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Construction and Characterization of Pyocin-Colicin Chimeric Proteins

MAKOTO KAGEYAMA,[1] MIEKO KOBAYASHI,[1] YUMIKO SANO,[1]* AND HARUHIKO MASAKI[2]

Mitsubishi Kasei Institute of Life Sciences, 11, Minamiooya, Machida-shi, Tokyo 194,[1] and Department of Biotechnology, The University of Tokyo, Bunkyo-ku, Tokyo 113,[2] Japan

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Chimeric proteins were constructed from pyocin S1 or S2 and colicin E3 or E2, and their characteristics were investigated with special reference to the domain structure. The nuclease domains were interchangeable between two bacteriocins so that a new kind of pyocin, with RNase activity, was created. A bacteriocin which can kill both *Pseudomonas aeruginosa* and *Escherichia coli* was also constructed. Investigations with various chimeric proteins indicate that the translocation domain as well as the receptor-binding domain is species specific. Inhibition of lipid synthesis, which is characteristic of pyocins, was also observed with chimeric pyocins carrying the DNase domain of colicin E2 but not with those carrying the RNase domain of E3. Thus, the DNase domain is responsible for the inhibition of lipid synthesis.

Pyocins S1, S2, and AP41 are protease-sensitive bacteriocins frequently found among *Pseudomonas aeruginosa* strains (20). We have recently cloned and sequenced their genes, purified the proteins, and investigated the modes of action (16, 18, 21). These pyocins are highly similar to each other in structure and function. They are complexes of two components, the killing and the immunity proteins. The killing proteins consist essentially of three domains, the receptor-binding, translocation, and DNase (with immunity protein-binding) domains, arranged from N terminus to C terminus. Whereas the amino acid sequences of the receptor-binding domains differ considerably, those of the translocation and nuclease domains and the immunity proteins are strikingly similar among these pyocins. Particularly, pyocins S1 and S2 have essentially the same sequences in these regions and they share complete immunity (21). Pyocins S1, S2, and AP41 provoke the breakdown of cellular DNA of indicator strains, and their killing proteins as well as their C-terminal peptides covering the nuclease domain (S1 thermolysin fragment, for instance) show DNase activity in vitro (16, 21). A peculiar feature of pyocins S1 and S2, but not of AP41, is that they inhibit the synthesis of phospholipid in the sensitive strain PML1516d (12, 16, 19, 21). However, we do not yet know which domain or which portion of the protein is responsible for the inhibition of lipid synthesis.

As reported previously, a high degree of sequence homology between these pyocins and E2 group colicins was found in the nuclease domain and the immunity protein (18, 21). Colicin E2 shows DNA endonuclease activity in vitro (22) and causes degradation of chromosomal DNA in vivo (5, 11). These findings suggest that the pyocins and the colicins are evolutionarily related, deriving from a common ancestor. However, the arrangements of domains are different between pyocins and colicins: from N to C terminus, translocation, receptor-binding, and nuclease (with immunity protein-binding) domains in the latter case (14). Furthermore, no inhibition of phospholipid synthesis was observed with colicin E2 (2). To study whether domains are exchangeable between pyocins and colicins, and to assign the function inhibiting lipid synthesis to a certain domain, construction of chimeric proteins made of these two bacteriocins was attempted. Colicins E2 and E3 possess the same translocation and receptor-binding domains (except for one amino acid replacement) but different nuclease domains: the latter has an RNase domain (3, 9, 14). So far, no pyocin with RNase activity has been reported. Therefore, chimera construction was started with colicin E3, to see if an RNase type of pyocin can be created. Thus, we constructed chimeras with various combinations of domains from two bacteriocins, S1 or S2 and E2 or E3, and investigated their functions to clarify the role of domains.

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* Corresponding author. Mailing address: Mitsubishi Kasei Institute of Life Sciences, 11, Minamiooya, Machida-shi, Tokyo 194, Japan. Phone: 81-427-24-6261. Fax: 81-427-29-6317. Electronic mail address: sano@libra.ls.m-kagaku.co.jp.

MATERIALS AND METHODS

Media. Bacteria were grown in YT medium containing 5 mM $MgSO_4$ (21). When necessary, 100 μg of carbenicillin per ml (for the maintenance of plasmids) or 0.2 mM α,α′-dipyridyl (for Fe limitation) was added. D medium (6) with 1 μM $FeSO_4$ was employed when leucine incorporation was investigated.

Bacterial strains and plasmids. For construction of chimeric plasmids and purification of proteins, *Escherichia coli* C600 and other strains described before (21) were used as host strains. For assay of killing activity, the following strains were employed: *E. coli* C600 and derivatives carrying either *btuB* (E2 and E3 resistant) or *tolA* or *tolB* for colicin (constructed by H. Masaki) and *Pseudomonas aeruginosa* PML1516d and its derivative PML1570 ($S1^r$) or PML1567 ($S2^r$) for pyocin (19). Plasmids pYMSS11 for pyocin S1 and pYMPS1 for pyocin S2 (both on pUC119) have been described previously (21). pPET2 for pyocin S2, pSH352 or pSH357 for colicin E3, and pSHME2 for colicin E2 were constructed in this study (Fig. 1). pPET2 was made from pYMPS1 by replacing its promoter part with that of pSH357. A 1.75-kbp sequence between two *Vsp*I sites (one in the *amp* gene of the vector and one in front of Shine-Dalgarno sequence of S2) was substituted with a 1.47-kbp *Vsp*I fragment of the corresponding region of pSH357 including the SOS promoter of E3. pSH352 and pSH357 are derivatives of pSH350, which carries the colicin E3 operon on pBR327 (8, 23). In pSH352, the 34-bp *Dra*I fragment at the junction of the receptor-binding and the nuclease domains was replaced by the blunt-ended 34-bp *Hin*dIII-*Eco*RI fragment of the M13mp9 multiple cloning site including the *Pst*I-*Sal*I (*Acc*I)-*Bam*HI-*Sma*I (*Xma*I) sites. This modified colicin E3 (with 10 amino acid changes) showed killing activity more than 10% that of the original E3 protein. In pSH357, an *Xho*II site near the end of the nuclease domain was modified to a *Bam*HI site without changing the encoded amino acid (8). pSHME2 was constructed from pSH357 by replacing its *Aat*II-*Pvu*II sequence ranging from the translocation domain to the lysis protein of colicin E3 with the corresponding *Aat*II-*Pvu*II fragment (1.71 kbp) from the plasmid Col E2-P9 (3). pS1I, a plasmid expressing the S1 immunity gene, was constructed by inserting the *Bam*HI-*Sac*I fragment (S1 nuclease domain and its immunity protein, 0.82 kbp) of pYMSS11 into the *Bam*HI-*Sac*I sites of pKT230 (1).

