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Increase of Sensitivity to Aminoglycoside Antibiotics by Polyamine-Induced Protein (Oligopeptide-Binding Protein) in *Escherichia coli*

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The sensitivity of *Escherichia coli* to several aminoglycoside antibiotics was examined with *E. coli* DR112 transformed by the gene for polyamine-induced protein (oligopeptide-binding [OppA] protein) or polyamine transport proteins. The results clearly showed that sensitivity to aminoglycoside antibiotics (gentamicin, isepamicin, kanamycin, neomycin, paromomycin, and streptomycin) increased due to the highly expressed OppA protein. When the gene for OppA protein was deleted, sensitivity to aminoglycoside antibiotics was greatly decreased. It was also shown that isepamicin could bind to OppA protein with a binding affinity constant of 8.5×10^3 M^{-1} under the ionic conditions of 50 mM K^+ and 1 mM Mg^{2+} at pH 7.5, and isepamicin uptake into cells was greatly stimulated by the OppA protein. These results, taken together, show that the OppA protein increases the uptake of aminoglycoside antibiotics. In addition, the OppA protein increased the transport of spermidine and an oligopeptide (Gly-Leu-Tyr). The uptake of isepamicin into cells was partially inhibited by spermidine, suggesting that the binding site for isepamicin overlaps that for spermidine on the OppA protein. Spermidine uptake activity by the OppA protein was less than 1% of that of the ordinary spermidine uptake system. Aminoglycoside antibiotics neither stimulated the synthesis of OppA protein nor increased spermidine uptake.

Polyamines are important for cell growth (36). We have previously found that synthesis of a protein of M_r 62,000, named polyamine-induced protein, was stimulated greatly by the addition of putrescine during growth of a polyamine-requiring mutant of *Escherichia coli* MA261 (28). Since this synthesis was followed by polyamine stimulation of cell growth, the physiological function of this protein may be important for cell growth. The protein was recently identified as a periplasmic oligopeptide-binding (OppA) protein (20).

Although aminoglycoside antibiotics are known to be accumulated in *E. coli* cells by an active transport system (1–3, 6, 30), the uptake of aminoglycoside antibiotics is a complex process and still a matter of controversy (7, 35). Streptomycin is thought to be taken up by *E. coli* in a process that may be subdivided into three consecutive phases: first, a rapid electrostatic binding to the cell; second, a slow rate of accumulation; and third, a much enhanced rate of uptake. There is also a report that the enhanced streptomycin uptake is explained by the induction of a polyamine transport system by streptomycin, which can be utilized by streptomycin itself (12). We recently isolated three clones carrying polyamine transport genes (pPT104, pPT79, and pPT71) and characterized them (9, 16, 19). To determine which protein is involved in the transport of aminoglycoside antibiotics, sensitivity to the antibiotics was examined with *E. coli* cells which overproduced transport proteins or polyamine-induced protein (OppA protein). The results indicate that aminoglycoside antibiotics were transported by the oligopeptide uptake system, whose synthesis was strongly regulated by polyamines, but not by the ordinary polyamine transport systems. We also found that spermidine was transported by the oligopeptide uptake system, although the rate of transport was much slower than that by the ordinary spermidine uptake system encoded by pPT104 (9, 16).

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MATERIALS AND METHODS

Bacterial strains, clones, and culture conditions. The polyamine-requiring mutants of *E. coli* MA261 (*speB speC thr leu ser thi* [5]) and *E. coli* DR112 (*speA speB thi* [24]) were kindly supplied by W. K. Maas and D. R. Morris, respectively. The polyamine transport-deficient mutant of *E. coli* NH1596 was isolated from *E. coli* MA261 as described previously (16). The clones containing the gene for polyamine-induced protein, i.e., OppA protein (pPI5.1 and pPI5), the spermidine and putrescine transport protein (pPT104), and the putrescine transport protein (pPT79 and pPT71) were isolated by using pACYC184 (4) as a vector, as described previously (16, 20). Transformation of *E. coli* DR112 and *E. coli* NH1596 with pACYC184 or the above clones was carried out by the method of Maniatis et al. (26).

