Figure 8). These residues may contribute directly to a binding site for putrescine and/or ornithine on PotE. A putative PotE in *Haemophilus influenzae* has been reported [50] that contains acidic residues at positions equivalent to Glu⁷⁷, Glu²⁰⁷ and Glu⁴³³ in *E. coli* PotE. The main functional amino acids of the lactose/H⁺ antiporter [51,52] and metal tetracycline/H⁺ antiporter [53] are also located on the cytoplasmic side of the protein.

1. <i>E. coli</i> PotD	-----MKKWSRHLAAGALALG-----MSAAHAD---DNNTLYEYFN	33
2. <i>H. influenzae</i> Pot2	-----MNKLRKFFAHSKLNFLSITTA-----LFAASAF---ANNKLYVYN	37
3. <i>H. influenzae</i> Pot1	-----MKKFAGLITASFVAATLITACNDKD---AKQETAKATAA---ANDTVVLYT	44
4. <i>A. actinomycetemcomitans</i> PotD	-----MKKFAGLFTASMIAVALTGCNDKDNKQQAQATPEAPKAEAP---ANDTVVLYT	49
5. <i>P. haemolytica</i> Lpp38	-----MKKLAGLFAAGLATAVALLTACNEEK---AAEPKATAAEAKPQATNQTVHLVT	48
6. <i>P. fluorescens</i> PotD	MVLKQARSVTLRCYRSSLNIGISTCLSAW-----ITTATAD---TPIVHVYN	44
7. <i>E. coli</i> PotF	-----MTALNKKLWLSGIVAGALMAVS-----VGTLAEE---QKTLHIYN	36
8. <i>B. burgdorferi</i> PotD	-----MKKIFILIVLITFACTN-----KD---TITLVNFN	28
9. <i>T. pallidum</i> PotD	-----MKRFCVSSRIISLLFLSLWVG-----SCLQTR---QDVLYLYN	36
#### # #		
1. <i>E. coli</i> PotD	WTEYVPPGLLEQFVKETGKIVTYSTYESNETMYAKLKYKDG---AVDLVVPSTIYVYDKMR	91
2. <i>H. influenzae</i> Pot2	WFDVVPDGLVAQFVKETGIEVIYSTFESNEBMYAKLKLTONTGSYDVLVFPSSVYVAKMI	97
3. <i>H. influenzae</i> Pot1	WTEYVPPDGLLEDFVKETGKIVTVSSLESNETMYAKIKTQCESG-GYDVLVAPSNYFVSKMA	103
4. <i>A. actinomycetemcomitans</i> PotD	WTEYVPPDGLLEDFVKETGKIVTVSSLESNETMYAKIKTQCAAG-GYDVLVAPSNYFVSKMA	108
5. <i>P. haemolytica</i> Lpp38	WTEYVPPGLLEDFVKETGKIVTVSSLESNETMYAKLKLQCKDG-GYDVLVAPSNYFVSKMA	107
6. <i>P. fluorescens</i> PotD	WYDYIGPTLQDFKRETFEEVYDTFDSCEVLEAKLMT-GHS--GYDVVVASNFILPALI	101
7. <i>E. coli</i> PotF	WSDYIAPDTVANFVKETGKIVVYVDFDSNEVLEKGLMA-GST--GDLVVPASFTLERQL	93
8. <i>B. burgdorferi</i> PotD	WAEYIDKTLQDFKRNKININYEIETHNEEMAKFNNTKNY---YDITVPSBYLQDEL	85
9. <i>T. pallidum</i> PotD	WYVYTFPSLTKKFEQQYVNVQVVDYASNEEDMFAKL SIGASG---YDLVVPSSGDFVSIK	93
