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Molecular Structures and Functions of Pyocins S1 and S2 in *Pseudomonas aeruginosa*

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Pyocins S1 and S2 are S-type bacteriocins of *Pseudomonas aeruginosa* with different receptor recognition specificities. The genetic determinants of these pyocins have been cloned from the chromosomes of *P. aeruginosa* NIH-H and PAO, respectively. Each determinant constitutes an operon encoding two proteins of molecular weights 65,600 and 10,000 (pyocin S1) or 74,000 and 10,000 (pyocin S2) with a characteristic sequence (P box), a possible regulatory element involved in the induction of pyocin production, in the 5′ upstream region. These pyocins have almost identical primary sequences; only the amino-terminal portions of the large proteins are substantially different. The sequence homology suggests that pyocins S1 and S2, like pyocin AP41, originated from a common ancestor of the E2 group colicins. Purified pyocins S1 and S2 make up a complex of the two proteins. Both pyocins cause breakdown of chromosomal DNA as well as complete inhibition of lipid synthesis in sensitive cells. The large protein, but not the pyocin complex, shows in vitro DNase activity. This activity is inhibited by the small protein of either pyocin. Putative domain structures of these pyocins and their killing mechanism are discussed.

Pyocins S1, S2, and AP41 are the most frequently found protease-sensitive bacteriocins among *Pseudomonas aeruginosa* strains (26). They are distinguished by their different receptor specificities. A peculiar feature of these bacteriocins is that their genetic determinants are located at definite sites on the chromosome, near *flaY* for pyocins S1 and S2 (10a) and between *lys-9015* and *argF* for pyocin AP41 (23). Recently, we have cloned and sequenced the genetic determinant of pyocin AP41 (23, 25). The determinant is present as a part of a transposon-like structure having terminal inverted repeats of the Tn*3* family of transposons. Pyocin AP41 is a complex of two proteins, the large component that is responsible for killing action and the small component that confers pyocin AP41 immunity. Pyocin AP41 kills sensitive cells by damaging chromosomal DNA through its inherent DNase activity (21, 22). The DNase domain, which is assigned to the carboxyl-terminal portion of the killing protein, and the immunity protein showed remarkable homology to those of the E2 group colicins (25).

Compared with pyocin AP41, we know much less about the biochemical properties of pyocins S1 and S2 and about their genetic determinants. Nothing is known about the molecular properties and function of pyocin S1, which is produced by strains PML28, NIH23, NIH-H, and QEA (26). Pyocin S2 is produced by strains PAO, NIH-O, and NIH18 (26). Ohkawa et al. reported that pyocin S2 is a bacteriocin with a molecular weight of approximately 75,000 that preferentially inhibits lipid synthesis in sensitive bacteria (17, 18). We have cloned and determined the nucleotide sequences of the determinants of pyocins S1 and S2 to elucidate their molecular structures and gene organization. We have also purified these proteins to investigate the biochemical characteristics of pyocin S1 and to reexamine those of pyocin S2.

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(Some of this work has been briefly described previously [26].)

MATERIALS AND METHODS

Bacterial strains and plasmids. *P. aeruginosa* NIH-H*leu-5*, a *leu* derivative strain of NIH-H (7) obtained by ethyl methanesulfonate mutagenesis, was used as a pyocin S1 producer. PML1516d (17) was used as the indicator strain for pyocins S1 and S2. The hosts and vectors used for gene cloning were *Escherichia coli* C600 (1), HB101 (2), JM109 (34), and MV1304 (31) and vector plasmids pMMB34 (8), pUC18 (34), and pUC118 and pUC119 (31).

Media. Nutrient broth (23) and YT broth and YT agar plates (34) were used for *E. coli* and *P. aeruginosa*. α, α′-Dipyridyl was added at 0.2 mM when required. G medium was used as a minimal medium for *P. aeruginosa* (22). When necessary, the $FeSO_4$ concentration was adjusted to 1 (Fe limited) or 36 (Fe rich) μM. Antibiotics were used at the concentrations described previously (23).

Construction of genomic library. A genomic library of *P. aeruginosa* NIH-H*leu-5* was constructed by the method of Frey et al. (8). A cosmid vector, pMMB34 (a derivative of RSF1010), was used as a shuttle vector between *P. aeruginosa* and *E. coli*. Chromosomal DNA was partially digested with *Sau*3A, after which size fractionation with sucrose-density gradient centrifugation was done, and fragments of 30 to 40 kb were ligated into the *Bam*HI sites of the vector plasmids. The ligated DNA mixture was packaged in phage λ particles and used to infect HB101. Kanamycin-resistant transformants thus obtained were screened for pyocin production. The genomic library of *P. aeruginosa* PAO3012, a pyocin S2 producer strain, was kindly supplied by T. Hoshino.

Screening of pyocin-producing clones. Kanamycin-resistant colonies of the genomic libraries were transferred with toothpicks onto YT agar plates in duplicate, and the plates were incubated at 37°C overnight. One of each pair of plates was exposed to chloroform vapor to prevent residual growth and then overlaid with soft agar containing the indicator

bacteria. After incubation at 30°C overnight, small clear zones were observed around pyocinogenic colonies. The colonies of interest were picked from the master plates and analyzed further.

Nucleotide sequencing. Nucleotide sequences were determined by the modified dideoxy chain termination method (9), using serial deletion plasmids generated by *Bal* 31 nuclease or exonuclease III. As the starting plasmids, we used pYMB51 and pYMB68 for pyocin S1 and pYMPS1 and pYMPS2 for pyocin S2.