E. coli CU103, an *oppA*::Km derivative of *E. coli* DR112 lacking the gene for OppA protein, was prepared as follows. The plasmid containing the gene for OppA protein at the polylinker cloning site of pMW119 (37) was partially digested with *Eco*RV, and the Km^r gene (a 1.5-kb *Cfr*13I fragment) from transposon Tn*903* was inserted into the *Eco*RV site. The linearized plasmid was then transformed into a *recD*::Tn*10* derivative of *E. coli* W3110, and Km^r transformants were isolated (*E. coli* CU102). Then, *E. coli* CU103, an *oppA*::Km derivative of *E. coli* DR112, was isolated by

TABLE 1. *E. coli* strains and plasmids used

Strain or plasmid	Relevant characteristics	Comments, source, or reference
E. coli strains		
MA261	*speB speC thr leu ser thi*	Polyamine-requiring mutant; W. K. Maas (5)
NH1596	MA261 *pot*	Polyamine transport-deficient mutant of MA261 (16)
DR112	*speA speB thi*	Polyamine-requiring mutant; D. R. Morris (24)
CU102	W3110 *recD*::Tn*10 oppA*::Km^r	Gene for OppA protein (polyamine-induced protein) disrupted by Km^r gene; this study
CU103	DR112 *oppA*::Km^r	Gene for OppA protein disrupted by Km^r gene; this study
Plasmids		
pACYC184	Vector	S. N. Cohen (4)
pPI5	*oppA*$^+$	Plasmid for OppA protein (polyamine-induced protein) gene (20)
pPI5.1	*oppA*$^+$	*Sal*I fragment-deleted pPI5 (20)
pPT104	*potA*$^+$ *potB*$^+$ *potC*$^+$ *potD*$^+$	Plasmid for spermidine and putrescine transport protein gene (9, 16)
pPT79	*potF*$^+$ *potG*$^+$ *potH*$^+$ *potI*$^+$	Plasmid for putrescine transport protein gene (16; unpublished results)
pPT71	*potE*$^+$ *speF*$^+$	Plasmid for putrescine transport protein gene and inducible ornithine decarboxylase gene (16, 19)

transduction with phage P1 (23) with *E. coli* CU102 as the donor.

E. coli DR112 and CU103 were cultured as described previously (17), and growth was monitored by measuring the A_{540}. *E. coli* NH1596 was cultured in medium B (13) without putrescine. When growth was sufficient to yield an A_{540} of 0.35, the cells were harvested by centrifugation at 12,000 × *g* for 10 min. The cells were washed once with 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 $MgSO_4$], centrifuged, and suspended in buffer A to yield a protein concentration of 0.1 mg/ml. The protein concentration was determined by the method of Lowry et al. (25).

The strains and plasmids used in this study are listed in Table 1.

Assay for uptake of antibiotics, spermidine, and tripeptide (Gly-Leu-Tyr) by intact cells. The cell suspension (0.48 ml), prepared as described above, was preincubated at 30°C for 5 min, and the reaction was started by the addition of 20 µl of 500 µM [^{14}C]isepamicin, [^{14}C]methylerythromycin, or [^{14}C] spermidine (925 MBq/mmol) or 500 µM Gly-Leu-[^{125}I]Tyr (approximately 2.5 × 10^7 cpm/ml). After incubation at 30°C, the cells were collected on membrane filters and washed as described previously (18). The radioactivity on the filters was assayed with a liquid scintillation spectrometer.

Assay for binding of isepamicin, spermidine, and tripeptide to OppA protein. This assay was performed by gel filtration as described previously (14). A column (0.5 by 11 cm) of Bio-Gel P-10 was equilibrated with a buffer containing 10 mM Tris-HCl (pH 7.5), 1 mM magnesium acetate, 50 mM KCl, and various concentrations of radioactive substrates (50 to 300 µM). OppA protein (1 mg) in 0.3 ml of the above buffer was applied to the column. The column was eluted with the same buffer, and the radioactivity was counted. Since the area of the radioactive peak should be equal to the area of the radioactive trough, the average of two interdependent measurements was used to estimate the amount of substrate bound to the OppA protein. The binding constant and the number of binding sites were calculated from a Scatchard plot (34) of the results.