# #		
1. <i>E. coli</i> PotD	REGMIQKIDKSKLINESNLDPDMLN--KPFDPNDYSPVYIAGATATGVNGDAVD---PK	146
2. <i>H. influenzae</i> Pot2	KKMLQPTDQSKLINTHOIPKHLIN--KPFDPENKYSLPVYVYGLTGLEVNADEID---PK	152
3. <i>H. influenzae</i> Pot1	REGMLKELDHSKLEPVLKELDPDMLN--KPYDKGNKYSLPQLLGAFLAINTNYK---CE	158
4. <i>A. actinomycetemcomitans</i> PotD	REGMLKELDHSKLEPVLKELDPDMLD--KPYDKGNKYSLPQLLGAFLAINTNYK---CS	163
5. <i>P. haemolytica</i> Lpp38	REGMLAELDHAQLEPVLKELDQDMLN--KPYDQGNKYSLPQLLGAFLAINTADYK---CE	162
6. <i>P. fluorescens</i> PotD	KACALQKLDKSLPNLKHLIDPILLEKLRANDPNDQVAPVYVWCTDGI GYVNEKVRAILD	161
7. <i>E. coli</i> PotF	TAGVFPQIDKSKLEPEMANLDPELKLKVAKHDPDKFAMPYMWATTGTGYNVDKVKAVLCE	153
8. <i>B. burgdorferi</i> PotD	DEKTEKLDYKSLPNVTKNITQNLIN--LEHDPENLYSVPAYWGLMGLIYNKTKID---LN	141
9. <i>T. pallidum</i> PotD	RKHLLEKIDLSKIPNVQFIKESVRAR-IAVDRKMEYSVPYVYLGAAGLAVNKKAVP---S	148
# #		
1. <i>E. coli</i> PotD	--SVTSPADLWKPEYKGS-----LILLDDAREVFQALRKLKLYSGNTTDFKELHE-AAYNE	198
2. <i>H. influenzae</i> Pot2	--TITSPADLWKPEFKGK-----VLMTSDAREVFHVALLLDCKSPNTNEEDIK-TAYER	204
3. <i>H. influenzae</i> Pot1	--DFTSPADLWKPEFANK-----VQLLDDAREVFNTALPKIGQDPNTQDFAIK-QAYBE	210
4. <i>A. actinomycetemcomitans</i> PotD	--DFTSPADLWKPEFANK-----VQLLDDAREVFNTALPKIGQDPNTQDFAIK-QAYBE	215
5. <i>P. haemolytica</i> Lpp38	--TFTSPADLWKPEFACK-----VQLLDDAREVFNTALPKLGNPNTINPEDEIK-BAFBE	214
6. <i>P. fluorescens</i> PotD	QAPVNSMDLLEKLEENLAKLSQCGVGLDAPAEIMPALHYLGLPPNSSNPDDMK-KAVAL	220
7. <i>E. coli</i> PotF	NAPVDSDLLKPPNLEKLEKSCGVSLDAPAEVVFATVIANLIGKDPNSTRADYTGATDL	213
8. <i>B. burgdorferi</i> PotD	--DMQGFDIENKRYKKE-----ITMLDSFKDNIGVALRKLKYSINHEHDTKIK-BAGEL	193
9. <i>T. pallidum</i> PotD	--YARITWISIFSRKELAYR-----MSMMDMREVMGAALASLGYVNVNWKNEQELAQATLV	201
# *		
1. <i>E. coli</i> PotD	LKKLMPNVAAFNSDNPA--NFYMEGEVNLGMIVNGSAFVARQ-----AGTPILVWVMPK-E	250
2. <i>H. influenzae</i> Pot2	LEKLLPNVATFNSDSPB-VFVYVQGEVALIGMIVNGSAYLAOK-----ENQSLCFVWPK-E	256
3. <i>H. influenzae</i> Pot1	LLKLRPNVLSFNSDNPA--NSFISGEVEVGLWNGSVRIAKK-----EKAPLDMVWPK-E	262
4. <i>A. actinomycetemcomitans</i> PotD	LLKLRPNVLSFNSDNPA--NSFISGEVEVGLWNGSVRIAKK-----EQAPLDMVWPK-E	267
