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Functional Domains of S-Type Pyocins Deduced from Chimeric Molecules

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Functional domain structures of pyocins AP41, S1, and S2 were assigned by examining the functions of chimeric pyocins and deletion derivatives. Pyocins AP41, S1, and S2 are essentially composed of three domains, the receptor-binding domain, the translocation domain, and the DNase domain, in that order from the N terminus to the C terminus. The alignment of these domains is distinct from that in E2-group colicins with functions similar to those of these pyocins. Pyocins AP41 and S2 have a fourth domain between the receptor-binding and the translocation domains, which is dispensable for their killing functions.

Pyocins S1, S2, and AP41 are the protease-sensitive bacteriocins most frequently found among *Pseudomonas aeruginosa* strains (19). They are distinguished by their different receptor specificities. Recently we have cloned and sequenced the genetic determinants for pyocins AP41, S1, and S2 (18, 20). Each determinant for these three pyocins constitutes an operon encoding two proteins of different sizes, one responsible for killing (the killing protein) and the other conferring immunity to its own pyocin (the immunity protein). In the 5′ upstream region of each operon, a characteristic sequence (a P box), a possible regulatory element for the induced pyocin production, is conserved (10, 20). The molecular weights of the killing proteins are different for the pyocins (84,000, 65,600, and 74,000 for pyocins AP41, S1, and S2, respectively), whereas the immunity proteins are of similar sizes, around 10,000. As described previously (20), the amino acid sequences of the C-terminal halves of the killing proteins of pyocins AP41, S1, and S2 are highly conserved. Those of pyocins S1 and S2 are identical except for one amino acid deletion (S1) or addition (S2) in this region. Furthermore, the distal ca. 130 amino acids show striking homology to the C-terminal sequences of E2-group colicins and possess DNase activity. The less highly conserved N-terminal halves have been suggested to be receptor-binding domains because these pyocins show different receptor specificities. In addition to causing breakdown of the chromosomal DNA, pyocins S1 and S2, but not pyocin AP41, inhibit lipid synthesis in the susceptible bacteria, although their susceptible strains are not the same (16, 18, 20).

In the study described here, we constructed various chimeric pyocins and domain-deleted pyocins on the basis of their sequence conservation and attempted to determine the functions of each domain by examining their functions in vivo and in vitro.

MATERIALS AND METHODS

Plasmids, bacterial strains, and media. For construction of plasmids, the following pUC plasmids containing the genes for pyocins AP41, S1, and S2 were used: for pyocin AP41, pYK211 (18); for pyocin S1, pYMSS11 (20); and for pyocin S2, pYMPS1 (20). *Escherichia coli* C600 (1), HB101 (3), JM109 (26), and MV1304 (25) were used as host strains. For preparation of pyocins and chimeric proteins, *E. coli* C600 carrying the appropriate plasmids was employed. *P. aeruginosa* PML1516d ($S1^s$ $S2^s$) (11) and its derivatives PML1567 ($S1^s$ $S2^r$) (13) and PML1570 ($S1^r$ $S2^s$) (this study) were used as indicators for pyocins S1 and S2, and PAO3092 (17) was used as an indicator for pyocin AP41. NIH3 (6) and its derivatives $NIH3S1^r$, $NIH3S2^r$, and $NIH3AP41^r$ (this study) were also used to determine the receptor specificity. *E. coli* cells were cultured with YT, and *P. aeruginosa* cells were cultured with G medium with 1 mM $FeSO_4$ or YT plus 5 mM $MgSO_4$ with or without 0.2 mM α,α′-dipyridyl (20). Dilution buffer was as described elsewhere (17).

Preparation of pyocins and their chimeras and the receptor substance. The purification procedure for pyocin proteins was described previously (20). For purification of chimeras TA3, TA4, TA5, AS7, and AT11, the final chromatography was done by using the fast protein liquid chromatography system (LCC500) with a Mono S column HR5/5 (Pharmacia LKB) instead of a carboxymethyl-Sepharose column.

The receptor substance for pyocin S1 was separated from PML1516d cells grown in YT with dipyridyl in accordance with Schnaitman's procedure (21, 22) except that cells were disrupted by sonication and 10 mM Tris-HCl (pH 7.5) was used as a buffer. Thus, the cell envelope fraction (fraction A), the 2% Triton-soluble fraction (fraction B), the 2%