Measurement of synthesis of OppA protein in the presence of aminoglycoside antibiotics. *E. coli* MA261 was cultured in medium B without putrescine until the A_{540} reached 0.2. Then, 1.85 MBq of [^{3}H]lysine (740 MBq/mmol) was added to each 5-ml aliquot in the absence or presence of an antibiotic or putrescine. The cells were allowed to grow for 30 min. The synthesis of OppA protein was determined by the method of Philipson et al. (32). To 400,000 cpm of [^{3}H]lysine-labeled protein in 0.5 ml of buffer containing 10 mM sodium phosphate (pH 7.4), 100 mM NaCl, 1% Triton X-100, and 0.1% sodium dodecyl sulfate (SDS) was added 0.25 mg of immunoglobulin for the OppA protein. After the preparation was agitated gently for 60 min at room temperature, 0.08 ml of 10% Formalin-fixed *Staphylococcus aureus* Cowan 1 cells was added. The precipitate was suspended in 0.04 ml of gel sample buffer (62.5 mM Tris-HCl [pH 6.8], 2% SDS, 10% glycerol, 5% 2-mercaptoethanol, 0.01% bromphenol blue). Gel electrophoresis (21) and fluorography (22) were performed with a 0.03-ml aliquot.

Other procedures and chemicals. OppA protein was purified by a previously published method (28). Immunoglobulin for the OppA protein was prepared as described previously (15) and was partially purified from the antiserum by precipitation with 40% saturated $(NH_4)_2SO_4$. Formalin-fixed *S. aureus* Cowan 1 was purchased from Calbiochem. [^{14}C]spermidine trihydrochloride was obtained from Amersham. Gentamicin, kanamycin A, neomycin B, and streptomycin were from Sigma Chemical Co. Paromomycin was kindly provided by Y. Yuda, Meiji Seika Co., Tokyo, Japan. Isepamicin (HAPA-B) (38) and [^{14}C]isepamicin were kindly supplied by S. Yamaji, Toyo Jozo Co., Shizuoka, Japan. Methylerythromycin (6-*O*-methylerythromycin B) (29) and [^{14}C]methylerythromycin were generously donated by K. Fukushima, Taisho Pharmaceutical Co., Saitama, Japan. Gly-Leu-[^{125}I]Tyr was prepared as described previously (20).

RESULTS

Effect of polyamine-induced protein (OppA protein) and polyamine transport proteins on sensitivity to aminoglycoside antibiotics. Sensitivity to aminoglycoside antibiotics was examined with *E. coli* DR112 transformed by the gene for OppA protein or polyamine transport proteins. The structures of the aminoglycoside antibiotics used are shown in Fig. 1. All antibiotics include the two guanidine groups (streptomycin) or propyl- or butyldiamine moiety (other antibiotics). The plasmids transformed into *E. coli* DR112 were pPI5.1, producing a large amount of OppA protein; pPI5, producing a small amount of OppA protein; pPT104,