Construction of chimeras. DNA manipulations were performed according to the method of Sambrook et al. (15). A DNA blunting kit and a DNA ligation kit from Takara (Kyoto, Japan) were used. The Geneclean kit from Bio 101 (La Jolla, Calif.) was employed for isolation of DNA fragments. The construction of plasmids encoding chimeric proteins was carried out by utilizing the cleavage sites of restriction enzymes. The actual processes are presented below (Fig. 1 and

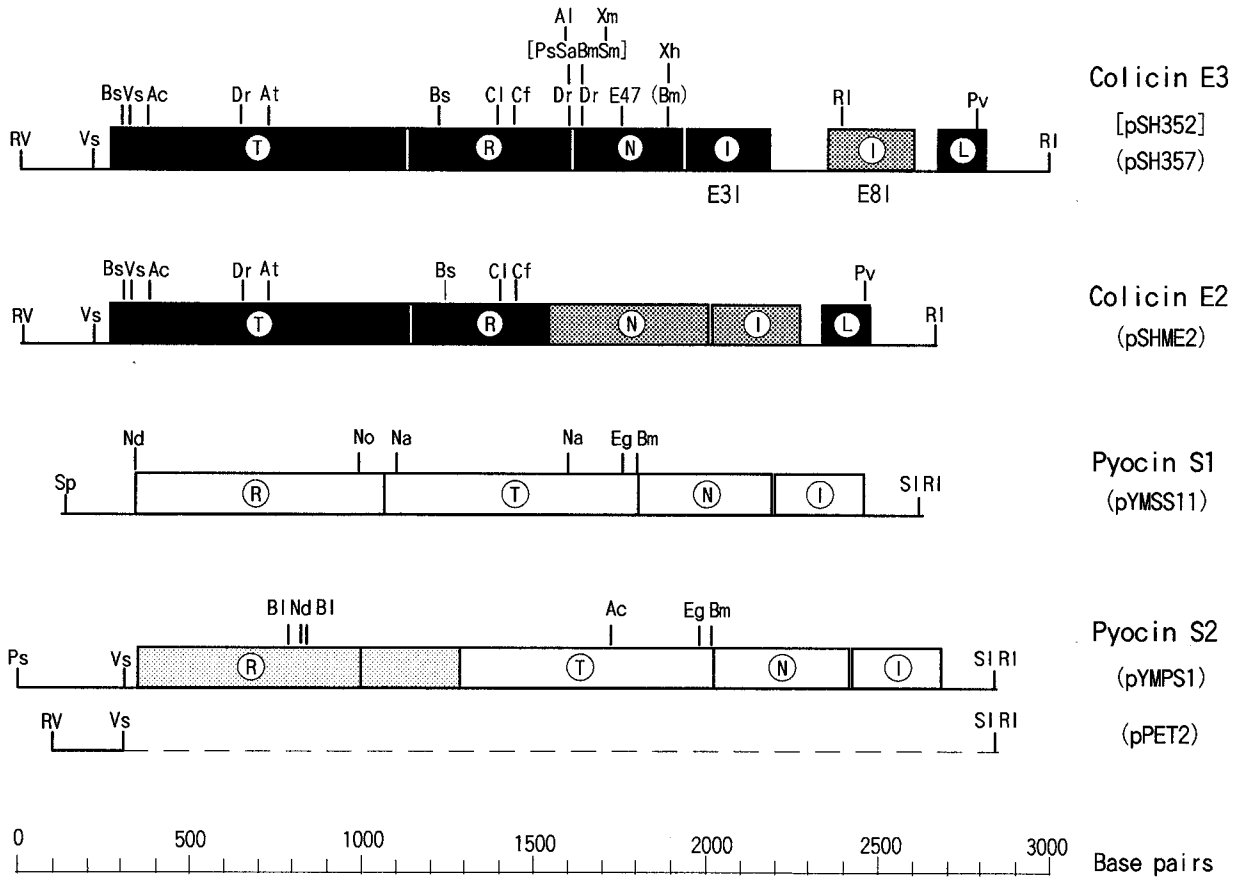
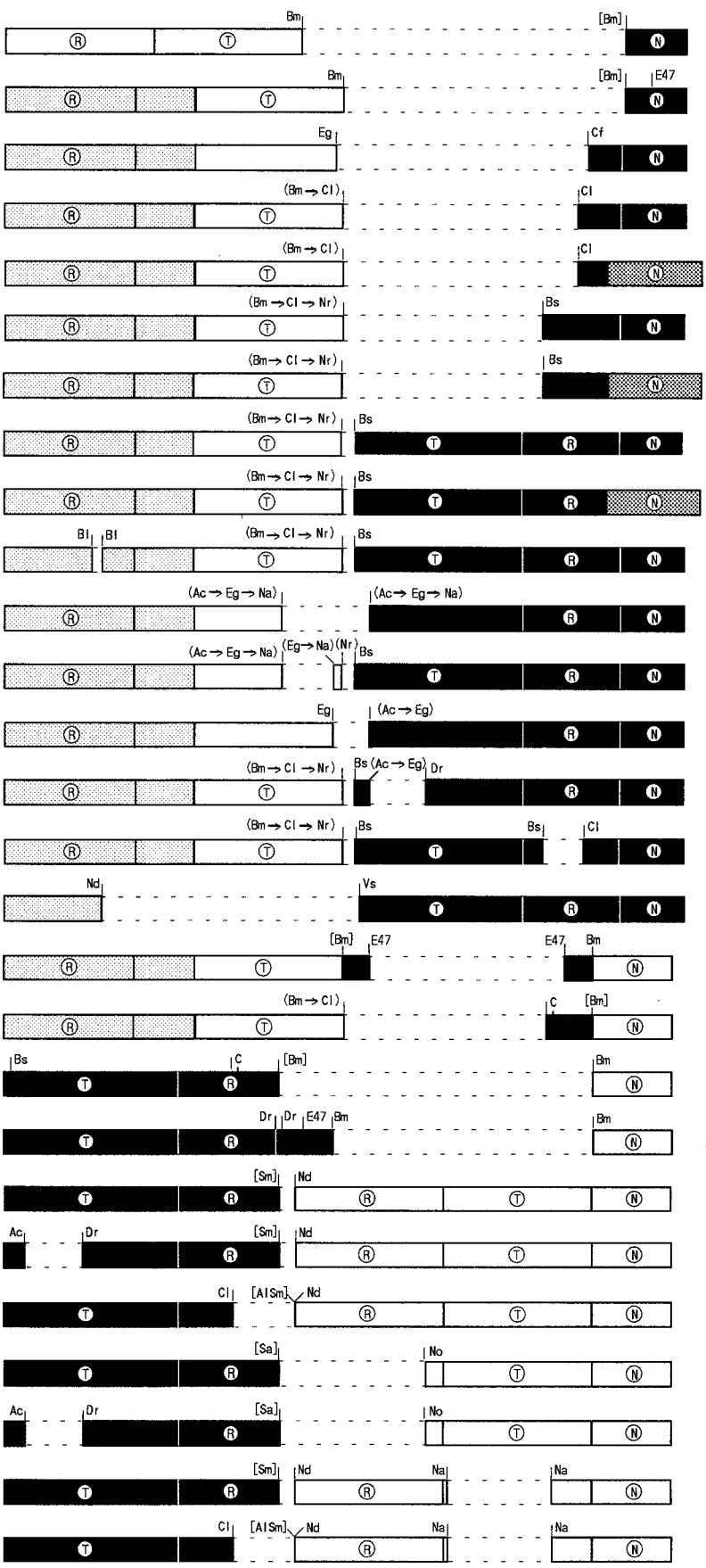