Purification of pyocins S1 and S2 and their subunits. Pyocins S1 and S2 were purified from *E. coli* C600 cells harboring either pYMSS11 (pyocin S1) or pYMPS1 (pyocin S2), since recovery of active pyocin proteins was better with *E. coli* cells carrying the cloned pyocin genes than with the original *P. aeruginosa* strains. The cells were harvested from overnight cultures in YT broth with carbenicillin (100 to 200 ml) and were lysed with lysozyme (120 μg/ml at 37°C for 30 min) in 20 ml of 25 mM Tris-HCl–1 mM EDTA (pH 7.5). The following procedures were carried out at 4°C. The lysate was centrifuged at 12,000 × *g* for 10 min, and the supernatant was treated for 1 h with 3 g of DEAE-cellulose prewashed with 50 mM Tris-HCl–1 mM EDTA (pH 7.5) (DE52; Whatman). The filtrate was dialyzed overnight against 1 liter of buffer (10 mM sodium phosphate, 0.5 mM EDTA [pH 6.1]). The dialyzed sample was applied to a CM-Sepharose column (1.2 by 9 cm; CL-6B; Pharmacia) equilibrated with 10 mM sodium phosphate (pH 6.1). Pyocin activities were recovered from the column by elution with a linear gradient of NaCl (0 to 0.25 M) in the phosphate buffer. When necessary, the CM-Sepharose chromatography was repeated to increase the purity. Pyocin S1 activity appeared in two peaks that eluted at about 0.07 and 0.12 M NaCl. The first peak was used since it was generally larger and purer. Pyocin S2 activity was recovered in a peak that eluted at about 0.13 M NaCl. Each pyocin was composed of two component proteins which behaved as a single complex by gel filtration, but they could be separated by treatment with 7.2 M urea and then Sephacryl S200 column filtration in the presence of urea. The pyocin S1 and S2 complexes therefore appeared more stable than that of AP41 (22), which dissociates at 6 M urea. The large components that appeared at K_{av} = 0.95 possessed killing activity, and the small components (K_{av} = 0.6) are considered to be immunity protein. Hereafter they shall be referred to as S1A and S2A (large) and S1I and S2I (small). S1I seems to be produced in much larger amounts than S1A in the cell, as it was also recovered in the passthrough fraction by CM-Sepharose chromatography.

Isolation of DNase-active fragment. Purified pyocin S1 complex (1.15 mg) was digested with 80 μg of thermolysin (*Bacillus thermoproteolyticum* protease; Seikagaku Corp., Tokyo, Japan) in 4.34 ml of 50 mM Tris-HCl (pH 7.5)–1 mM $CaCl_2$ for 1 h at 40°C, and then EDTA was added at 5 mM. Urea was dissolved into this mixture to a final concentration of 7.2 M. This sample was chromatographed with a CM-Sepharose CL-6B column (1.2 by 9 cm) equilibrated with 7.2 M urea–10 mM sodium phosphate (pH 6.1). The column was first washed with the same urea buffer and then with 10 mM sodium phosphate (pH 6.1), and then it was eluted with a linear gradient of NaCl up to 0.4 M in the phosphate buffer. A peptide with a molecular weight of around 15,000 appearing at about 0.27 M NaCl was concentrated by Centricon-10 filtration (Amicon, Grace, Japan).

DNA decomposition in vivo and in vitro. DNA breakdown in pyocin-treated cells was tested as follows. About 5×10^7 cells were collected and suspended in 50 μl of 0.1 M Tris-HCl–0.01 M EDTA (pH 8.5). To this suspension, 12.5 μl of 5% sodium dodecyl sulfate (SDS) in 0.1 M EDTA (pH 8) was added, and then the mixture was heated at 95°C for 5 min. A 16-μl portion of this lysate was loaded onto 1% agarose gels with 4 μl of gel loading buffer (40% sucrose, 0.1% bromophenol blue, 0.1 M EDTA [pH 8], 0.3% SDS). After electrophoresis, the gels were stained with ethidium bromide. Conditions of electrophoresis were as described before (23). DNase activity in vitro was measured with pBR322 covalently closed circular DNA as a substrate. The reaction mixture (10 μl) contained 200 ng of DNA in 50 mM Tricine buffer (pH 7.4)–4 mM $MnCl_2$ and enzyme. After 1 h at 37°C, samples were mixed with 2.5 μl of gel loading buffer without bromophenol blue and electrophoresed on 1% agarose with ethidium bromide.

Lipid synthesis. Incorporation of [2-^{3}H]glycerol into the acid-insoluble fraction was measured as reported before (18).

Other methods. Pyocin units were determined by the critical dilution method or by the survivor count method as described before (16, 17, 22). Protein concentrations were calculated from the A_{280}, assuming $A^{1\%}_{280}$ values to be 10. SDS-polyacrylamide gel electrophoresis (PAGE) was carried out by the method of Laemmli with slight modifications (22) or by the Pharmacia Phast Gel System (gradient, 10 to 15% gel). This system was also used to determine isoelectric points of the pyocins (IEF 3-9 gel). N-terminal amino acid sequences were determined with a pulse-liquid phase sequencer (477A; Applied Biosystems).

Nucleotide sequence accession numbers. The nucleotide sequences of the genes for pyocins S1 and S2 have been deposited in the DDBJ/EMBL/GenBank DNA data bases under accession numbers D12707 and D12708, respectively.

RESULTS

Cloning of the pyocin S1 and S2 genes. The *P. aeruginosa* cosmid genomic libraries of NIH-H*leu-5* (pyocin S1 producer) and PAO3012 (pyocin S2 producer) were screened for pyocinogeny. Of 600 colonies tested, we isolated three clones as pyocin S1 producers in *E. coli* and designated them pYS190, pYS266, and pYS299. In the same way, we obtained two pyocin S2-producing clones, pYS424 and pYS510, by screening 100 colonies. Restriction enzyme analysis showed that each plasmid had a chromosomal segment of about 30 kb. To localize the pyocin S1 and S2 genes, genomic fragments of pYS299 and pYS424 were recloned into pUC plasmids, using appropriate restriction enzymes, and pyocin-producing clones were picked up. We used a *Bam*HI partial digest of pYS299 to reclone the pyocin S1 gene(s) and thereby obtained two pyocin-producing clones, pYMB68 and pYMB51, each of which carries two *Bam*HI fragments of 3.5 and 1.4 kb. The pyocin S1 gene(s) was finally localized to the 2.5-kb *Sph*I-*Sst*I fragment by deletion mapping as shown in the upper part of Fig. 1. We isolated a pyocin S2-producing plasmid, pYMS105, by using a *Sal*I partial digest of pYS424. As shown in the lower part of Fig. 1, the 2.8-kb *Pst*I-*Sst*I fragment was found to confer the ability to produce pyocin S2. Some restriction enzyme sites (*Kpn*I, *Bam*HI, and *Sst*I) seemed to be common to both pyocin S1 and S2 determinants.