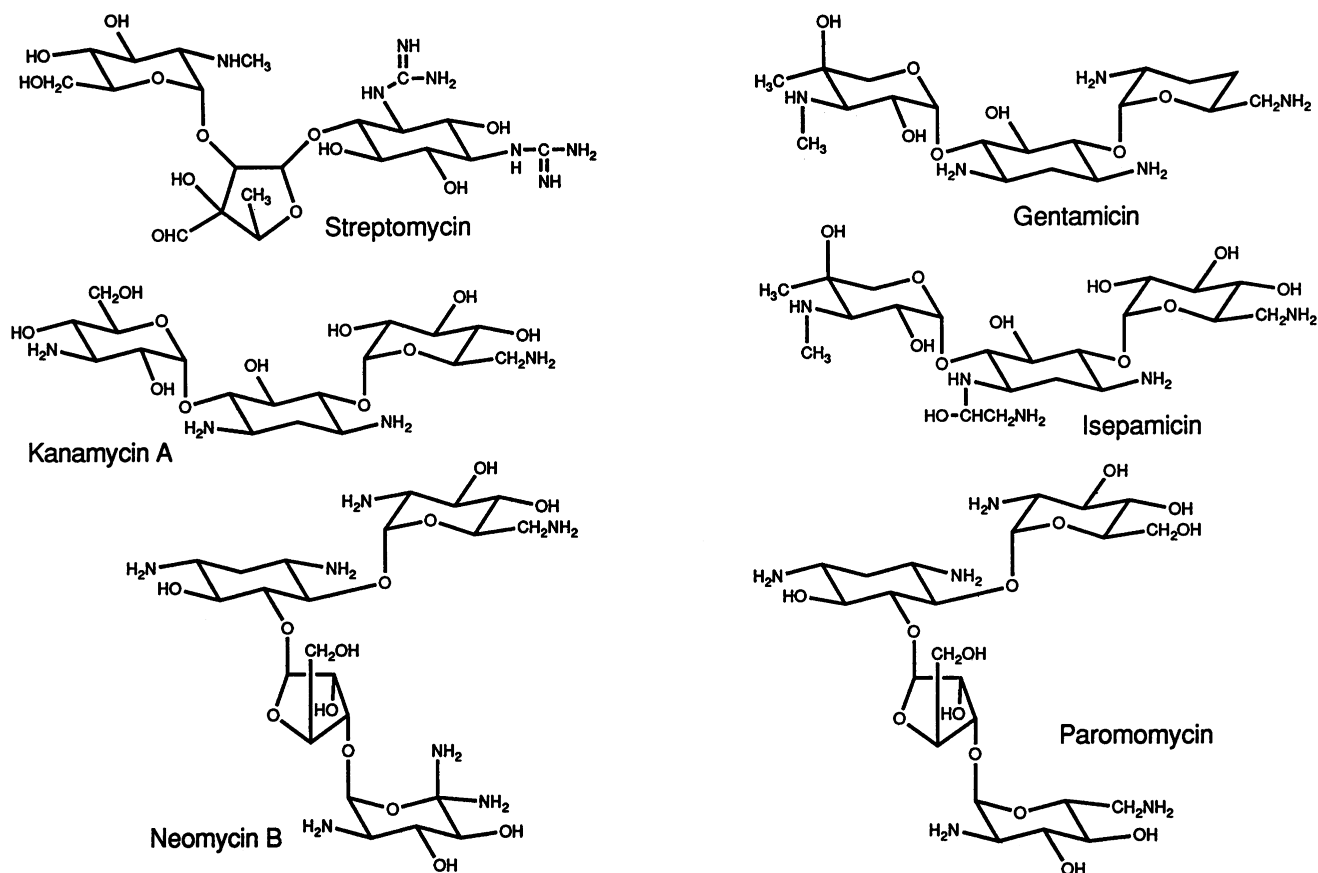

FIG. 1. Structures of aminoglycoside antibiotics.

producing spermidine and putrescine transport protein; pPT79, producing putrescine transport protein with high activity; and pPT71, producing putrescine transport protein with low activity (9, 16, 19, 20).

As shown in Fig. 2 and Table 2, expression of OppA protein greatly increased the sensitivity to streptomycin, gentamicin, isepamicin, kanamycin, neomycin, and paromomycin, but sensitivity to methylerythromycin, one of the macrolide antibiotics, was not increased by the protein. The sensitivity of *E. coli* DR112 transformed with pPI5.1 to aminoglycoside antibiotics was always greater than that of *E. coli* DR112 transformed with pPI5, indicating that the degree of inhibition of cell growth by these antibiotics was proportional to the amount of OppA protein synthesized. Then, the gene for OppA protein was deleted in *E. coli* DR112, and sensitivity to aminoglycoside antibiotics was examined. As shown in Fig. 3, sensitivity to neomycin, paromomycin, and streptomycin was greatly decreased by deletion of the gene for OppA protein. These results indicate that aminoglycoside antibiotics may be transported at least in part by the oligopeptide transport system.