FIG. 1. Physical map of genetic determinants for colicins and pyocins. The protein structures and the restriction sites used for the construction of chimeras are shown. Regions with identical or nearly identical amino acid sequences are shaded in the same pattern. Circled letters T, R, N, I, and L signify the translocation domain, the receptor-binding domain, the nuclease domain, the immunity protein, and the lysis protein, respectively. Colicin genes are cloned on pBR327. The colicin E3 plasmid carries an extra immunity gene, E8I, which shows homology with E2I (24). Restriction sites in brackets are in pSH352, and the site in parentheses is in pSH357. Pyocin genes are cloned on pUC119. In pPET2, the promoter region of pYMPS1 is replaced with that of pSH357. The region indicated by the dotted line is the same as pYMPS1. The scale shows size in nucleotide base pairs. Abbreviations: Ac, *Acc*III; AI, *Acc*I; At, *Aat*II; BI, *Bal*I; Bm, *Bam*HI; Bs, *Bss*HII; Cf, *Cfr*I; Cl, *Cla*I; Dr, *Dra*I; E47, *Eco*47III; Eg, *Eag*I; Na, *Nae*I; Nd, *Nde*I; No, *Not*I; Ps, *Pst*I; Pv, *Pvu*II; RI, *Eco*RI; RV, *Eco*RV; Sa, *Sal*I; SI, *Sac*I; Sm, *Sma*I; Sp, *Sph*I; Vs, *Vsp*I; Xh, *Xho*II; Xm, *Xma*I.

2). For convenience, each plasmid is designated by prefixing the name of the protein with the letter p so that, e.g., pSE3 is the plasmid encoding a chimeric protein named SE3.

pSE3 or pTE3 was constructed by replacing the *Bam*HI-*Eco*RI sequence of pYMSS11 or pYMPS1 with the 0.77-kbp *Bam*HI-*Eco*RI fragment of pSH352 and restoring the reading frame by utilizing *Xma*I, cut and ligated after filling in the ends. With pES1 or pET1, the *Acc*I (in *Sal*I) site of pSH352 was first cut, end filled, and ligated and its *Bam*HI-*Eco*RI region was replaced by that of pYMSS11 or pYMPS1 (0.82 kbp). Similarly, pES2 or pET2 was made by exchanging the *Bam*HI-*Eco*RI region of pSH357 with that of pYMSS11 or pYMPS1. pTE40 was constructed by placing the 0.96-kbp *Cfr*I-*Eco*RI fragment of pSH357 in the *Eag*I-*Eco*RI position of pYMPS1, as both fragments had the same cohesive ends. To construct pTE52 or pTE22, pYMPS1 was first cut with *Bam*HI enzyme and then end filled and ligated, which resulted in a *Cla*I site. The *Cla*I-*Eco*RI sequence of this plasmid was replaced by the 1-kbp *Cla*I-*Eco*RI fragment of pSH357 to form pTE52 or by that of pSHME2 (1.27 kbp) to form pTE22. Likewise, pTET4 was made by substituting the 1.05-kbp *Cla*I-*Eco*RI fragment from pET1. pTET1 was constructed from pTE3 by exchanging its *Eco*47III-*Eco*RI region with that of pET2 (0.95 kbp). pTE53, pTE23, pTE54, and pTE24 were made from pPET2. pPET2 was first cut with *Bam*HI enzyme and then end filled and ligated to create a *Cla*I site, which was then converted to an *Nru*I site in the same way. Next, this plasmid was cut with *Nru*I and *Eco*RI enzymes and the longer fragment was saved. Meanwhile, pSH357 was cut with *Bss*HII enzyme and the sticky ends were filled in. The 1.16-kbp *Bss*HII-*Eco*RI fragment was isolated and placed between the *Nru*I-*Eco*RI sites of the former plasmid, resulting in pTE53. The *Bss*HII site was regenerated. pTE54 was obtained by inserting the 0.94-kbp *Bss*HII-*Bss*HII fragment of pSH357 into the *Bss*HII site of pTE53 in the correct order. pTE24 was derived from pTE54 by exchanging its *Cla*I-*Eco*RI fragment with that of pSHME2 (1.27 kbp). pTE23 was obtained by deleting the *Bss*HII-*Bss*HII region from pTE24. pTE54ΔBI was made by deleting a sequence between two *Bal*I sites. pTE54ΔAA, pTE54ΔAE, and pTE54ΔEA were constructed as follows. pTE54 was cleaved with *Acc*III enzyme and end filled and ligated to make an *Eag*I site (pTE54ΔApE), and then this site was similarly changed to an *Nae*I site, resulting in TE54ΔAA. The 2.02-kbp *Eag*I-*Eco*RI fragment was isolated from pTE54ΔApE and inserted to replace the *Eag*I-*Eco*RI portion of pTE54, giving pTE54ΔEA. Conversely, the *Eag*I-*Eco*RI region of pTE54ΔApE was replaced with that of pTE54 (2.13 kbp) and the *Eag*I site was converted to an *Nae*I site, resulting in pTE54ΔAE. For the construction of pTE54ΔAD, pSH352ΔAD was made in advance. pSH352 was cut at the *Acc*III site and the sticky ends were filled in, and then the plasmid was digested with *Bam*HI enzyme. A 0.98-kbp *Dra*I-*Bam*HI fragment was isolated from another preparation of pSH352 and put in place between the *Acc*III (filled)