5. <i>P. haemolytica</i> Lpp38	LKLRPNVLSFNSDNPA--NSFIAGEVSVGLWNGSVRIAKK-----ENAPVDMVWPK-E	266
6. <i>P. fluorescens</i> PotD	MLKLRPNVITFNSKTIK--SDLANGDTCVALTMSGAVFGAKQAANEAGKGVKTEYSTIPV-E	278
7. <i>E. coli</i> PotF	LLKLRPNVIRVHSSQYK--NDLANGDTCVALTGACDVMQASNRRAKEAKGVNVSFSTIPK-E	271
8. <i>B. burgdorferi</i> PotD	LKIQNELLIGYFSDVPAKSLMLNGEASTQLTMSCEAQSAML-----KDKNLDVYAP-E	245
9. <i>T. pallidum</i> PotD	TDHAKPNLVKHDSDGYA-KSFASCFVVAHGFAEAFEAETP-----EAMHEHIDFFIPQDV	256
# #		
1. <i>E. coli</i> PotD	GCIFWMSLAI PANAKNKECAIKLINFLLRPDVAKOVAETIGYPTPNLAARLKLSPPEVAN	310
2. <i>H. influenzae</i> Pot2	GAIFWMDNYAIPITAKNVECAHKFIDELLRPENAKIVIPRMCFESMPNNGAKLILSAEVAN	316
3. <i>H. influenzae</i> Pot1	GPVLWVDLAI PANAKNSECARHLINNYMKGKTAERLTLAIGYPTNIBAKRLPKETITE	322
4. <i>A. actinomycetemcomitans</i> PotD	GPVLWVDLAI PVTSKNEDCAHKLINNYMKGAKAAEKLTLAIGYPTNIBAKRLPKETITE	327
5. <i>P. haemolytica</i> Lpp38	GPVLWVDLAI PANAKNKENAHLINYLLESAKVAEKTLAIGYPTSNVDAIKVLPKETITE	326
6. <i>P. fluorescens</i> PotD	GAPLWSDNTLVKDGSHPEHGLTFINMYLRPEVIAPATDLALTANANKDATALVSAEVND	338
7. <i>E. coli</i> PotF	GAMAFEPVFAFADAKNKDPAVQFLNYLLRPDVVAHISDHFVYANANKAATFVSAEVRE	331
8. <i>B. burgdorferi</i> PotD	NINLWIDAFVFPIDAFNKNLAKRFINFLYENPSPYKFKETRMNSKNKVIKRIIEEAKN	305
9. <i>T. pallidum</i> PotD	ASPVYVDSFCIPKGAARNRDLAHAFINFFLEPAHYAEFLDTFEPSTIHRERAAAYQKKT--	314
#		
1. <i>E. coli</i> PotD	DK--TLYPDABETKNGE-WQNDVGAASSTIYEBYQKTKAGR--	348
2. <i>H. influenzae</i> Pot2	DP--KLFPPABEVEKGI-MQGDVGEAVDIYERVWGLKRTN--	353
3. <i>H. influenzae</i> Pot1	DP--AIYPSADILKNSH-WQDDVGDALQFVEYVYQBLKAAK--	360
4. <i>A. actinomycetemcomitans</i> PotD	DS--AIYPSADVLCKSY-WQDEVDGALQVYEQYVYQBLKAAK--	365
5. <i>P. haemolytica</i> Lpp38	DT--AIYPSAEVLEKQSK-WQDDVGDALQFVEYVYQBLKAAK--	364
6. <i>P. fluorescens</i> PotD	NP--NIYPTAQLVISTLFTLQPSHAVDVRVTRFRWNSIKNGN--	377
7. <i>E. coli</i> PotF	NP--GIYPTADVRAKLFITLVQDPKIDRVTRRAWTKVKSQK--	370
8. <i>B. burgdorferi</i> PotD	NPENKLVLEEKFLKDFSKFEIFPKIKPKIKKEILKIYLNLS	348
9. <i>T. pallidum</i> PotD	-F----YVSEHDLERGT-LKT DVGAALAHNAHNAVRF--	348