As shown in Table 2, the spermidine and putrescine transport protein encoded by pPT104 increased the sensitivity to gentamicin but not to the other aminoglycoside antibiotics. The putrescine transport protein encoded by pPT71 increased the sensitivity to isepamicin but not to the other aminoglycoside antibiotics. A part of the structure of gentamicin and isepamicin may be similar to that of spermidine and putrescine, respectively. When sensitivity to gentamicin and isepamicin was examined with *E. coli* CU103, lacking the gene for OppA protein, the decrease in sensitivity to these antibiotics was small compared with that to neomycin, paromomycin, and streptomycin (data not shown), also suggesting that there are other transport systems for gentamicin and isepamicin.

Increase in [^{14}C]isepamicin uptake into *E. coli* cells by OppA protein and [^{14}C]isepamicin binding to the protein. We next examined whether the increase in sensitivity to aminoglycoside antibiotics is due to their accumulation in cells. As shown in Fig. 4, the uptake of [^{14}C]isepamicin was increased by the OppA protein, but the uptake of [^{14}C]methylerythromycin was not. The results indicate that aminoglycoside antibiotics are transported by the OppA protein and accumulate in cells. This was confirmed by the result that isepamicin bound to the protein with a binding affinity constant of 8.5×10^3 M^{-1} under the ionic conditions of 50 mM K^+ and 1 mM Mg^{2+} at pH 7.5 (Table 3).

Spermidine and Gly-Leu-Tyr uptake into *E. coli* cells by OppA protein. Streptomycin has been reported to be transported by an inducible polyamine transport system (12), and the uptake of aminoglycoside antibiotics was stimulated by OppA protein. Thus, we examined whether spermidine and Gly-Leu-Tyr were transported by OppA protein. The uptake of spermidine and Gly-Leu-Tyr was catalyzed by the protein, but the rate of tripeptide uptake was very slow compared with that of isepamicin and spermidine (Fig. 4). This slow rate was reflected by the binding constants for spermidine and Gly-Leu-Tyr binding to OppA protein (Table 3).

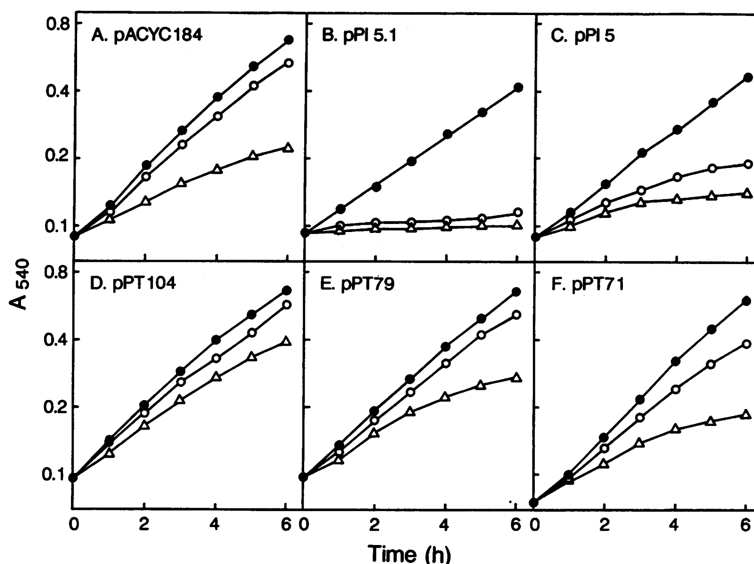

FIG. 2. Effect of streptomycin on growth of *E. coli* DR112 carrying various plasmids. *E. coli* DR112 containing pACYC184 (A), pPI5.1 (B), pPI5 (C), pPT104 (D), pPT79 (E), or pPT71 (F) was cultured under standard conditions. ●, no streptomycin; ○, streptomycin (10 μg/ml); △, streptomycin (30 μg/ml).