FIG. 2. Construction of chimeric proteins and deletion derivatives and their activities. Joining restriction sites are connected with dotted lines. Arrows (Bm→Cl→Nr, etc.) show the modification of restriction sites by end filling and ligation. Numbers for amino acid residues include the N-terminal Met. Specific activities (units per milligram of protein) are given for samples purified to homogeneity. Relative activities in increasing order (− to +++) are given to less-purified samples. The numbers of amino acid residues and the specific activities of original colicins and pyocins are as follows: E3, 551 and 5×10^6 (*E. coli* C600); E2, 581 and 10^7 (C600); S1, 618 and 3×10^6 (*P. aeruginosa* PML1516d); S2, 690 and 6×10^6 (PML1516d). Nr, *Nru*I. Other symbols and abbreviations are as described in the legend for Fig. 1.



Chimeric protein	Amino acid residues	Killing activity against PML1516d	Killing activity against E.coliC600
SE3	585	$2x10^5$	–
TE3	656	$5x10^5$	–
TE40	702	–	–
TE52	732	$2x10^5$	–
TE22	762	$5x10^5$	–
TE53	784	$3x10^5$	–
TE23	814	$4x10^5$	–
TE54	1,097	$6x10^4$(++)	$2x10^5$(+++)
TE24	1,127	++	+++
TE54ΔBI	1,080	–	+++
TE54ΔAA	975	–	–
TE54ΔAE	1,013	–	+++
TE54ΔEA	1,058	–	–
TE54ΔAD	1,008	++	–
TE54ΔBC	1,047	++	–
TE33	690	–	+++
TET1	780	$4x10^5$	–
TET4	768	$2x10^5$	–
ES1, ET1	586, 587	–	$2x10^5$
ES2, ET2	674, 675	–	10^5
ES3	1,073	–	$2x10^6$
ES3ΔAD	985	+++	–
ES3ΔCA	999	+++	–
ES3ΔSN	853	–	$1.6x10^6$
ES3ΔAD, SN	764	–	–
ES3ΔNa	908	–	++
ES3ΔCA, Na	834	–	–

and *Bam*HI sites of the pSH352 fragment described above. The *Acc*III site restored was then converted to an *Eag*I site. From pSH352ΔAD thus obtained, the 0.67-kbp *Bss*HII-*Bss*HII fragment was isolated. This fragment was inserted in the *Bss*HII site of pTE53, giving pTE54ΔAD. pTE54ΔBC was constructed as follows. The sequence between the *Bss*HII and *Cla*I sites was removed from pTE54ΔApE, and the sticky ends were filled in and ligated to regenerate the *Bss*HII site. From this plasmid, a fragment between two *Aat*II sites (one in the translocation domain of E3 and the other in the pUC119 vector, 1.97 kbp) covering the deleted E3 domain was isolated and inserted in place of the corresponding two *Aat*II sites of pTE54. To construct pTE33, a segment from the *Nde*I site to the *Eco*RI site of pYMPS1 was replaced by the 2.07-kbp *Vsp*I-*Eco*RI fragment of pSH357 carrying the same cohesive ends. pES3 was constructed by substituting the *Sma*I-*Eco*RI sequence of pSH352 with the 2.28-kbp *Nde*I (filled)-*Eco*RI fragment of pYMSS11. pES3ΔAD was made in a way similar to that used for pTE54ΔAD, by replacing the *Bss*HII-*Bss*HII region with that of pSH352ΔAD. pES3ΔCA was produced by cutting pES3 with *Cla*I and *Acc*I enzymes (at the *Sal*I site), filling in the ends, and ligating. An *Nru*I site was created at the junction. pES3ΔSN was obtained by cleaving pES3 with *Sal*I and *Not*I enzymes, filling in the ends, and ligating. Likewise, pES3ΔAD,SN was derived from pES3ΔAD. pES3ΔNa or pES3ΔCA,Na was simply made by deletion with *Nae*I enzyme. Every chimera thus constructed carries an immunity gene corresponding to the nuclease domain.

Purification of bacteriocin proteins. The purification procedure was essentially the same as described previously (19, 21), except that cells were broken by a Branson sonifier and the final step was conducted with the fast-protein liquid chromatography (FPLC) system LCC500 by using a Mono S column (HR5/5; Pharmacia LKB). For colicin E3 and ES3ΔCA,Na, Mono Q column (HR5/5) chromatography was applied (in 0.01 M Tris-HCl [pH7.5]; NaCl gradient elution, 0 to 0.3 M). When the bacteriocin production was under the control of the SOS promoter (colicins E3 and E2, etc.), the culture was treated with mitomycin C (1 μg/ml) at mid-log phase and the cells were harvested 2 h later. Separation of the killer (A) and the immunity (I) proteins was carried out under the conditions described before (21), except that a column of Superose 12 (HR10/30) with FPLC (Pharmacia LKB) was employed and proteins were recovered and concentrated with Centricon 10 or Centricon 30 tubes (Amicon, Grace Japan, Tokyo, Japan). Separated proteins are designated by the suffix A (killing activity) or I (immunity), such as, e.g., SE3A or ET1I. ET1I signifies the immunity protein prepared fom ET1, although it is nothing but S2I. DNase-active S1 thermolysin fragment (peptide of the DNase domain) was prepared as described before (21).