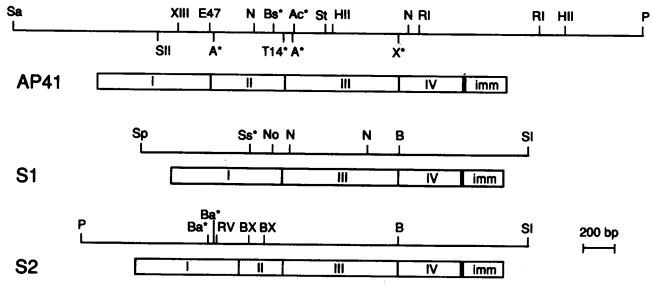

FIG. 1. Physical map of the pyocin determinants with their protein structures. The restriction sites indicated with an asterisk are relevant sites used among their multiple sites for the construction of chimeras. Abbreviations: A, *Alu*I; Ac, *Acy*I; B, *Bam*HI; Ba, *Bal*I; Bs, BssHII; BX, *Bst*XI; E47, *Eco*47III, *Hin*cII; N, *Nae*I; No, *Not*I; P, *Pst*I; SI, *Sac*I; SII, *Sac*II; RI, *Eco*RI; RV, *Eco*RV; Sa, *Sal*I; Sp, *Sph*I; Ss, *Ssp*I; St, *Stu*I; XIII, *Xma*III; imm, immunity protein.

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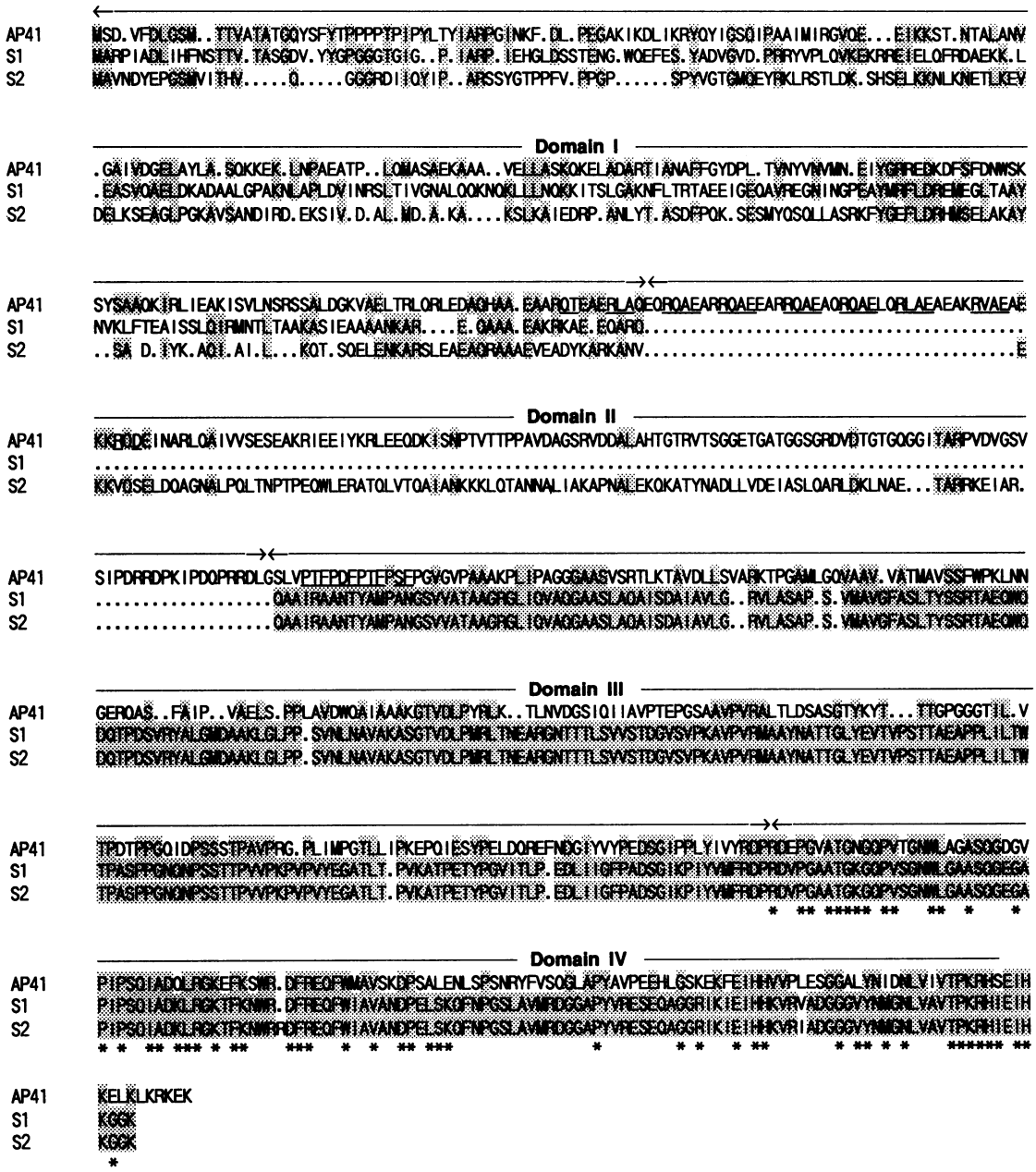

FIG. 2. Assignment of the putative domains of pyocins by their sequence homology. Identical amino acids conserved among these pyocins are shaded. The homology of domains III and IV has been reported previously (20). Asterisks indicate the identical residues commonly conserved in colicins E2, E7, E8, and E9 (20).