However, the binding constant for Gly-Leu-Tyr binding to OppA protein increased from 0.97×10^3 to 5.1×10^3 M^{-1} when the protein was dialyzed against a buffer containing 6 M guanidine-HCl at pH 7.2 (Table 3). Thus, the apparent slow speed of tripeptide uptake may be due to the dilution of Gly-Leu-[^{125}I]Tyr with bound oligopeptides on the OppA protein. When the rate of spermidine uptake by OppA protein was compared with that by the ordinary spermidine uptake system encoded by pPT104 (16), the former rate was judged to be less than 1% of the latter rate.

The rate of Gly-Leu-Tyr uptake by *E. coli* NH1596 observed in the present study was very much lower than that by *E. coli* MA261 reported in the previous work (20). This may be explained as follows. (i) *E. coli* NH1596 was obtained from *E. coli* MA261 by *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine treatment. During the treatment, *E. coli* cells may be damaged. (ii) In the previous experiment (20), *E. coli* MA261 was grown in the presence of the tripeptide, which may induce the oligopeptide transport system.

The uptake of isepamicin was partially inhibited by spermidine but not by Gly-Leu-Tyr, and that of spermidine was partially inhibited by the antibiotic but not by the tripeptide. Neither isepamicin nor spermidine inhibited the uptake of Gly-Leu-Tyr (Fig. 5). These findings indicate that the binding site of the antibiotic on OppA protein partially overlapped that of spermidine but not that of Gly-Leu-Tyr.

Effect of aminoglycoside antibiotics on synthesis of OppA protein. It has been reported that spermidine transport is increased in the presence of streptomycin (5 μg/ml) (12). Since both aminoglycoside antibiotics and spermidine were recognized by OppA protein, the effect of the antibiotics on the synthesis of OppA protein was examined (Fig. 6). It was observed that synthesis of the protein was not stimulated significantly by 30 μg of streptomycin or 3 μg of gentamicin per ml, which caused 50% inhibition of cell growth of *E. coli* MA261. It was not stimulated even when the concentration of streptomycin was changed from 5 to 60 μg/ml. Under these conditions, spermidine uptake activity was not increased (data not shown).

DISCUSSION

It has been reported that streptomycin is taken up by *E. coli* via an inducible polyamine transport system (12). We recently isolated three polyamine transport clones (carried by pPT104, pPT79, and pPT71) and characterized them (16).

TABLE 2. Effect of OppA protein and polyamine transport proteins on inhibition of cell growth by various antibiotics[a]

Antibiotic (μg/ml)	% Inhibition of growth					
	Control (pACYC184)	OppA protein		Polyamine transport protein		
		pPI5.1	pPI5	pPT104	pPT79	pPT71
Gentamicin (0.3)	12	88	54	89	9	7
Isepamicin (3)	14	95	71	3	11	73
Kanamycin (1)	3	95	28	7	5	4
Neomycin (3)	15	93	74	18	14	15
Paromomycin (10)	14	95	68	16	15	19
Methylerythromycin (30)	60	71	62	64	65	68

[a] *E. coli* DR112 carrying various plasmids was cultured in the presence and absence of aminoglycoside antibiotics, and percent inhibition was calculated from their generation times.