Assay methods. Methods for measuring killing activity (units), the receptor-binding assay, and the translocation assay (receptor bypass killing) have been described previously (19). Specific activity is expressed as the killing units per milligram of protein. DNA decomposition in vivo and DNase activity in vitro were assayed as described before (21). RNase activity was measured by using 16S-23S rRNA of *E. coli* (Boehringer Mannheim) as a substrate. The reaction mixture (10 μl) contained 400 ng of rRNA in 50 mM Tris-HCl buffer (pH 7.5), 50 mM NaCl, 5 mM EDTA, and the sample to be tested. After 2 h at 37°C, 2.5 μl of gel-loading buffer (40% sucrose, 0.125 M EDTA, and 0.5% sodium dodecyl sulfate, pH 8) was added and the mixture was heated at 95°C for 2 min and electrophoresed on a 1% agarose gel with ethidium bromide (0.5 μg/ml). Lipid synthesis was measured by incorporation of [2-^{3}H]glycerol as reported before (12). The conditions for measuring protein and lipid syntheses at the same time were as follows. PML1516d cells were cultured in D medium (Fe concentration, 1 μM). When the cell density became 2×10^8 to 3×10^8/ml, [2-^{3}H]glycerol and [^{14}C]leucine were added at the final concentrations of 2 μCi (0.2 μmol) and 0.4 μCi (0.1 μmol)/ml, respectively, and then bacteriocin was added. At appropriate times, 50-μl portions were removed and dried on 24-mm-diameter filter paper disks. After serial washing with 10% trichloroacetic acid and water, radioactivities on the disks were counted differentially for ^{3}H and ^{14}C with a Beckman scintillation counter.

Other methods. Estimations of concentration, size, and purity of proteins were made as described previously by UV absorption and by using the Pharmacia Phast Gel System (21). Agarose gel electrophoresis assays for DNA and RNA were done as reported before (16, 21).

RESULTS AND DISCUSSION

Killing properties of chimeras with exchanged nuclease domains. In the chimeric proteins SE3, TE3, ES1, ES2, ET1, and ET2, the DNase domain of S1 (133 amino acid residues) or S2 (134 amino acids) and the RNase domain of E3 (100 amino acids) were exchanged, along with the immunity gene (Fig. 1 and 2). These proteins showed killing activities as represented in Tables 1 and 2 and Fig. 2. Chimeric proteins SE3 and TE3, composed of the receptor-binding and the translocation domains of pyocins S1 and S2, respectively, and the nuclease domain of colicin E3, killed *Pseudomonas* strains with the receptor specificities of S1 and S2, respectively, but did not kill *E. coli* (Table 1 and Fig. 2). Therefore, these chimeras are

TABLE 1. Killing specificities of pyocins and chimeras[a]

Sample	Killing activity against *P. aeruginosa* strain[b]:				Specificity	
	PML1516d	PML1570 (S1^r)	PML1567 (S2^r)	PML1516d (pS1I)	Killing	Immunity
S1	+	−	+	−	S1	S1 and S2
S2	+	+	−	−	S2	S1 and S2
SE3	+	−	+	+	S1	E3
TE3	+	+	−	+	S2	E3

[a] Specific activities (units per milligram of protein) of purified samples are shown in Fig. 2.
[b] +, killing; −, no killing.

kinds of pyocins. The susceptibility of the *Pseudomonas* strain to either chimera was higher when the cells were cultivated under conditions of iron deficiency (data not shown), as was the case with S1 or S2 (21). SE3 and TE3 killed cells expressing the S1 immunity gene, indicating that the immunity specificities are not of the S1 or S2 type but probably of the E3 type (see below). These findings indicate that the nuclease domain of colicin E3 can function in *Pseudomonas* strains as a part of a pyocin, although specific activities of these chimeric proteins were about 1/10 of those of original pyocins (Fig. 2). On the other hand, chimeras ES1, ES2, ET1, and ET2, consisting of the translocation and the receptor-binding domains of colicin E3 and the nuclease domain of pyocin S1 or S2, killed *E. coli* but not *P. aeruginosa*. Properties of these chimeras are compared with those of colicins E2 and E3 in terms of killing and immunity specificities (Table 2). A receptor substance for colicin E3 (*btuB*), as well as *tolA* and *tolB* functions, was required for killing. These chimeras killed pCol E3-bearing cells, and colicin E3 killed cells bearing these chimera plasmids but not those bearing pSE3 or pTE3. These results indicate that the nuclease domain of pyocin can function in *E. coli* as a part of colicin and that killing specificities are determined by the receptor-binding domains and immunity specificities are determined by the nuclease domains. As described in a previous paper (19), receptorless (resistant) cells can be killed by the bacteriocins under certain conditions of osmotic shock. Under such receptor bypass conditions, chimeras SE3 and TE3 as well as S1 and S2 killed *Pseudomonas* strains but not *E. coli* and ET1 and ET2 as well as E3 and E2 killed *E. coli* but not *Pseudomonas* strains (data not shown). Thus, the translocation specificities were not modified by replacing the nuclease domains.

Compared with ES1 or ET1, ES2 and ET2 carry a longer stretch of E3 peptide extending into the RNase domain, lacking only 10 amino acids from the C terminus (Fig. 2). In ES2 and ET2, the pyocin DNase domain only, but not the trimmed colicin RNase domain, seems to be functional for killing. Unlike colicin E3, ES2 and ET2 do not kill the cells carrying pES1 or pET1, which are devoid of the E3I gene (Table 2). Specific activities of the fresh samples of ES2 and ET2 were essentially the same as those of ES1 and ET1 (Fig. 2). However, ES2 and ET2 were less stable, being liable to decompose and lose activity upon storage (data not shown).

Nuclease activities of chimeras with exchanged nuclease domains. Pyocins S1 and S2 caused breakdown of chromosomal DNA of the indicator cells under growing conditions (21). However, SE3 and TE3 did not show such action, while the killing was efficient. On the other hand, ET1, ET2, and E2, but not E3, caused DNA degradation in *E. coli* cells (data not shown). These findings suggest that chimeras ET1 and ET2 possess DNase activity, and SE3 and TE3 might show RNase

TABLE 2. Killing specificities of colicins and chimeras[a]

Sample	Killing of *E. coli* strains carrying[b]:										Specificity	
	No plasmid	*btuB*	*tolA*	*tolB*	pCol E2	pCol E3	pES1 or pET1	pES2 or pET2	pSE3	pTE3	Killing	Immunity
E2	+	−	−	−	−	+	+	+	+	+	E2	E2
E3	+	−	−	−	+	−	+	+	−	−	E3	E3
ES1	+	−	−	−	+	+	−	−	+	+	E2 or E3	S1 and S2
ES2	+	−	−	−	+	+	−	−	+	+	E2 or E3	S1 and S2
ET1	+	−	−	−	+	+	−	−	+	+	E2 or E3	S1 and S2
ET2	+	−	−	−	+	+	−	−	+	+	E2 or E3	S1 and S2

[a] Specific activities (units per mg of protein) of purified samples are shown in Fig. 2.
[b] +, killing; −, no killing.