Triton-insoluble fraction (fraction C) containing outer membrane proteins (13), the EDTA-Triton-soluble fraction (fraction D), and the residual fraction (fraction E) were obtained. Fractions A, C, and E were finally suspended in dilution buffer prior to use.

Measurement of killing activity and adsorption of pyocins and their derivative proteins. Killing activity was measured by counting surviving cells or by the spot method described previously (17). Adsorption to cells or isolated membrane fractions was assayed by competition against active pyocins. PML1516d cells grown in YT with dipyridyl were harvested, washed, and suspended in dilution buffer at about 10^8 cells per ml. To 40 μl of the cell suspension, 40 μl of YT plus dipyridyl and 20 μl of pyocin derivatives in dilution buffer were added, and the mixture was incubated for 15 min at 37°C (adsorption). Next, 10 μl of pyocin S1 or S2 was added to the mixture at a concentration of about 20 to 30 U/ml. Surviving cells were counted after incubation at 37°C for 30 min. Adsorption to the receptor substance was estimated in essentially the same way, except that the membrane fractions (A through E) were used instead of cells and residual pyocin activity was titrated by the spot method 15 min after incubation with about 200 to 300 U of pyocin S1 or S2 per ml.

Translocation assay by receptor bypass. The procedure to bypass the receptor-mediated process in *E. coli*, described previously (5, 23), was modified to increase the efficiency of

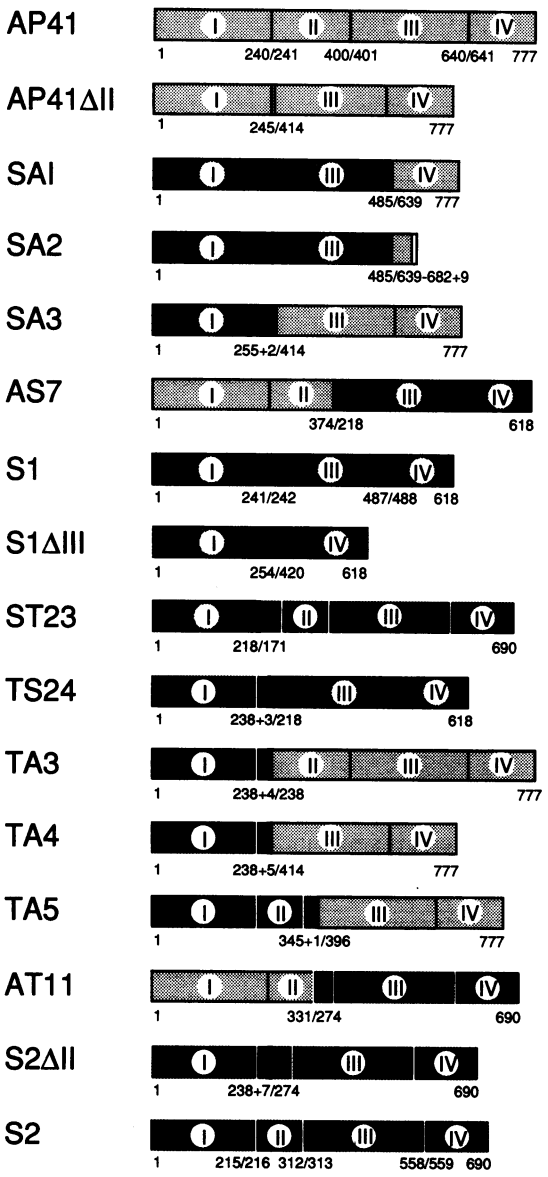

FIG. 3. Construction of the chimeric pyocins and deletion derivatives. Chimeric pyocins were constructed by exchanging domains. Each domain is shown boxed and assigned a domain number. Numbers below the domains indicate amino acid residues; 1 is the first methionine, although it is processed in the mature proteins in all three pyocins. Light, medium, and dark boxes indicate domains derived from pyocins AP41, S1, and S2, respectively.

killing as well as the reproducibility in *P. aeruginosa*. Pyocin-resistant cells (PML1567 or PML1570) cultured in YT plus 5 mM $MgSO_4$ were harvested, washed with a buffer (85 mM NaCl–10 mM potassium phosphate [pH 7]–1 mM $MgCl_2$), and suspended in the same buffer at about 2×10^9 cells per ml at room temperature. To 500 μl of this suspension, 500 μl of 1.2 M glucose was added, and then this mixture was cooled to O°C in ice water. After 5 min, 50-μl portions of this mixture were dispersed into 450 μl of ice-cold 10 mM sodium acetate–1 mM $MgCl_2$ (pH 4.3) containing appropriate amounts of pyocin proteins and mixed thoroughly. Surviving cells were counted after incubation on ice for 15 min.