Nucleotide sequences of the pyocin S1 and S2 genes. The nucleotide sequences of the region of about 2.7 kb which includes the *Sph*I site to the *Sst*I site (for pyocin S1) and the 2.8-kb *Pst*I-*Sst*I fragment (for pyocin S2) were determined (Fig. 2). We found that the nucleotide sequences of the

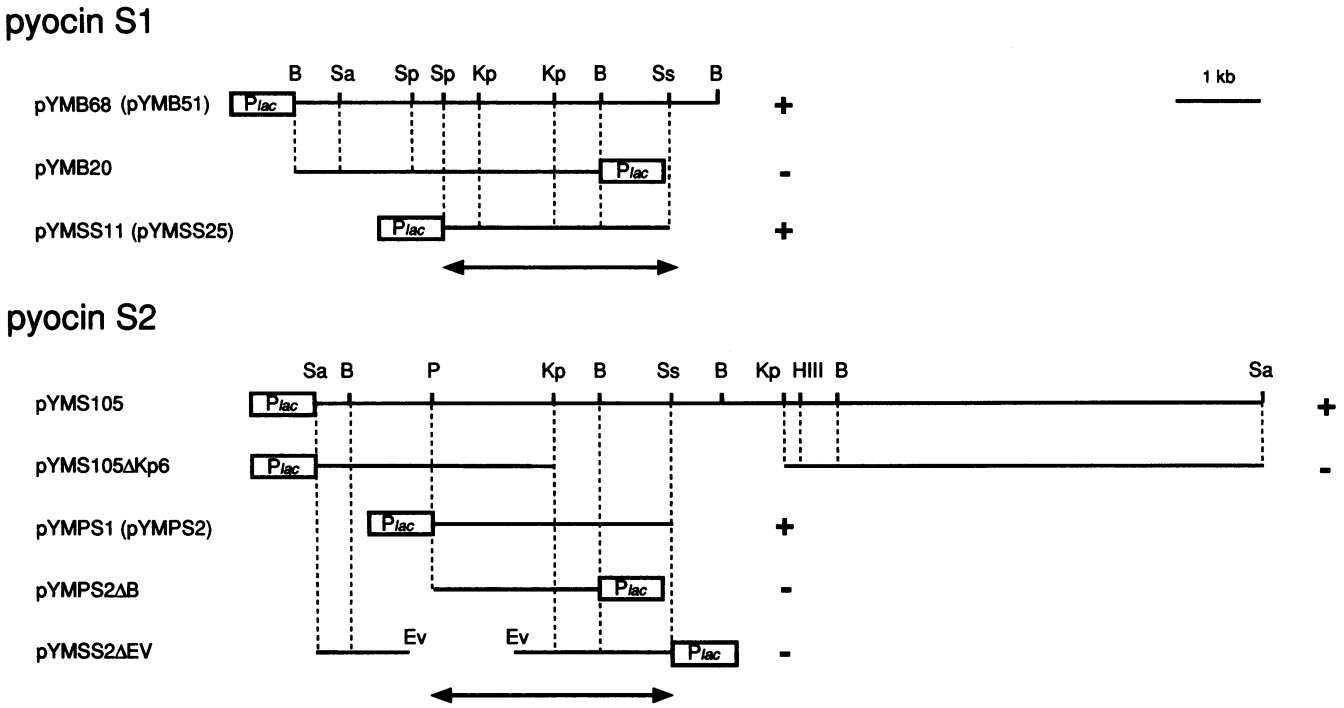

FIG. 1. Localization of the genes for pyocins S1 and S2 by deletion mapping. P*lac* in the box indicates the position of the *lacZ* promoter of the pUC plasmid. Transcription from P*lac* on the left proceeds from left to right and vice versa. The plasmid in parentheses carries the same restriction fragments inserted in the opposite direction from those of the plasmids shown. Pyocin production of each plasmid is shown by the symbol + or − on the right. Arrows indicate the regions conferring the production of pyocins. B, *Bam*HI; Ev, *Eco*RV; HIII, *Hin*dIII; Kp, *Kpn*I; P, *Pst*I; Sa, *Sal*I; Sp, *Sph*I; Ss, *Sst*I.

determinants of pyocins S1 and S2 are nearly identical in two-thirds of the entire region, as suggested by restriction analysis. Specifically, the sequences starting at nucleotides 1093 of pyocin S1 and 1290 of pyocin S2 are essentially the same up to the 3′ ends. In these regions, two sequences differ at seven points: one is the deletion or addition of three bases, and six are substitutions of one base at each point.

Similarities in the nucleotide sequence were also found at about 150 bp in the 5′ ends and in the regions preceding the ribosome-binding site, shown underlined in Fig. 2. The latter sequence was also conserved in the pyocin AP41 gene (25). Figure 3 schematically represents the structure of these sequences, which we designate P boxes. The P box is located approximately 60 to 100 bp upstream of the ribosome-binding site and is composed of four repeats of 10 to 11 nucleotides each. Each repeat has a consensus sequence of ATTGnn(n)GTnn(n).