activity in response to the altered nuclease domains. These points were verified by in vitro experiments. ET1A, a chimeric protein with the immunity protein removed, had DNase activity, as shown in Fig. 3. This activity, as well as that of the S1 thermolysin fragment which shows DNase activity higher than that of S1A (21), was inhibited by the addition of the immunity protein S1I or ET1I (same as S2I). Both the S1 thermolysin fragment and ET1A did not degrade RNA under the RNase assay conditions. Thus, by substituting its nuclease domain with that of pyocin, colicin E3 became an E2 type colicin with the immunity of pyocin S1 and S2. On the contrary, SE3A exhibited the activity of decomposing RNA in vitro (Fig. 4). The level of this activity was not very high, but it was of the same level as that of colicin E3A. According to previous authors, the RNase activity of colicin E3 was inhibited by the addition of E3I (13). Under our assay conditions, mere addition of the immunity protein to the reaction mixture was not effective for inhibition. However, by preincubation of SE3A or E3A with SE3I or E3I at 75°C for 10 min, the RNase activity was neutralized. Furthermore, SE3I or E3I was more effective when either was preheated at 95°C for 10 min. SE3A or E3A itself became more active by 75°C treatment but was inactivated at 95°C (Fig. 4). The thermal effects suggest that some steric structures are required for the interaction, enzyme-RNA or enzyme-inhibitor. Neither SE3A nor E3A decomposed DNA (Fig. 3). Thus, SE3 is a new kind of pyocin carrying RNase activity with E3 immunity. The above results indicate that the nuclease domains of colicin E3 and pyocin S1 or S2 can function independently of other domains of the molecule in either microorganism.

Properties of chimeras with various combinations of domains of pyocins and colicin E3 or E2. Construction of more chimeras made from these pyocins and colicins E2 and E3 was attempted to see the roles of domains in more detail and to investigate the effects of these chimeras on the biosynthesis of phospholipid. Chimeras constructed in the present study and their killing activities are shown in Fig. 2. Pairs of chimeras, TE52 and TE22 or TE53 and TE23, were used to compare the effects of nuclease domains of two colicins, E3 and E2, set downstream of the receptor-binding and the translocation domains of pyocin. Their killing activities were all very high with *Pseudomonas* strains, essentially the same level as that of TE3, irrespective of the size of the colicin part. However, TE40 was completely devoid of activity. This chimera lacks a small region (*Eag*I-*Bam*HI fragment, 15 amino acids) which corresponds to a common peptide found at the end of translocation domains

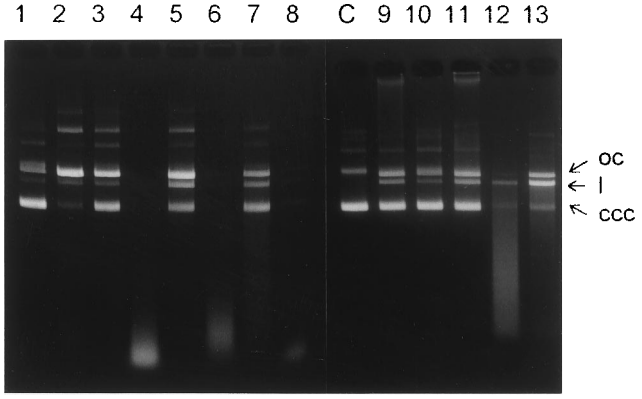


FIG. 3. DNase activity of chimeric proteins and S1 thermolysin fragment and effects of immunity proteins. pUC119 DNA was used as a substrate. The reaction mixture was incubated for 1 h at 37°C and analyzed by agarose gel electrophoresis. oc, l, and ccc signify open circular, linear, and covalently closed circular DNA, respectively. C, control (DNA only). ET1A (88 ng) was added to lanes 1 to 4. S1 thermolysin fragment (30 ng) was added to lanes 5 to 8. ET1I was added to lanes 1 and 5 at 800 ng each and to lanes 2 and 6 at 200 ng each. S1I (400 ng) was added to lanes 3 and 7. No immunity protein was added to lanes 4 and 8. In lanes 1 to 8, reactions were started upon addition of DNase. SE3A at 900 ng each was added to lanes 9 and 10, E3A at 920 ng was added to lane 11, and ET1A (44 ng) was added to lanes 12 and 13. Proteins added to lanes 10, 11, and 13 were preheated at 75°C for 10 min. In lanes 9 to 13, reactions were started upon addition of DNA.

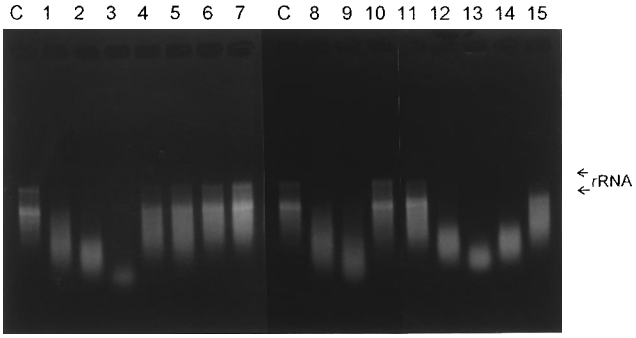


FIG. 4. RNase activity of chimeric proteins and E3A and effects of immunity proteins. rRNA was added last to start the reaction. C, control (no enzyme). Effects of heat treatment on the activities of enzymes and immunity proteins are shown in lanes 1 to 11, where 900 ng of SE3A (each) was added (except in lane 7). SE3I (450 ng) was added to lanes 2, 4, 6, and 7. SE3I at 450 ng and E3I at 860 ng, both preheated at 95°C for 10 min, were added to lanes 10 and 11, respectively. Reaction mixtures were treated for 10 min at the following temperatures before addition of the substrate rRNA: lanes 1, 2, and 8, 37°C; lanes 3, 4, 9, 10, and 11, 75°C; lanes 5, 6, and 7, 95°C. Lane 7, SE3I only. Thermal effects upon E3A (920 ng) are shown in lanes 12 to 15. E3I preheated at 95°C was added to lane 14 at 290 ng and to lane 15 at 860 ng. Reaction mixtures were treated for 10 min at 37°C (lane 12) or 75°C (lanes 13 to 15) before addition of rRNA.