Other methods. Amounts of proteins in the membrane fractions were determined by the method of Lowry et al., with bovine serum albumin as the standard (8). Lipid synthesis as determined by incorporation of [2-^{3}H]glycerol and sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) were done as described before (12, 17). DNA manipulations and DNA sequencing were performed in accordance with the manual of Sambrook et al. (15).

RESULTS AND DISCUSSION

Putative domain structures of S-type pyocins. The structures of the genetic determinants for pyocins AP41, S1, and S2 are summarized in Fig. 1 (18, 20). Each determinant encodes two protein components, the killing protein and its immunity protein. The putative domains, domains I to IV, of the killing protein were deduced from the sequence conservation (Fig. 2). Pyocins AP41 and S2 are composed of four domains, whereas pyocin S1 lacks domain II. Domain II of pyocin AP41 forms an α-helix with the repeated RQAE motifs (18). Biochemical studies have shown that domain IV functions as DNase (16, 18, 20).

Construction of chimeric pyocins and domain-deleted pyocins. The strategies used for the construction of plasmids encoding chimeric and domain-deleted pyocins are presented briefly below. The restriction enzyme sites used for the construction are shown in the physical maps in Fig. 1, together with the putative domain structures of the pyocins encoded. For convenience, unless indicated otherwise, we designate each plasmid by prefixing the name of the pyocin with the letter p, so that pSA1 is the plasmid encoding a chimeric pyocin named SA1. The structures of the chimeric and domain-deleted pyocins finally obtained are schematically shown in Fig. 3.

pAP41ΔII was obtained by removing the 508-bp *Alu*I fragment, which covers almost the whole of domain II and a small portion of domain III of pyocin AP41. Ligation of these *Alu*I sites created a *Hin*dIII site (pAP3). To make the sequence in frame, the newly created *Hin*dIII site was cut and ligated after its ends had been filled in (AP41ΔII). In pSA1, the 820-bp *Bam*HI-*Sst*I fragment of the pyocin S1 clone, including domain IV and the immunity protein, was replaced with the corresponding 896-bp *Xho*II-*Eco*RI segment of the pyocin AP41 gene. Deletion of the C-terminal part of SA1 by using the *Eco*RI sites in the coding region for domain IV and in the 3′ noncoding region of pSA1 produced SA2. SA2 had nine additional amino acids at its C-terminal end (indicated by a white box in Fig. 3), since the open reading frame for SA2 lost the original stop codon of the pyocin AP41 gene. To produce SA3, the domain I coding region was removed from pAP3 by cutting at the *Bam*HI site in the multiple cloning sites in the pUC vector and at the *Hin*dIII site and ligated after their ends had been filled in (pAP3ΔBH). The 5′ portion from the first *Nae*I site of the pyocin S1 gene, including the coding region for domain I, was inserted at the *Sma*I site at the 5′ end adjacent to the fused *Bam*HI-*Hin*dIII site of pAP3ΔBH in the correct direction (pSA3). pAS7 was constructed by removing the 5′ segment from the *Not*I site in the pyocin S1 clone and replacing it with the 5′ segment from the *Bss*HII site in the pyocin AP41 gene. In this case, ligation of both the filled *Bss*HII and *Not*I ends generated the in-frame sequence. pS1ΔIII was simply obtained by removing the segment between the two *Nae*I sites in the coding region for domain III. In pST23, the 5′ end from the *Eco*RV site of the pyocin S2 clone (domain I) was removed and in its place the 5′

TABLE 1. Properties of pyocins and their chimeras

Pyocin	MW[a] (10^3)	Killing activity[b] against:			Receptor specificity
		PML1516d	PAO3092	NIH3	
S1	65	3×10^6	−	6×10^4	S1
S2	74	6×10^6	−	6×10^5	S2
AP41	84	8×10^2	8×10^4	8×10^3	AP41
SA1	67	2×10^6	2×10^6	5×10^5	S1
SA2	58	−	−	−	S1[c]
SA3	66	1×10^6	1×10^6	1×10^5	S1
TA3	84	3×10^5	3×10^5	1×10^5	S2
TA4	70	3×10^5	3×10^5	1×10^5	S2
TA5	79	5×10^5	5×10^5	1×10^5	S2
AS7	83	±	−	10^2	AP41
AT11	80	±	−	10^2	AP41
S1ΔIII	48	−	−	−	S1[c]
S2ΔII	72	++	−	+	S2
AP41ΔII	65	+	++	+	AP41
ST23	77	++	−	+	S1
TS24	67	++	−	+	S2

[a] MW, molecular weight as estimated by SDS-PAGE.
[b] Killing activities for purified proteins are shown in units per milligram of protein. For partially purified proteins killing activity is shown as follows: −, no activity; ±, +, and ++, relative activities in increasing order.
[c] Determined by protection of adsorption (see Table 2).