We identified two tandem open reading frames (ORFs) in each determinant. In the pyocin S1 determinant, the first ORF encodes a protein of 618 amino acids and the second ORF specifies a protein of 87 amino acids. The predicted molecular weights of these proteins are 65,600 and 10,000, respectively. The ORFs in the pyocin S2 determinant encode proteins with molecular weights of 74,000 (690 amino acids) and 10,000 (87 amino acids). In both cases, the second ORF is followed by the inverted repeated sequence, which could function as a transcription terminator. The stop codon (TGA) of the first ORF is separated by a single base (T) from the start codon (ATG) of the second ORF, and the putative ribosome-binding site of the gene for the small protein is located in the region encoding the carboxyl-terminal portion of the large protein. This gene organization suggests the translational coupling of two components.

Molecular structures of pyocins S1 and S2 and evolutionary relationship. By comparing the amino acid sequences of the large proteins, we found that the carboxyl-terminal halves are nearly identical in pyocins S1 and S2 (Fig. 2). Of six nucleotide differences in these regions, two cause the replacement of valine (pyocin S1) by isoleucine (pyocin S2) and the deletion or addition of one arginine residue, while the other four nucleotide differences are silent at the amino acid level. In the small protein, one amino acid substitution (glutamic acid to lysine) in pyocin S2 is observed. As the large protein probably forms a complex with the small protein, these altered sites between pyocins S1 and S2 might contribute to the interaction of the two components. Figure 4 shows the alignment of the amino acid sequences of the two components of pyocins S1 and S2 with those of pyocin AP41 and E2 group colicins. We found that the homologous regions of pyocins S1 and S2 (the shaded regions of the large protein and the small protein) are also conserved in pyocin AP41. The homology to pyocin AP41 is, however, less pronounced than that between pyocins S1 and S2, and we could detect little homology at the nucleotide level.

The large component of pyocin AP41 is responsible for killing action, and the small component confers immunity to bacteriocin. Pyocin AP41 kills sensitive bacteria by causing the breakdown of chromosomal DNA by its inherent DNase activity, which is assigned to the carboxyl-terminal portion of the large component (21, 22, 25). Furthermore, homology in this region and in the next immunity protein extended to colicins E2, E7, E8, and E9 (Fig. 4). The presence of the

pyocin S1

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CGGCTTGCAATCAAAAACGCTGGGCTCGCAATCAGGGTGGTTTTGTCCGGAAACTCGCGAAGGGGCGGCATGGATGAATTTTGAAGGTGG      90

GACTTCGTTAATGAACCGAAGGTATGCGGCTTCCGTGACTGGCAGGGGTAGATGAGGTTTTCGTGCAGAAAATGACCGCTGGATGGGCGC     180
SphI                                  BalI
ATGCGCAAACGAGGTCGTTTCGTAGAAGGGAATGGCCAAGGATGGGCCTTCTGGGTCATTGGATACGGGATATTGCAGTTTGATTGCTGT     270

GTATTTCCAGTGTATTGGCGTTCATTTGGCAAGGGGCGTTTAAAAAGTCTAATTCGACGTAAATAATAAGCCATGCACTCATGGAAGTAG     360

GAGATACATATGGCACGACCCATTGCTGACCTTATCCACTTCAACTCTACAACTGTCACGGCAAGCGGAGACGTTTATTACGGCCCTGGG     450
          M  A  R  P  I  A  D  L  I  H  F  N  S  T  T  V  T  A  S  G  D  V  Y  Y  G  P  G      27
    KpnI
GGAGGTACCGGCATTGGCCCCATTGCCAGACCTATAGAGCACGGCTTGGATTCGTCCACTGAAAATGGCTGGCAAGAGTTTGAAAGTTAT     540
G  G  T  G  I  G  P  I  A  R  P  I  E  H  G  L  D  S  S  T  E  N  G  W  Q  E  F  E  S  Y      57

GCTGATGTGGGCGTTGACCCCAGACGCTATGTTCCTCTTCAGGTTAAAGAAAAACGCAGGGAGATCGAGCTTCAGTTCCGAGATGCCGAG     630
A  D  V  G  V  D  P  R  R  Y  V  P  L  Q  V  K  E  K  R  R  E  I  E  L  Q  F  R  D  A  E      87

AAAAAACTTGAGGCGTCGGTACAAGCCGAGCTGGATAAGGCTGATGCCGCTCTTGGTCCGGCAAAGAATCTTGCACCATTGGACGTCATC     720
K  K  L  E  A  S  V  Q  A  E  L  D  K  A  D  A  A  L  G  P  A  K  N  L  A  P  L  D  V  I     117

AACCGCAGTCTGACCATCGTTGGAAACGCCCTCCAGCAAAAGAATCAAAAACTACTGCTGAATCAGAAGAAGATTACCAGCCTGGGTGCA     810
N  R  S  L  T  I  V  G  N  A  L  Q  Q  K  N  Q  K  L  L  L  N  Q  K  K  I  T  S  L  G  A     147

AAGAATTTCCTTACCCGTACGGCGGAAGAGATCGGTGAACAAGCGGTGCGAGAAGGCAATATTAACGGGCCTGAAGCCTATATGCGCTTC     900
K  N  F  L  T  R  T  A  E  E  I  G  E  Q  A  V  R  E  G  N  I  N  G  P  E  A  Y  M  R  F     177

CTCGACAGGGAAATGGAAGGTCTCACGGCAGCTTATAACGTAAAACTCTTCACCGAAGCGATCAGTAGTCTCCAGATCCGCATGAATACG     990
L  D  R  E  M  E  G  L  T  A  A  Y  N  V  K  L  F  T  E  A  I  S  S  L  Q  I  R  M  N  T     207

TTGACCGCCGCCAAAGCAAGTATTGAGGCGGCCGCAGCAAACAAGGCGCGTGAACAAGCAGCGGCTGAGGCCAAACGCAAAGCCGAAGAG    1080
L  T  A  A  K  A  S  I  E  A  A  A  A  N  K  A  R  E  Q  A  A  A  E  A  K  R  K  A  E  E     237
                .  .        .        .
CAGGCCCGCCAGCAAGCGGCGATAAGAGCTGCCAATACCTATGCCATGCCGGCCAATGGCAGCGTTGTCGCCACCGCCGCAGGCCGGGGT    1170
Q  A  R  Q  Q  A  A  I  R  A  A  N  T  Y  A  M  P  A  N  G  S  V  V  A  T  A  A  G  R  G     267