Figure 7 Alignment of the deduced amino acid sequence of *potD* or *potF* gene products

The conserved amino acid residues are shown by black boxes with white lettering. The amino acid residues involved in the spermidine binding to PotD are indicated with a hash sign (#), and Asp²⁴⁷ involved in the putrescine binding to PotF is indicated with an asterisk (*). Accession numbers for the proteins 1–9 are gi:130691, 1172561, 1172560, 3341857, 1142681, 3097812, 1787078, 2688565 and 3322956 respectively. The full specific names of organisms not already mentioned are *Actinobacillus actinomycetemcomitans*, *Pasteurella haemolytica*, *Pseudomonas fluorescens* and *Borrelia burgdorferi*.

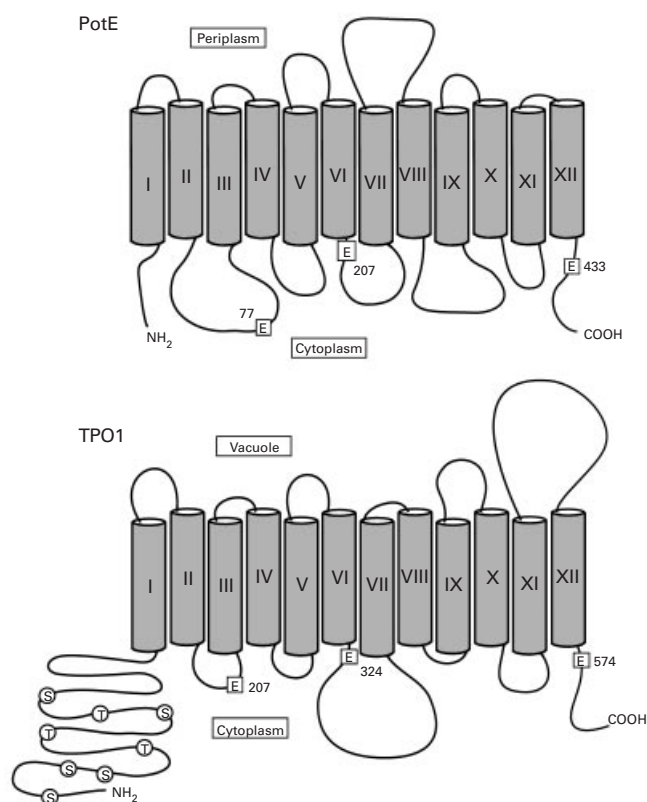


Figure 8 Model of secondary structure of PotE and TPO1

The putative 12 transmembrane segments are shown as columns. Glu⁷⁷, Glu²⁰⁷ and Glu⁴³³ are involved in both uptake and excretion of putrescine by PotE. Glutamic acid, serine and threonine residues which may be involved in the polyamine transport activity and the phosphorylation by protein kinase(s) in TPO1 are also shown.

In *E. coli*, there are two other antiporters for amino acids and their decarboxylated amines (i.e. the polyamine-like molecules cadaverine and agmatine). These are antiporters for lysine/cadaverine [54] and arginine/agmatine [55], which are expected to have cadaverine- and agmatine-uptake activities. The existence of multiple transport systems supports the hypothesis that polyamine contents are strictly regulated by transport in addition

to biosynthesis and degradation. The antiporters also play important roles in the generation of a protonmotive force [56], neutralization of low extracellular pH [54] and supply of CO₂ [57].

POLYAMINE TRANSPORT SYSTEMS IN OTHER BACTERIA

Genes for several kinds of putative polyamine transport systems have been described in bacteria, in addition to *E. coli*, in which the whole genome has been sequenced (Table 2). It is noteworthy that a polyamine transport system exists even in Archaea. Thus polyamines may be used as biomodulators or metabolites, even in archaeobacteria. In this regard, unique polyamines such as thermopenta-amine and tetrakis-(3-aminopropyl)ammonium are found, together with normal polyamines, in one of the archaeobacteria, *Thermus thermophilus* [58]. These unusual amines are more effective than normal polyamines as biomodulators, and probably play important roles in cell survival at high temperatures in *Thermus thermophilus*.

A polyamine transport system exists without polyamine-biosynthetic enzymes in *Mycoplasma genitalium* [59]. Mycoplasmas are reduced genomes, with reduction in genome size being a consequence of their parasitic life cycle. It is therefore likely that Mycoplasmas have lost many essential biosynthetic pathways, including amino-acid-biosynthetic pathways, and have evolved to rely upon transport of many essential metabolites. Although *Haemophilus influenzae* has an ABC transporter as well as a PotE-like protein [45], other bacteria have only one transport system, either an ABC transporter or a PotE-like protein (Table 2). In *Archaeoglobus fulgidus*, *Mycoplasma genitalium* and *Mycoplasma pneumoniae*, genes with detectable similarity to polyamine-binding proteins like PotD or PotF are lacking. So, in these bacteria, polyamines may be recognized solely by channel-forming proteins, analogous to PotB and PotC. In *Synechocystis* PCC6803, only a PotD-like protein is detectable. The PotD-like protein may share channel-forming proteins and membrane-associated ATPase with the other uptake system in *Synechocystis* PCC6803. Another possibility is that the similarity between *E. coli* and *Synechocystis* genes may be too low to easily detect equivalents of the *pot* genes.