TABLE 2. Protective effects of defective pyocins against the killing action of pyocins S1 and S2[a]

Treatment	Pretreatment[b]				
	None	S1ΔIII		SA2	
		100 ng	360 ng	100 ng	1,100 ng
None (control)	100	−	112	−	86
Pyocin S1, 1.5 ng (20 U/ml)	9	95	93	73	84
Pyocin S2, 1 ng (30 U/ml)	10	2.2	9.5	11.5	5.5

[a] The indicator cell suspensions were first treated with the indicated amounts of defective pyocins (S1ΔIII or SA2) for 10 min at 37°C and then treated with pyocin S1 or pyocin S2. After 30 min, numbers of colony formers were determined.
[b] Values are shown as percentages of the control value (100%), which was 4.1×10^7 cells per ml. −, not tested.

segment from *Not*I of the pyocin S1 gene was inserted after its end had been filled in. To construct the clones encoding TS24, TA3, and S2ΔII, two deletion derivatives of the pyocin S2 clone, pPBX and pBXB, were made in advance. pPBX had lost the upstream segment from the second *Bst*XI site in domain II by ligation of the *Pst*I site at the 5′ end and the *Bst*XI site in domain II, whereas the 3′ portion from the first *Bst*XI site of the pyocin S2 gene was deleted in pBXB by ligation of the *Bst*XI site and the *Bam*HI site of the vector at the 3′ end after the ends had been trimmed and filled in, respectively. The insert of pBXB, containing domain I of pyocin S2, was ligated by joining its *Sma*I site at the 3′ end, just outside the *Bam*HI site, with the end-filled *Not*I site of the pyocin S1 clone, resulting in pTS24. Similarly, by using the filled *Xma*I site instead of the *Sma*I site of pBXB, the 5′ portion of the pyocin S2 gene was ligated with the *Eco*47III site in the pyocin AP41 gene (pTA3). pTA4 was constructed by using the *Xma*I sites of pAP3ΔBH and pTA3. The ligation of the end-filled *Acy*I site at the head of domain III of pyocin S2 and the end-filled *Eco*T14 site at the end of domain II of pyocin AP41 made pTA5. pAT11 was generated by connecting the *Nae*I site in the middle of domain II of pyocin AP41 and the trimmed *Bst*XI site, the second of the two sites of the pyocin S2 gene. For deleting domain II of pyocin S2, the multiple cloning sites of the vector, the *Bam*HI site at the 3′ end of pBXB, and the *Hin*dIII site upstream of the *Pst*I site of pPBX were used to connect the 5′ and 3′ segments of pyocin S2 after both ends had been filled in.

Properties of pyocins and their derivatives. The killing activities and the receptor specificities of pyocins and their derivatives are summarized in Table 1. For those which were purified to homogeneity, specific activities (units per milligram of protein) are given, and for those partially purified, relative activities are shown. Receptor specificity was determined by using PML1570 ($S1^r$ $S2^s$) and PML1567 ($S1^s$ $S2^r$) or NIH3S1[r] ($S2^s$ $AP41^s$), NIH3S2[r] ($S1^s$ $AP41^s$), and NIH3 $AP41^r$ ($S1^s$ $S2^s$). It is clear from Table 1 and Fig. 3 that pyocin S1 receptor specificity is carried on the peptide of 1 to 218 amino acid residues (ST23), receptor specificity of S2 is carried on the peptide of 1 to 238 amino acid residues (TS24, TA3, and TA4), and receptor specificity of AP41 is carried on the peptide of 1 to 331 amino acid residues (AT11) or probably on that of 1 to 245 amino acid residues (AP41ΔII). Attempts to reduce the sizes of receptor domains were so far unsuccessful. Trimming of the S1 portion of ST23 to 1 to 168 amino acids (the *Ssp*I site) or deletion of two *Bal*I sites (resulting in deletion of amino acids 148 to 164) of pyocin S2, or shortening of the AP41 portion of AS7 to 1 to 168 amino acids (the *Xma*III site) resulted in inactivation of these proteins (data not shown). Therefore these proteins with defective receptor domains were poorly produced. Therefore these proteins were not purified. In pyocins AP41 and S2, amino acid stretches in domain II could be eliminated without loss of killing activity (AP41ΔII and S2ΔII). Chimeric proteins between pyocins S1 or S2 and AP41 (SA1, SA3, TA3, TA4, and TA5) showed slightly reduced activities compared with S1 and S2 on PML1516d but higher activities on PAO3092 than pyocin AP41, indicating that PAO3092 carries the receptors for pyocins S1 and S2, in addition to that for AP41. PAO3092 is insensitive to pyocins S1 and S2 by immunity, since it harbors the pyocin S2 gene on its chromosome (20).