CTGATCCAGGTCGCACAAGGCGCCGCATCCCTTGCTCAAGCGATCTCCGATGCGATTGCCGTCCTGGGCCGGGTCCTGGCTTCAGCACCC    1260
L  I  Q  V  A  Q  G  A  A  S  L  A  Q  A  I  S  D  A  I  A  V  L  G  R  V  L  A  S  A  P     297

TCGGTGATGGCCGTGGGCTTTGCCAGTCTGACCTACTCCTCCCGGACTGCCGAGCAATGGCAGGACCAAACGCCCGATAGCGTTCGTTAC    1350
S  V  M  A  V  G  F  A  S  L  T  Y  S  S  R  T  A  E  Q  W  Q  D  Q  T  P  D  S  V  R  Y     327
                                                                        KpnI
GCCCTGGGCATGGATGCCGCTAAATTGGGGCTTCCCCCAAGCGTAAACCTGAACGCGGTTGCAAAAGCCAGCGGTACCGTCGATCTGCCG    1440
A  L  G  M  D  A  A  K  L  G  L  P  P  S  V  N  L  N  A  V  A  K  A  S  G  T  V  D  L  P     357

ATGCGCCTGACCAACGAGGCACGAGGCAACACGACGACCCTTTCGGTGGTCAGCACCGATGGTGTGAGCGTTCCGAAAGCCGTTCCGGTC    1530
M  R  L  T  N  E  A  R  G  N  T  T  T  L  S  V  V  S  T  D  G  V  S  V  P  K  A  V  P  V     387

CGGATGGCGGCCTACAATGCCACGACAGGCCTGTACGAGGTTACGGTTCCCTCTACGACCGCAGAAGCGCCGCCACTGATCCTGACCTGG    1620
R  M  A  A  Y  N  A  T  T  G  L  Y  E  V  T  V  P  S  T  T  A  E  A  P  P  L  I  L  T  W     417

ACGCCGGCGAGTCCTCCAGGAAACCAGAACCCTTCGAGTACCACTCCGGTCGTACCGAAGCCGGTGCCGGTATATGAGGGAGCGACCCTT    1710
T  P  A  S  P  P  G  N  Q  N  P  S  S  T  T  P  V  V  P  K  P  V  P  V  Y  E  G  A  T  L     447

ACACCGGTGAAGGCTACCCCGGAAACCTATCCTGGGGTGATTACACTACCGGAAGACCTGATCATCGGCTTCCCGGCCGACTCGGGGATC    1800
T  P  V  K  A  T  P  E  T  Y  P  G  V  I  T  L  P  E  D  L  I  I  G  F  P  A  D  S  G  I     477
                BamHI
AAGCCGATCTATGTGATGTTCAGGGATCCGCGGGATGTACCTGGTGCTGCGACTGGCAAGGGACAGCCCGTCAGCGGTAATTGGCTCGGC    1890
K  P  I  Y  Y  V  M  F  R  D  P  R  D  V  P  G  A  A  T  G  K  G  Q  P  V  S  G  N  W  L  G     507
                                                                              ↓
GCCGCCTCTCAAGGTGAGGGGGCTCCAATTCCAAGCCAGATTGCGGATAAACTACGTGGTAAGACATTCAAAAACTGGCGGGACTTTCGG    1980
A  A  S  Q  G  E  G  A  P  I  P  S  Q  I  A  D  K  L  R  G  K  T  F  K  N  W  R  D  F  R     537
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FIG. 2. Nucleotide sequences of the genes for pyocins S1 and S2 and predicted amino acid sequences of both pyocins. Singly and doubly underlined DNA sequences indicate the P box (Fig. 3) and a Shine-Dalgarno sequence, respectively. Putative transcription terminators are shown by bidirectional horizontal arrows. Nucleotides differing in the conserved regions encoding pyocins S1 and S2 are marked by dots. The vertical arrow shows the position of the deletion or addition of three bases. Underlined amino acid sequences were determined with purified proteins.

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GAACAATTCTGGATAGCTGTGGCTAATGATCCTGAGTTAAGTAAACAGTTTAATCCTGGTAGTTTAGCTGTAATGAGAGATGGAGGGGCT  2070
E  Q  F  W  I  A  V  A  N  D  P  E  L  S  K  Q  F  N  P  G  S  L  A  V  M  R  D  G  G  A     567

CCTTATGTCAGAGAGTCAGAACAGGCTGGCGGGAGAATAAAGATCGAAATCCACCACAAGGTTCGAGTAGCAGATGGAGGCGGCGTTTAC  2160
P  Y  V  R  E  S  E  Q  A  G  G  R  I  K  I  E  I  H  H  K  V  R  V  A  D  G  G  G  V  Y     597

AATATGGGGAACCTTGTTGCAGTAACGCCAAAACGTCATATAGAAATCCACAAGGGAGGGAAGTGATATGAAGTCCAAGATTTCCGAATA  2250
N  M  G  N  L  V  A  V  T  P  K  R  H  I  E  I  H  K  G  G  K  *  M  K  S  K  I  S  E  Y     618/8

TACGGAAAAAGAGTTTCTTGAGTTTGTTGAAGACATATACACAAACAATAAGAAAAAGTTCCCTACCGAGGAGTCTCATATTCAAGCCGT  2340
T  E  K  E  F  L  E  F  V  E  D  I  Y  T  N  N  K  K  K  F  P  T  E  E  S  H  I  Q  A  V     38