REGULATION OF GENE EXPRESSION OF THE POLYAMINE TRANSPORT OPERON

The uptake of polyamines decreases following their accumulation in *E. coli*. The mechanism for the inhibition of spermidine uptake

Table 2 Genes for polyamine transport

Bacterium or archaeon	Size of chromosome (kbp)	Number of genes coded	Gene for polyamine transport	Reference
Archaea				
<i>Archaeoglobus fulgidus</i>	2178	2407	<i>potABC</i>	[93]
Bacteria				
<i>Bacillus subtilis</i>	4215	4100	<i>blt</i>	[72]
<i>Borrelia burgdorferi</i>	911	850	<i>potABCD</i>	[45]
<i>Escherichia coli</i>	4639	4289	<i>potABCD, potFGHI, potE</i>	[17,18,47]
<i>Haemophilus influenzae</i>	1830	1709	<i>potABCD, potD, potE</i>	[43]
<i>Mycoplasma genitalium</i>	580	467	<i>potABC</i>	[59]
<i>Mycoplasma pneumoniae</i>	816	677	<i>potABC</i>	[94]
<i>Rickettsia prowazekii</i>	1112	834	<i>potE</i>	[95]
<i>Synechocystis</i> PCC6803	3573	3169	<i>potD</i>	[96]
<i>Treponema pallidum</i>	1138	1031	<i>potABCD</i>	[46]

was studied using the spermidine uptake operon consisting of *potABCD* [60]. Transcription of *potABCD* operon was inhibited by PotD, usually found in the periplasm, and the inhibitory effect of PotD was increased by spermidine. A 50% inhibition of transcription was observed with a molar ratio of approx. 1:500 of template DNA/PotD in the presence of spermidine. PotD bound to regions -258 to -209 nucleotides upstream and +66 to +135 nucleotides downstream of the ATG initiation codon of the *potA* gene. Binding of PotD to the downstream site was stimulated by spermidine. Overexpression of PotD in *E. coli* inhibited the uptake of spermidine and the synthesis of PotABCD mRNA. In cells overexpressing PotD, a large amount of PotD existed as PotD precursor in spheroplasts. Thus transcription of the spermidine transport operon (*potABCD*) is inhibited *in vivo* by PotD precursor through its binding to two regions close to the transcriptional initiation site of the operon.

To confirm that PotD precursor functions as a regulator of the spermidine uptake operon, it was important to determine the number of PotD precursors in the spheroplast. Since the number of molecules of the major σ subunit of RNA polymerase (σ^{70}) is constant during the exponential phase of cell growth and is estimated to be about 700 molecules/cell [61], the number of PotD precursor existing in spheroplasts of *E. coli* cells overexpressing PotD was estimated by comparison with the number of RNA polymerase σ^{70} molecules. It was about 5×10^3 – 2.5×10^4 molecules/cell. The values can explain about 70–80% inhibition of spermidine uptake by excess PotD. It is well known that some ribosomal proteins function as translational repressors [62]. It has been reported that PutA also functions as membrane-bound dehydrogenase as well as the repressor of the *put* operon [63]. In summary, PotD, or, more likely, a PotD precursor, is a new type of transcriptional regulator.

Since the *speF-potE* operon encoding inducible ODC and PotE is inducible at acidic pH, a gene encoding a protein involved in the enhancement of expression of the operon was sought. Using a fused gene containing the upstream sequence of the *speF-potE* operon and the open reading frame of β -galactosidase as a reporter gene, a clone which caused an increase of β -galactosidase activity at acidic pH was isolated. The clone was identified as a gene encoding RNase III [64]. Our results suggest that the initiation codon AUG and Shine–Dalgarno sequence are exposed on the surface of the mRNA by the RNase III processing of the 5'-untranslated region of mRNA. Although this is the first example of mRNA derived from *E. coli* gene, stimulation of the synthesis of λ N protein and T7 0.3 gene protein by RNase III has been reported [65,66]. It is noteworthy that expression of the *speF-potE* operon is positively regulated at the translational level.