The receptor specificities of the proteins lacking the killing activity (SA2 and S1ΔIII) were determined by their interaction with cells or isolated receptor substances. SA2 and S1ΔIII could bind to the cell surface receptor, protecting cells from being killed by pyocin S1 (Table 2). S1ΔIII protein as well as SA2 protein at 1 μg per ml gave protection against pyocin S1 but not against pyocin S2, suggesting that the receptors for pyocins S1 and S2 are different. This was further confirmed by studies with isolated crude receptor substances. Although susceptibility of PML1516d to both pyocins S1 and S2 increased under iron-limited conditions (13, 20), the two receptor substances behaved differently during isolation (Table 3). Thus, the cell envelope fraction (A) and the 2% Triton-insoluble fraction (C) neutralized both pyocins S1 and S2, but upon extraction with EDTA-Triton, receptor activity for pyocin S1 was solubilized (fraction D), while that for S2 disappeared (Table 3, experiment 1). Additions of fractions D and E did not restore the S2 receptor activity. Fraction D showed pyocin S1-neutralizing activity, which was completely inhibited by the addition of S1ΔIII or SA2 (Table 3, experiment 2). The 2% Triton-soluble fraction (B) (data not shown) and the EDTA-Triton-insoluble, residual fraction (E) did not neutralize either pyocin. These findings indicate that S1ΔIII and SA2 carry

TABLE 3. Interaction of the cell envelope proteins with pyocin S1 and defective pyocins

Substance (amt)	Residual activity (U) of pyocin:	
	S1	S2
Expt 1[a]		
None (control)	300	300
Fraction A (33 μg)	0	30
Fraction C (11 μg)	0	10
Fraction D (11 μg)	0	300
Fraction E (8 μg)	300	300
Expt 2[b]		
Pyocin S1 only (control)	240	
Fraction D (5.5 μg) and pyocin S1	80	
Fraction D (11 μg) and pyocin S1	0	
Fraction D (11 μg) + S1ΔIII (10 ng) and pyocin S1	0	
Fraction D (11 μg) + S1ΔIII (20 ng) and pyocin S1	3	
Fraction D (11 μg) + S1ΔIII (40 ng) and pyocin S1	240	
Fraction D (11 μg) + S1ΔIII (360 ng) and pyocin S1	240	
Fraction D (11 μg) + SA2 (230 ng) and pyocin S1	240	

[a] Pyocin S1 or S2 (about 10 ng) was incubated with the indicated amounts of the cell envelope proteins (fractions A to D) for 15 min at 37°C, and the residual activities were assayed.

[b] Fraction D was first incubated with the indicated amounts of S1ΔIII or SA2 for 15 min at 37°C and then incubated with pyocin S1 (about 10 ng) for 15 min at 37°C.

TABLE 4. Receptor-bypass killing by osmotic shock treatment[a]

Substance (amt [ng])	% Survival[b]
Expt 1	
None (control)	31
S1 (0.6, 6, 60)	14, 4.7, 1.2
S1ΔIII (500)	34
SA1 (500)	0.02
SA2 (580)	16
SA3 (500)	0.08
Expt 2	
None (control)	23.5
S2 (0.06, 0.6, 6, 60)	19, 11, 3.5, 0.8
Expt 3	
None (control)	10
S2 (60)	0.14
AT11 (60, 180, 600)	1.6, 0.52, 0.2
AS7 (60, 180, 600)	1.5, 0.49, 0.2
Expt 4	
None (control)	17.5
TA3 (500)	2.6
TA4 (500)	2.4
TA5 (500)	3.1
Expt 5	
None (control)	28.5
S2 (60)	0.14
TA5 (50, 150, 500)	0.5, 0.2, 0.08
Expt 6	
None (control)	18.5
AP41 (210, 700, 2,100)	2.4,1.0, 1.1

[a] PML1570 cells ($S1^r$) (experiment 1) or PML1567 cells ($S2^r$) (experiments 2 through 6) were osmotically shocked in the presence of the indicated amounts of pyocin or related proteins at 0°C.

[b] Number of colony formers expressed as a percentage of initial input.

the receptor-binding region of pyocin S1. In summary, the receptor-binding activity resides in the N-terminal region of all three pyocins, illustrated as domain I in Fig. 1 and 3. In pyocins AP41 and S2, an extra domain (domain II) which seems to be dispensable for the activity is present next to the receptor-binding domain. The function of this region remains unknown.