GCTTGAATTTAAAAAACTAACGGAACACCCAAGCGGCTCAGACCTTCTTTACTACCCCAACGAAAATAGAGAAGATAGCCCAGCTGGAGT  2430
L  E  F  K  K  L  T  E  H  P  S  G  S  D  L  L  Y  Y  P  N  E  N  R  E  D  S  P  A  G  V     68
                                                                BalI
TGTAAAGGAAGTTAAAGAATGGCGTGCTTCCAAGGGGCTTCCTGGCTTTAAGGCCGGTTAGTTGGCCATCAACAAGCCCCGTTTCGACGG  2520
V  K  E  V  K  E  W  R  A  S  K  G  L  P  G  F  K  A  G  *                                    87

GGCTTGTTCCATGTTGACGCTGCTCTATCTTGACAGTCTGTCCTGAATATTCTTGAAGACAATCACATTATGAGCCGCAACCACCGCAAA  2610
                                SstI
ACACCCATCGTCGGGCGCACGAGCTGCCGTAGCGAGCTCTCAGTTGGCAGCAGCCACTTCCTATGGAA  2678
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pyocin S2

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PstI
CTGCAGCGGCTTGCAATCAAAAACGCTGGGCTCGCAATCAGCGTGGCTTTGTCCGGAAGCTCGCGAAGGGGCGGCATGGATGAATTTTGA   90

AAGTGGGAGTTCGTTAATGAACCGAAGGTATGCGGCTTCCGTGACTGGCCGGGGTAGATGAGGTGTGCGTTAAGGAAATGACCGTTTGAC  180

GCAGGCGCGATTATAAGAGGCGAGAGAGCGCACCTTCTGGATCATCTAAACACGGGATATTGAAGTTGATTGCAGTGTATTGCCGATGCA  270

TTGGGGCTTATTGACTGGGAGAGGGTTCTCTAGGTGGGAGTCGCTCAATACATTACACTTTCAAATTAATGTAGGGGCGATCTATGGCTG  360
                                                                          M  A  V                3

TCAATGATTACGAACCTGGTTCGATGGTTATTACACATGTGCAGGGTGGTGGGCGTGACATAATCCAGTATATTCCTGCTCGATCAAGCT  450
N  D  Y  E  P  G  S  M  V  I  T  H  V  Q  G  G  G  R  D  I  I  Q  Y  I  P  A  R  S  S  Y     33

ACGGTACTCCACCATTTGTCCCACCAGGACCAAGTCCGTATGTCGGTACTGGAATGCAGGAGTACAGGAAGCTAAGAAGTACGCTTGATA  540
G  T  P  P  F  V  P  P  G  P  S  P  Y  V  G  T  G  M  Q  E  Y  R  K  L  R  S  T  L  D  K     63

AGTCCCATTCAGAACTCAAGAAAAACCTGAAAAATGAAACCCTGAAGGAGGTTGATGAACTCAAGAGTGAAGCGGGGTTGCCAGGTAAAG  630
S  H  S  E  L  K  K  N  L  K  N  E  T  L  K  E  V  D  E  L  K  S  E  A  G  L  P  G  K  A     93

CGGTCAGTGCCAATGACATCCGCGATGAAAAGAGTATCGTTGATGCACTCATGGATGCCAAAGCAAAATCGCTAAAGGCCATTGAGGATC  720
V  S  A  N  D  I  R  D  E  K  S  I  V  D  A  L  M  D  A  K  A  K  S  L  K  A  I  E  D  R     123

GCCCGGCCAATCTTTATACGGCTTCAGACTTTCCTCAGAAGTCAGAGTCGATGTACCAGAGTCAGTTGCTGGCCAGCCGAAAATTCTATG  810
P  A  N  L  Y  T  A  S  D  F  P  Q  K  S  E  S  M  Y  Q  S  Q  L  L  A  S  R  K  F  Y  G     153

GAGAGTTCCTGGATCGCCATATGAGTGAGCTGGCCAAAGCGTACAGCGCCGATATCTATAAGGCGCAAATCGCTATCTTGAAACAAACGT  900
E  F  L  D  R  H  M  S  E  L  A  K  A  Y  S  A  D  I  Y  K  A  Q  I  A  I  L  K  Q  T  S     183

CTCAAGAGCTGGAGAATAAAGCCCGGTCATTGGAAGCAGAAGCCCAGCGAGCCGCTGCTGAGGTGGAGGCGGACTACAAGGCCAGGAAGG  990
Q  E  L  E  N  K  A  R  S  L  E  A  E  A  Q  R  A  A  A  E  V  E  A  D  Y  K  A  R  K  A     213