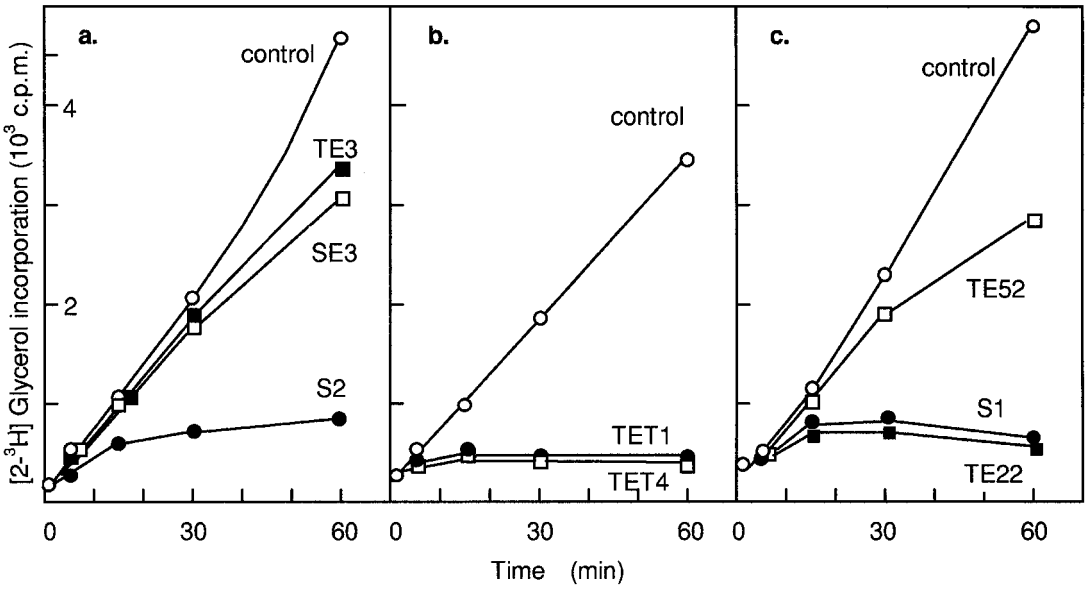


FIG. 5. Effects of pyocins and their chimeras on lipid synthesis. [2-^{3}H]glycerol was added to a culture of PML1516d in YT with dipyridyl 3 min before bacteriocin addition. At 0 min, pyocin or chimera was added. Portions of 50 μl were removed at the indicated times, and the radioactivities in the acid-insoluble fraction were counted. Amounts of bacteriocin added (units per milliliter) and the numbers of colony formers at 60 min relative to the control value (100%) were as follows: (a) S2, 100, 1.8%; SE3, 400, 1%; TE3, 400, 5%; (b) TET1, 100, 1.8×10^{-2}%; TET4, 400, 4.4×10^{-3}%; (c) S1, 200, 0.8%; TE52, 100, 5%; TE22, 200, 3.4×10^{-2}%.

of various pyocins and colicins (19). TE54 and TE24 showed a peculiar activity, killing both *E. coli* and *Pseudomonas* strains. These chimeras consist of the receptor-binding and the translocation domains of pyocin S2 followed by virtually the entire colicin E3 or E2 molecule. When deletions were introduced into each domain of TE54, the resulting proteins had either pyocin (TE54ΔAD or TE54ΔBC) or colicin (TE54ΔBI or TE54ΔAE) activity, depending upon the position of deletion: a lesion in one domain caused the loss of the corresponding bacteriocin activity (Fig. 2). On the other hand, ES3 carrying the entire pyocin S1 molecule downstream of the translocation and the receptor-binding domains of E3 killed only *E. coli* and not *Pseudomonas* strains. However, by introducing deletions into ES3, pyocin (ES3ΔAD or ES3ΔCA) as well as colicin (ES3ΔSN or ES3ΔNa) activities appeared (Fig. 2). In ES3 the activity against *Pseudomonas* strains was probably hindered by some steric structure, and the hidden pyocin activity was disclosed by deleting colicin sequences from ES3. These results indicate that although the nuclease domain can function in either species, the translocation domain, as well as the receptor-binding domain, is species specific and that the steric structure of the molecule modulates the activity. The breakdown of cellular DNA was observed not with the proteins TE52, TE53, and TE54 but with their counterparts carrying E2 peptide (TE22, etc.). That the colicin activity remains unchanged when its N terminus is trimmed down to the *Vsp*I site (20 amino acids, TE33) but not to the *Acc*III site (37 amino acids, TE54ΔEA) is also indicated.

In a previous paper, we described that the receptor substance for pyocin S1 neutralized the killing action of pyocin S1 and that some defective pyocin proteins blocked the neutralizing ability of the receptor substance (19). Under the same assay conditions, chimeric proteins ES3 and ES3ΔCA,Na were effective and ES3ΔSN and ES3ΔAD,SN were ineffective in the blocking reaction in response to the presence or absence of the receptor-binding domain of S1 (data not shown). Under receptor bypass conditions, ES3ΔSN killed both *Pseudomonas* strains and *E. coli* whereas ES3ΔAD,SN killed *Pseudomonas* strains only. The translocation domain of colicin is apparently required to kill *E. coli.*

Effects of chimeras on phospholipid synthesis. As reported before, pyocins S1 and S2 and their chimeras with pyocin AP41 inhibited the phospholipid synthesis of the indicator strain PML1516d, whereas these chimeras and pyocin AP41 did not inhibit phospholipid synthesis of strain PAO3092, a good indicator for pyocin AP41 but immune to S1 or S2 (12, 16, 19, 21). So far, the inhibition seems to be specific for strain PML1516d. Chimeras constructed from pyocins and colicins were examined in this respect. SE3 and TE3, chimeric pyocins with the RNase domain of colicin E3, did not inhibit lipid synthesis (Fig. 5a). TET1, carrying the DNase domain of S2 downstream of almost the entire molecule of TE3, recovered the inhibitory function (Fig. 5b). TE52 and TE22 exhibited contrasting effects. The chimera with the E3 RNase domain did not show inhibition, but that with the E2 DNase domain did show inhibition (Fig. 5c). TET4, in which the RNase domain of TE52 was replaced with the DNase domain of S2, again recovered inhibitory activity (Fig. 5b). These findings suggest that the DNase domain, either of pyocin or colicin, is responsible for the inhibition of lipid synthesis. Next we investigated whether this inhibition is directly linked to the killing. Figure 6 shows the effects of various amounts of TE22 on the lipid synthesis and viability of cells. There was a linear correlation between residual synthetic rates and surviving fractions of the culture; the extent of inhibition was parallel to that of killing. It is interpreted that the inhibition of lipid synthesis is an all-or-none process.