POLYAMINE TRANSPORT IN YEAST

To try to obtain a clone of a eukaryotic polyamine transport gene, we studied polyamine transport in yeast. Polyamine uptake was energy-dependent and the K_m values for spermidine and spermine were in the range 5–100 μ M. It seems that there are at least two polyamine transport systems that can recognize all three polyamines, although the K_m value for each polyamine is different. A distinctive feature of the polyamine transport system in *Saccharomyces cerevisiae* is that it is strongly inhibited by Mg^{2+} [67]. Thus, in a Mg^{2+} -limited medium, polyamines, especially spermine, overaccumulate in cells and are toxic for growth. We isolated a mutant (YTM22-8) whose growth was tolerant to spermine in a Mg^{2+} -limited medium. This mutant was defective in polyamine uptake and did not accumulate spermine.

We obtained clones of two yeast genes that restore spermine sensitivity to YTM22-8. Those genes were *PTK1* and *PTK2*, which encode putative serine/threonine protein kinases [68,69]. This suggests that spermine uptake in yeast is regulated by phosphorylation and dephosphorylation. Poulin and co-workers recently reported that another putative serine/threonine protein kinase, NPR1, essential for the re-activation of several nitrogen permeases, is also involved in the activation of spermidine uptake in addition to PTK1 (= STK1) and PTK2 (STK2) [70,71]. However, a gene for the polyamine transporter on the plasma membrane of *Saccharomyces cerevisiae* has not yet been isolated.

It has been reported that excretion of spermidine can be catalysed by the *Bacillus subtilis* multidrug transporter Blt [72]. Thus we reasoned that the polyamine transporter in yeast may have some sequence similarity to Blt, and we searched for such amino acid sequence between Blt and proteins encoded by yeast genome [73]. We detected four candidate genes (*YLL028W*, *YBR180W*, *YKR105C* and *YCR023C*) and subsequently studied polyamine toxicity and transport activity using yeast transformed with these genes. One gene (*YLL028W*) encodes a protein found on the membrane of vacuoles that catalyses proton-gradient-dependent polyamine transport [74]. This gene was named *TPO1* (transporter for polyamine 1) [14].

The *TPO1* gene is located on chromosome XII and encodes a membrane protein consisting of 586-amino-acid residues [73]. The protein has 12 putative transmembrane segments. Three glutamic acid residues, which may interact with polyamines, are located in positions similar to those of the key residues in PotE (Figure 8). Polyamine transport is positively regulated by protein kinases. When the amino acid sequences of TPO1 and PotE were compared, TPO1 possessed a longer hydrophilic N-terminal region in which many serine and threonine residues are included. Thus the N-terminal region of the protein may be important for regulation by protein kinases.

CONCLUDING REMARKS AND FUTURE PERSPECTIVES

Many properties of the polyamine transport systems in *E. coli* have been clarified and studied in detail. However, many characteristics still remain unknown or poorly understood. This is especially true for the membrane-associated proteins such as PotA and transmembrane proteins such as PotB, PotC and PotE. The crystal structures of PotD and PotF defined the characteristics of the polyamine-binding sites on these proteins. The arrangement and the chemical properties of amino acids in PotD and PotF may be used as a template for studies of other polyamine-binding proteins. One encouraging example is that a sequence comparison with PotD revealed several amino acid residues crucial for polyamine binding to the *N*-methyl D-aspartate subtype of glutamate receptor [75–78].

In eukaryotic cells, including yeast, the gene(s) that encode polyamine transporters on the plasma membrane have not yet been isolated. However, it is known that polyamine uptake is positively regulated by serine/threonine protein kinases in yeast and negatively regulated by antizyme in animal cells [79–83]. Judging from the properties of polyamine transport in yeast and animal cells, we expect that the polyamine transporter on the plasma membrane is structurally similar to the polyamine transporter on the yeast vacuolar membrane.

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