CAAATGTCGAGAAAAAAGTGCAGTCCGAGCTTGACCAGGCTGGGAATGCTTTGCCTCAACTGACCAATCCAACGCCAGAGCAGTGGCTTG  1080
N  V  E  K  K  V  Q  S  E  L  D  Q  A  G  N  A  L  P  Q  L  T  N  P  T  P  E  Q  W  L  E     243
```

FIG. 2—*Continued.*

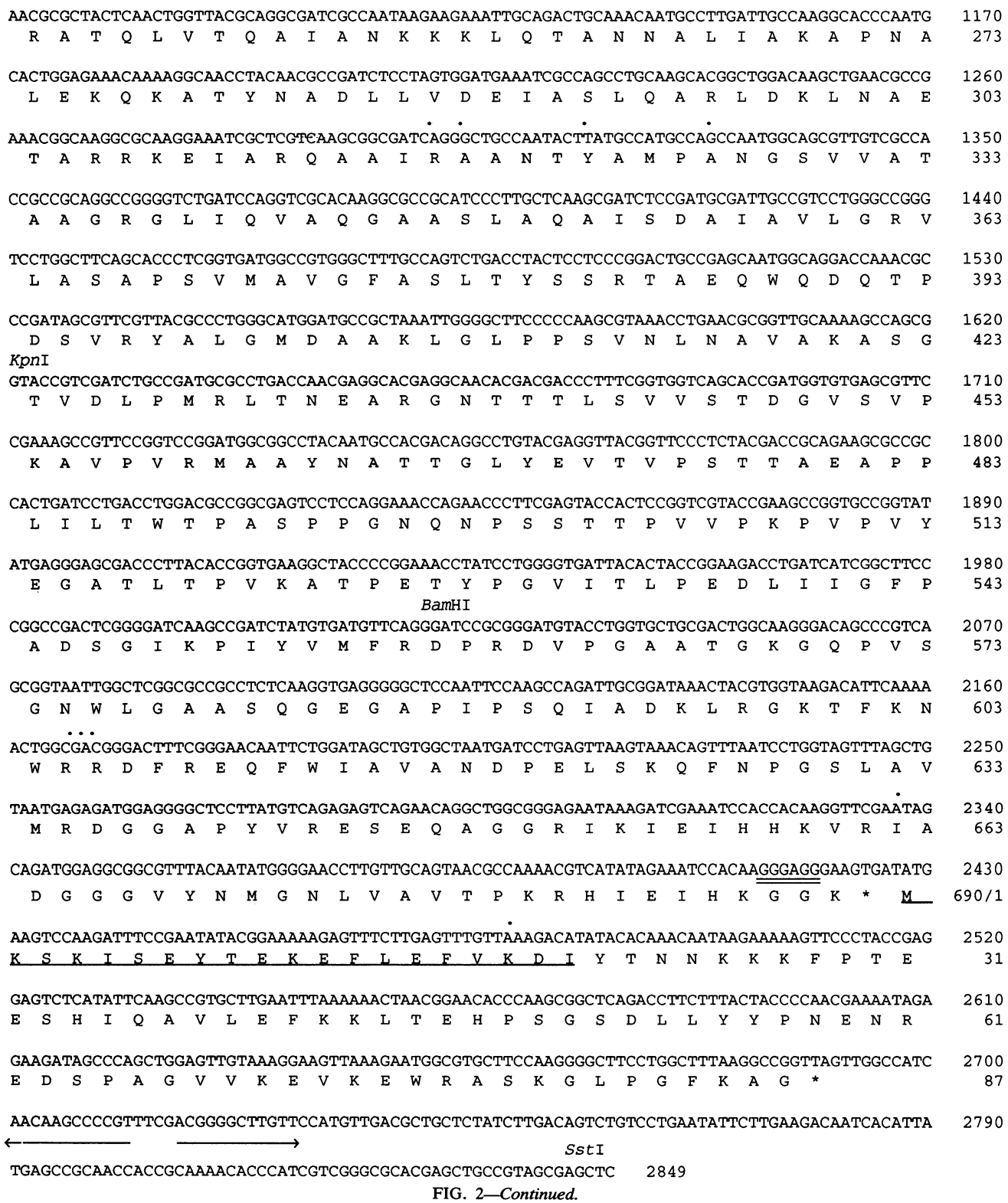

FIG. 2—*Continued.*

regions homologous to the DNase domain in pyocins S1 and S2 suggests that pyocins S1 and S2, like pyocin AP41, might actually possess DNase activity. In a previous report (18), pyocin S2 was shown to cause the preferential inhibition of lipid synthesis prior to its effect on DNA synthesis. Thus, we examined biological functions of pyocins S1 and S2 with the purified proteins.

Properties of purified pyocin proteins. Pyocins were puri-

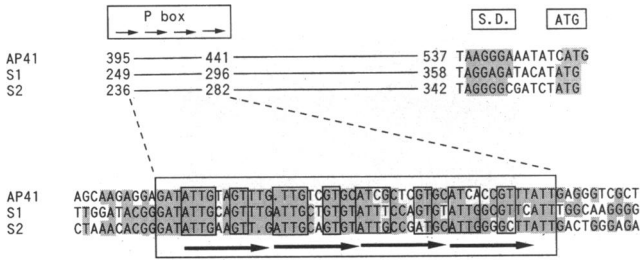

FIG. 3. Structure of the P box in the 5′ noncoding regions of the genes for pyocins AP41, S1, and S2 (25). Numbers indicate the positions of nucleotides from the 5′ end. Arrows indicate the repeated motif in the P box. S. D., Shine-Dalgarno sequence.

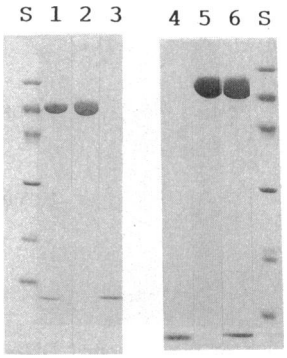

FIG. 5. SDS-PAGE of purified pyocin S1 and S2 proteins. S, size markers (from the top): 94,000, 67,000, 43,000, 30,000, 20,000, and 14,400, respectively. Lane 1, S1 complex; lane 2, S1A; lane 3, S1I; lane 4, S2I; lane 5, S2A; lane 6, S2 complex.

fied as a complex of two components having molecular weights of 66,000 (S1A) and 10,000 (S1I) for pyocin S1 and 74,000 (S2A) and 10,000 (S2I) for pyocin S2 (Fig. 5). These subunit proteins could be separated under denaturation conditions, using 7.2 M urea. The N-terminal amino acid sequences determined for the isolated proteins, as well as their molecular sizes, agreed with those predicted from the nucleotide sequences, except that the N-terminal methionine was removed in S1A and S2A (Fig. 2). Isoelectric points determined for these proteins were as follows: S1 complex, pH 8; S1A, >pH 9; S1I, pH 5.4; S2 complex, >pH 9; S2A, >pH 9; S2I, pH 6.85. The specific activities of purified samples by the spot method against PML1516d were about 3×10^6 U/mg of protein for pyocin S1 and about 6×10^6 U/mg of protein for pyocin S2. These values are much higher than that for AP41, 8×10^4 U/mg of protein (22), although the

FIG. 4. Alignment of the amino acid sequences of pyocins S1 and S2 with those of related bacteriocins. (a) Carboxyl-terminal halves of the killing (large) proteins. The positions in the proteins are as follows: S1, 242 to 618; S2, 313 to 690; AP41, 401 to 777. E2, E7, E8, and E9, DNase domains. (b) Immunity proteins. Shaded amino acids are conserved between pyocins S1 and S2 and other bacteriocins. References for the sequences are as follows: pyocin AP41, Sano and Kageyama (25); colicin E2, Lau et al. (12), Cole et al. (5), and Masaki et al. (14); colicin E7, Chak et al. (4) and Soong et al. (28); colicin E8, Uchimura and Lau (30) and Toba et al. (29); colicin E9, James et al. (10), Lau and Condie (11), and Eaton and James (6).

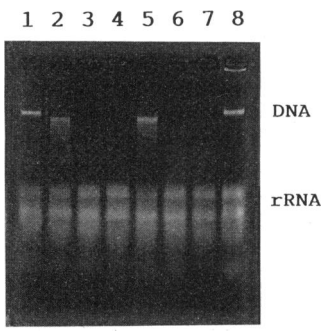

FIG. 6. Breakdown of chromosomal DNA caused by pyocin treatment as revealed by agarose gel electrophoresis. PML1516d cells were cultured in YT medium supplemented with 5 mM $MgSO_4$–0.2 mM α,α′-dipyridyl at 37°C under constant shaking. Pyocin S1 (50 U/ml) or S2 (30 U/ml) was added at 0 min. Portions of 200 μl (about 5×10^7 cells) were withdrawn at the times indicated and analyzed further. Lane 1, control sample without pyocin at 0 min; lanes 2 to 4, samples with pyocin S1 at 15, 30, and 60 min, respectively; lanes 5 to 7, samples with pyocin S2 at 15, 30, and 60 min, respectively; lane 8, control sample at 60 min. Numbers of colony formers at 30 min were 0.004% (pyocin S1) and 0.006% (S2) of the control.