With pyocin S2, inhibition of protein synthesis follows that of lipid synthesis (12). Colicin E3 has been known to inhibit protein synthesis of *E. coli* cells (10). We examined the effects of chimeric proteins on the syntheses of lipid and protein at the same time in the same cultures. TE53 with the RNase domain inhibited protein synthesis but not lipid synthesis, whereas TE23 with the DNase domain inhibited both processes (Fig. 7). The results with TE23 were essentially the same as those obtained with pyocin S2 reported before (12). Thus, TE53 be-

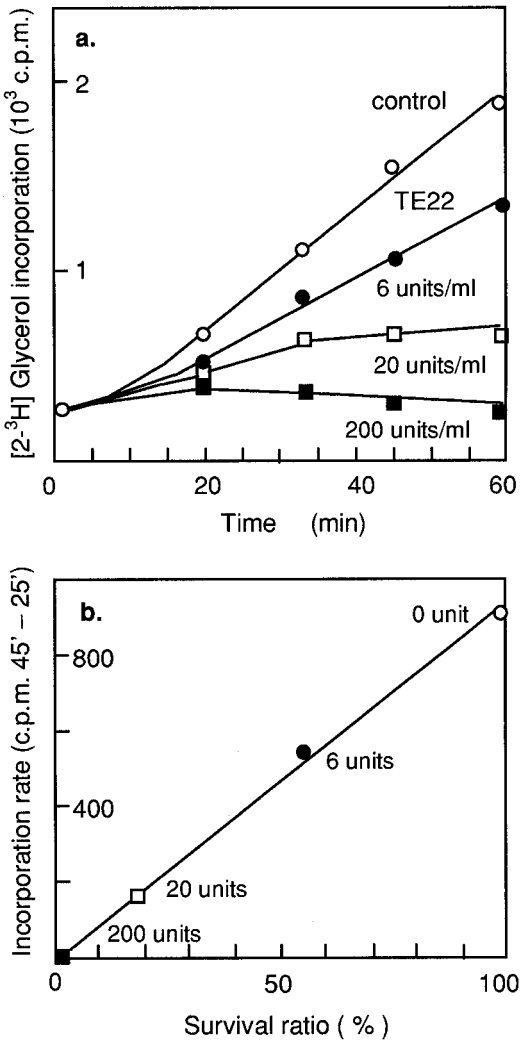


FIG. 6. Relationship between the inhibition of lipid synthesis and viability of *P. aeruginosa* PML1516d cells as investigated by varying the amounts of bacteriocin. (a) Inhibition of lipid synthesis with indicated amounts (units per milliliter) of TE22; (b) rates of lipid synthesis versus ratios of surviving cells. Rates of synthesis are expressed as the difference between incorporated counts at 20 and 45 min. The survival ratio with 200 U of TE22 per ml was 0.75%.

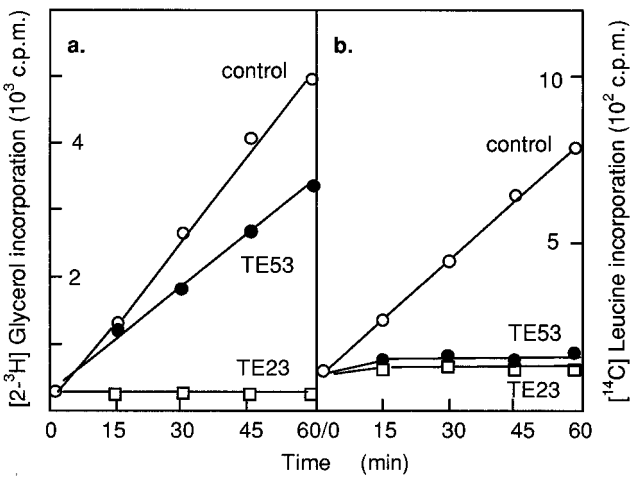


FIG. 7. Effects of chimeric proteins TE53 and TE23 on the syntheses of lipid and protein of PML1516d. Cells were cultured in D medium (1 μM Fe). Incorporations of [2-^{3}H]glycerol (a) and [^{14}C]leucine (b) into the acid-insoluble fractions were counted differentially at the times indicated. Units of bacteriocin per milliliter added and the cell survival ratio at 60 min were as follows: TE53, 320, 1%; TE23, 100, 0.8%.

haves like colicin E3 in *Pseudomonas* strains and TE23 behaves like pyocin S2. On the other hand, colicins E2 and E3 as well as chimeras ET1, ET2, and ES3 did not inhibit the lipid synthesis of *E. coli* (data not shown), in accord with an earlier report (2).

In summary, we have confirmed the previously proposed domain structure of pyocins as well as colicins (14, 19, 21) and found that the nuclease domains are interchangeable between two species of bacteriocins. Thus, a new type of pyocin with RNase activity was created. On the other hand, the translocation and the receptor-binding domains are species specific. Pyocins and their chimeras with the DNase domain of colicin E2 show two functions: inhibition of lipid synthesis and breakdown of DNA. Both activities are assigned to the DNase domain. The inhibition of lipid synthesis by chimeric proteins with the DNase domain occurs very early and completely, suggesting that the inhibition is the primary event, as reported before (12). However, this phenomenon is observed only with strain PML1516d. The same chimeric bacteriocins showed no such inhibition in *P. aeruginosa* PAO (19) or in *E. coli* C600, while efficient killing with DNA breakdown was observed. It has been known that DNA damage induces a series of gene expressions called the SOS response (25) and that colicin E2 (4) and pyocin AP41 (17), as well as mitomycin C, are the SOS inducers. An unpublished observation of ours showed that pyocins S1 and S2, as well as mitomycin, induced prophage production in PML1516d, one of the typical SOS responses. Therefore, the effects of mitomycin C on the lipid synthesis of PML1516d were examined. The incorporation of [2-^{3}H]glycerol was not inhibited at all for 60 min after mitomycin treatment, while the killing was efficient (data not shown). Accordingly, the DNA damage inducing the SOS function is not the cause of the inhibition of lipid synthesis. Therefore, the two functions, decomposition of DNA and inhibition of lipid synthesis, seem to be independent. However, we do not know whether these functions are separable in the protein structure. The relationship between the two functions in the killing process remains unclear. Inhibition of lipid synthesis by DNase type bacteriocins may be a special feature of strain PML1516d.

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