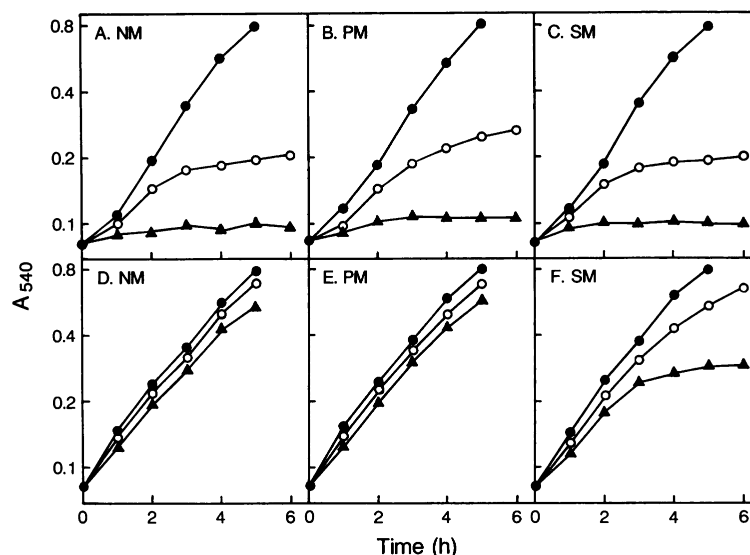

FIG. 3. Effect of aminoglycoside antibiotics on growth of *E. coli* CU103 lacking the gene for OppA protein. *E. coli* DR112 (A through C) or *E. coli* CU103 (D through F) was cultured under standard conditions. ●, no antibiotics added. A and D, neomycin (NM; ○, 3 μg/ml; ▲, 10 μg/ml); B and E, paromomycin (PM; ○, 10 μg/ml; ▲, 20 μg/ml); C and F, streptomycin (SM; ○, 10 μg/ml; ▲, 30 μg/ml).

The system encoded by pPT104 can catalyze both spermidine and putrescine transport, but the systems encoded by pPT79 and pPT71 can catalyze only putrescine transport. In addition, polyamine-induced protein (OppA protein) is induced strongly by polyamines (20, 28). Thus, the sensitivity of *E. coli* to several aminoglycoside antibiotics was examined by using *E. coli* DR112 transformed by the gene for polyamine transport protein or OppA protein. The results clearly show that aminoglycoside antibiotics are transported by the oligopeptide transport system (11), in which the synthesis of OppA protein is stimulated by polyamines. This was supported by the results that sensitivity to the antibiotics decreased in cells lacking the gene for OppA protein.

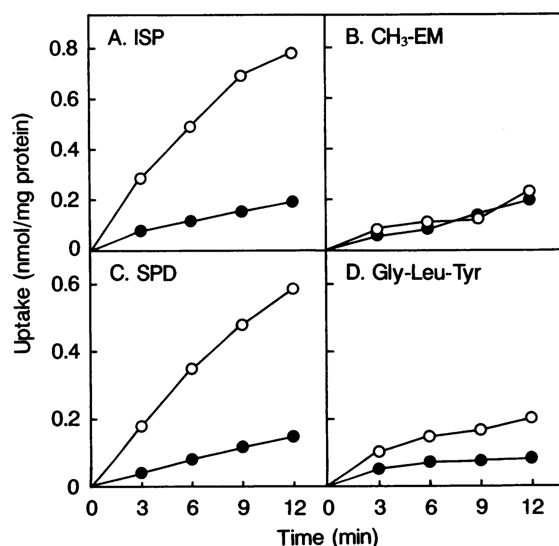

FIG. 4. Uptake of isepamicin, spermidine, and Gly-Leu-Tyr in *E. coli*. The assay for uptake of isepamicin, spermidine, and Gly-Leu-Tyr was performed with *E. coli* NH1596 (●) or *E. coli* NH1596 carrying pPI5.1 (○) as described under Materials and Methods. (A) Isepamicin (ISP) uptake; (B) methylerythromycin (CH_3-EM) uptake; (C) spermidine (SPD) uptake; (D) Gly-Leu-Tyr uptake.

The oligopeptide transport system consisted of five proteins (OppA, -B, -C, -D, and -F proteins). Our results indicate that overproduction of the OppA protein is sufficient to increase the uptake of aminoglycoside antibiotics. According to the results in Table 3, the apparent K_m of OppA protein for isepamicin was 120 μM, which is relatively high. The overproduction of OppA protein may increase the interaction between substrates (aminoglycoside antibiotics) and the membrane-associated components (OppB, -C, -D, and -F proteins).

It seems that most of the aminoglycoside antibiotics are not transported by the polyamine transport systems. Only gentamicin and isepamicin were taken up by the pPT104- and pPT71-encoded systems, respectively. It remains to be clarified how the antibiotics were recognized by the polyamine transport systems.