Translocation across the membrane measured by receptor bypass killing. Tilby et al. (23) reported that receptorless (resistant) *E. coli* cells could be killed by colicin E3 under conditions of osmotic shock (receptor bypass killing). Later, Eick-Helmerich and Braun (5) and Benedetti et al. (2) reported similar conditions for killing by other colicins. We investigated such conditions with *P. aeruginosa* and pyocins. The procedure described in Materials and Methods gave satisfactory results, as presented in Table 4. The osmotic shock alone reduced the viability of cells to 10 to 31%, which is higher than the viability of 5% (23) or 10% (5) reported previously. The amounts of pyocin proteins required to kill the osmotically shocked cells are also smaller than those reported for colicin E3 (23). PML1570 cells resistant to pyocin S1 or PML1567 cells resistant to pyocin S2 were killed by each pyocin, depending on the amounts of pyocins added (Table 4, experiments 1 and 2). Chimeric proteins SA1, SA3, TA3, TA4, and TA5 killed resistant cells under this condition (experiment 1, 2, and 5). Pyocin AP41, chimera AT11, and chimera AS7 killed PML1567 only poorly under normal conditions but more efficiently under bypass conditions. The efficiency of killing of resistant cells by pyocin S1 or S2 under conditions of osmotic shock was about 10% of the efficiency of killing of sensitive cells (PML1516d) under normal conditions. Among the proteins tested, S1ΔIII and SA2 showed no killing activity under conditions of osmotic shock (Table 4, experiment 1). Deletion of domain III in S1ΔIII should be responsible for the loss of activity, probably because of loss of translocation. The results obtained with SA2, lacking domain IV, suggest that the actual killing requires domain IV. One of the features of the present procedure is that an acidic medium (pH 4.3) was used with osmotic shock. The reason why the acidic medium gives efficient killing is not clear. The charge of the pyocin molecule and that of the membrane lipid may be important factors for the process. In the case of colicin A action on the phospholipid bilayers, insertion of the colicin into the membrane occurs preferentially in acidic conditions (7).

Effects of pyocins and chimeras on lipid synthesis. As reported previously, pyocins S1 and S2 inhibit the lipid synthesis of sensitive cells but AP41 does not, although different strains, PML1516d (for pyocins S1 and S2) and PAO3295 (for pyocin AP41), were used (16, 20). Since the chimeras between pyocins AP41 and S1 or S2 (SA1, SA3, TA3, TA4, and TA5) can kill either PML1516d or PAO3092, their effects on lipid synthesis were investigated. As shown in Fig. 4, SA1 and TA5 inhibited the incorporation of [2-^{3}H]glycerol into the lipid fraction in PML1516d (a) but not in PAO3092 (b). In PAO3092, incorporation in the cells treated with pyocin AP41, SA1, or TA5 increased linearly, whereas that in nontreated cells increased exponentially since the measurements were carried out on growing cells. In other words, growth of the treated cells was inhibited but

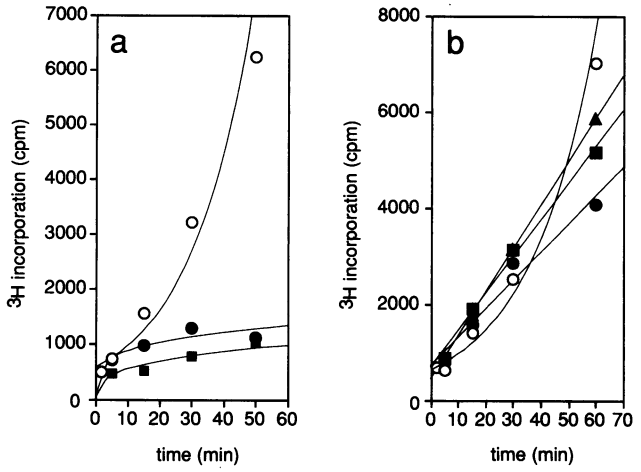

FIG. 4. Effects of pyocin AP41 and its chimeric proteins on lipid synthesis, shown by incorporation of [2-^{3}H]glycerol into the acid-insoluble fraction. (a) PML1516d cells in G medium with 1 μM $FeSO_4$. Symbols: ○, control without pyocin (ratio of surviving cells, 1); ●, cells with TA5 at 400 U/ml (4×10^{-3} at 50 min); ■, cells with SA1 at 200 U/ml (4×10^{-3} at 50 min). (b) PAO3092 cells in YT plus 5 mM $MgSO_4$. Symbols: ○, control without pyocin (ratio of surviving cells, 1); ▲, cells with AP41 at 200 U/ml (2.5×10^{-3} at 60 min); ●, cells with TA5 at 50 U/ml (1.3×10^{-3} at 60 min); ■, cells with SA1 at 100 U/ml (1×10^{-1} at 60 min). Activity units were determined with PML1516d for panel a and PAO3092 for panel b.

their lipid synthesis continued at the same rate for at least 60 min in PAO3092. Similar results were obtained with SA3, TA3, and TA4 used with PAO3092. The different results shown in panels a and b of Fig. 4 are not attributable to differences in the media, since chimeras, as well as pyocin S2, also inhibited lipid synthesis of PML1516d in YT medium (Table 5). Thus, the inhibition of lipid synthesis is observed in PML1516d but not in PAO3092 with chimeras of various combinations of domains. Differences in the sensitive strains, not in the pyocin proteins, probably give the different effects, although the mechanism remains unclear.