It was also reported that streptomycin increases spermidine transport 10-fold (12). Since the increase in transport activity was inhibited by chloramphenicol, this augmentation was thought to be the result of a streptomycin-induced synthesis of the transport system. We could not confirm those results. Furthermore, gentamicin and streptomycin did

TABLE 3. OppA protein binding constant (K) and number of substrate-binding sites

Substrate	K (M^{-1})	No. of substrate-binding sites (no./molecule of OppA protein)
Isepamicin	8.5×10^3	1.6
Spermidine	8.5×10^3	2.2
Gly-Leu-Tyr[a]		
Not dialyzed	0.97×10^3	3.2
Dialyzed	5.1×10^3	1.3

[a] The binding of Gly-Leu-[^{125}I]Tyr to OppA protein was performed with undialyzed protein and protein dialyzed against 6 M guanidine-HCl at pH 7.2 (27).

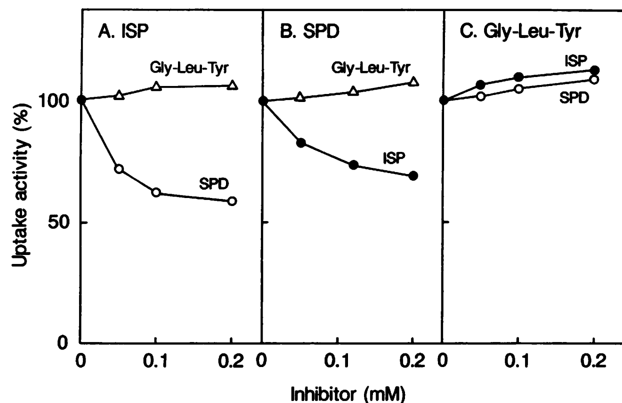

FIG. 5. Effect of other substrates on uptake of isepamicin, spermidine, and Gly-Leu-Tyr. The assay was performed under standard conditions with NH1596 carrying pPI5.1. (A) [^{14}C]isepamicin (ISP) uptake (100% activity, 105 pmol/min/mg of protein); (B) [^{14}C]spermidine (SPD) uptake (100% activity, 67 pmol/min/mg of protein); (C) Gly-Leu-[^{125}I]Tyr uptake (100% activity, 32 pmol/min/mg of protein). Unlabeled isepamicin (●), spermidine (○), or Gly-Leu-Tyr (△) was added as indicated.

not significantly influence the synthesis of OppA protein (Fig. 6) or the proteins encoded by pPT104 (unpublished results). The enhanced effect of streptomycin on its own uptake may be explained by assuming that the membrane is damaged by the incorporation of misread protein (8). Spermidine uptake may also be stimulated by membrane damage. Under our experimental conditions, membrane damage may not occur, since the cells were incubated for only 30 min with streptomycin or gentamicin.

Spermidine was also transported by the oligopeptide transport system, although the rate of transport was extremely slow. This suggests that OppA protein recognizes the aliphatic amines of several compounds, and thus the system has a broad substrate specificity. Our results also suggest that the binding site of aminoglycoside antibiotics and spermidine may be different from that of oligopeptides. There are several reports thus far on the function of the oligopeptide transport system: supply of nutrients (20), recycling of cell wall peptides (10), and involvement in sporulation (31, 33). The variety of functions of the oligopeptide transport system may be the result of the broad substrate specificity of the OppA protein.

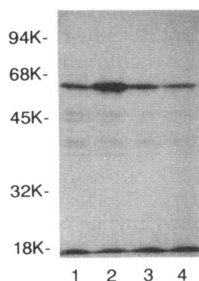

FIG. 6. Effect of putrescine and aminoglycoside antibiotics on synthesis of OppA protein. Fluorography of [^{3}H]lysine-labeled OppA protein was performed as described in Materials and Methods. Lane 1, OppA protein from polyamine-deficient *E. coli* MA261; lane 2, OppA protein from putrescine (100 μg/ml)-supplemented *E. coli* MA261; lane 3, OppA protein from streptomycin (30 μg/ml)-supplemented *E. coli* MA261; lane 4, OppA protein from gentamicin (3 μg/ml)-supplemented *E. coli* MA261. Numbers on the left represent molecular weight (in thousands), and the arrow on the right indicates the position of OppA protein.

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