There is another difference between two strains. Susceptibility to pyocin S1 or S2, as well as to the chimeras, increased under iron-limited conditions in PML1516d (13), but susceptibility to these chimeras or to pyocin AP41 in PAO3092 did not change in accordance with the iron concentration. Pyocin receptors might be constitutively derepressed in PAO3092.

TABLE 5. Inhibition of lipid synthesis by pyocins[a]

Expt no.	Pyocin	Amt of pyocin added (U/ml)	Counts of ^{3}H incorporated (%)	Surviving cell ratio
1	None (control)		6,500 (100)	1
	TA4	100	1,660 (25.5)	7.7×10^{-3}
	TA5	25	1,150 (17.7)	9.1×10^{-3}
	SA1	25	1,170 (18)	3.5×10^{-2}
2	None (control)		8,500 (100)	1
	S2	800	370 (4.4)	2×10^{-4}
	TA3	200	1,530 (18)	2.4×10^{-3}
	SA3	400	700 (8)	4.4×10^{-4}

[a] Counts per minute of [2-^{3}H]glycerol incorporated in the acid-insoluble fraction and the surviving cell ratios at 60 min are shown. PML1516d cells cultured with YT-$MgCl_2$-α,α-dipyridyl were used.

AP41	619DGIYVYPEDSGIPPLYIVY637
S1	466DLIIGFPADSGIKPIYVMF484
S2	537DLIIGFPADSGIKPIYVMF555
E2	269DAVIRFPKDSGHNAVYVSV287
E3	269DAVIRFPKDSGHNAVYVSV287
DF13	227EAVIRFPKETGQKPVYVSV295

FIG. 5. Homology of the translocation domains of pyocins AP41, S1, and S2, colicins E2 and E3, and cloacin DF13. Numbers indicate amino acid residues, and shading indicates identical amino acids conserved among these bacteriocins. References for these sequences are as follows: pyocin AP41, reference 18; pyocins S1 and S2, reference 20; colicin E2, reference 4; colicin E3, reference 9; and cloacin DF13, reference 24.

Domain structures and functions of pyocins. The present study defined the domain structure of S-type pyocins by examining the functions of each domain. The receptor-binding domain was assigned to the N-terminal polypeptide of about 240 amino acids (domain I). This was deduced from the receptor specificity of chimeric proteins constructed by exchanging this domain. In the case of pyocin S1, actual binding to the cells and the receptor substance confirmed this point further.

The receptor-mediated process could be bypassed by osmotic shock in *P. aeruginosa* too. Pyocins and chimeras could kill otherwise resistant cells under the bypass condition. Translocation across the outer membrane (and presumably insertion into the cytoplasmic membrane) seemed to require domain III. Benedetti et al. defined a region responsible for translocation in colicins by a similar experiment (2).

DNase activity has been assigned to the C-terminal domain, domain IV (16, 18, 20). This was also confirmed by using chimeric pyocins, SA1, SA3, TA3, TA4, and TA5. Breakdown of chromosomal DNA with these chimeric proteins was observed by the procedure described before (20).

In summary, pyocins are composed of three functional domains, the receptor-binding domain, the translocation domain, and the DNase domain, in that order from their N termini. In pyocins AP41 and S2, extra stretches of peptides of unknown function are found as the second domain. The overall arrangement of domains is different in E2- and E3-group colicins with functions similar to those of S-type pyocins. In these colicins, the order is translocation domain, receptor-binding domain, and nuclease domain (14). The difference in the location of each domain is interesting in view of evolution. Besides the DNase domain highly conserved between S-type pyocins and E2-group colicins, we also found a conserved region at the end of the translocation domain (domain III), as shown in Fig. 5. Our unpublished observation indicates that this region is required for the killing activity.

Although our receptor bypass system worked well for the killing of *E. coli* by colicins E2 and E3, these colicins did not kill PML1516d cells, nor did pyocins S1 and S2 kill *E. coli* C600 cells, even under these conditions (data not shown). Apparently, some components required for translocation are different in the membranes of the two species.

We have not yet decided which domain(s) is responsible for the inhibition of lipid synthesis, since all the chimeras that are active on PML1516d showed inhibition of lipid synthesis and breakdown of the chromosomal DNA. Re-

cently we have constructed chimeras between pyocins and colicin E2 or E3, which will give more information about the structure and function of bacteriocins.

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