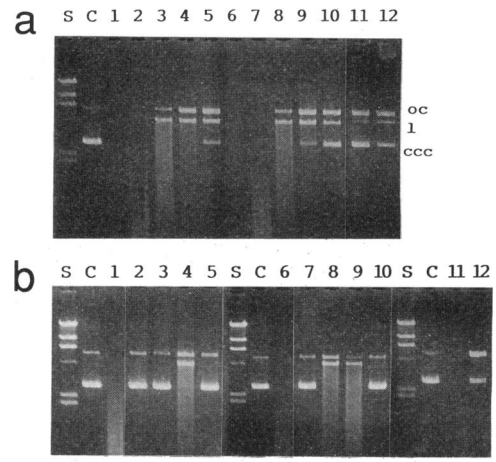

FIG. 7. (a) DNase activity of pyocins. pBR322 DNA was incubated with pyocin samples. S, size markers, λ DNA *Hin*dIII fragments. C, control, DNA only. Lanes 1 to 5, S1A at 340, 170, 85, 43, and 21 ng, respectively; lanes 6 to 10, S2A at 290, 145, 72, 36, and 18 ng, respectively; lane 11, pyocin S1 complex at 625 ng; lane 12, pyocin S2 complex at 470 ng. CCC, OC, or l signifies covalently closed circular, open circular, or whole linear DNA, respectively. (b) Inhibition of DNase activity by small proteins. Effects on DNase activity of S1A, S2A, and S1 thermolysin fragment are shown. S1A (170 ng) was added to lanes 1 to 5. Lane 1, no small protein; lanes 2 to 4, 115, 57, and 29 ng of S1I, respectively; lane 5, 57 ng of S2I. S2A (145 ng) was added to lanes 6 to 10. Lane 6, no small protein; lanes 7 to 9, 57, 29, and 14 ng of S1I, respectively; lane 10, 50 ng of S2I; Lanes 11 and 12, 15 ng of thermolysin fragment without or with 57 ng of S1I, respectively. S, size markers. C, control (DNA only).

indicators are different. The large subunit showed essentially the same killing activity as its complex in either pyocin. Details of killing action were investigated in liquid media, counting the number of cells surviving after pyocin treatment. Similar to that of pyocin S2 (19), the killing efficiency of pyocin S1 was higher when the indicator PML1516d was cultured under iron deficiency, in G medium with 1 μM Fe^{2+} or YT broth with dipyridyl. Viabilities of the cells treated with pyocin S1 (40 U/ml) were 8×10^{-4} in G medium with 1 μM Fe^{2+} and 6.4×10^{-1} in G medium with 36 μM Fe^{2+} after 60 min, whereas values were 9×10^{-4} and 4×10^{-1}, respectively, with pyocin S2 (80 U/ml). The logarithm of the surviving cell number after 40 min decreased linearly with the amount of pyocin, indicating that the killing occurred in a single-hit manner. From the amount of pyocin required to give cell survival to e^{-1} (37%), the number of pyocin molecules necessary to kill a single cell (lethal unit) was determined to be 50 for pyocin S1 or S2 under iron-limited conditions. Similar values, 40 to 300, were reported for colicin E2 (13).

DNA breakdown in vivo and in vitro. Following addition of pyocin S1 or S2 complex, the growth of the culture stopped after 20 min, and then its turbidity decreased gradually. The breakdown of DNA was observed within 15 min and thereafter (Fig. 6), suggesting that pyocins S1 and S2 possess DNase activity. Actually, DNase activity in vitro was found in both large components, S1A and S2A, similar to pyocin AP41 (21) or colicin E2 (27). Pyocin S1 or S2 complex showed essentially no DNase activity, whereas S1A or S2A degraded DNA. Covalently closed circular DNA was converted to an open circle and subsequently depolymerized through a linear form (Fig. 7a). The activity increased two to four times when Mn^{2+} was used instead of Mg^{2+}. With Mn^{2+}, Tricine buffer gave higher activity than Tris buffer. Yet the activity was not very strong. About 150 ng of S1A or S2A was required to decompose 200 ng of covalently closed circular DNA into smaller fragments in 60 min. This activity is about the same level that was reported for E2A (20). However, this process is specific to S1A or S2A since either S1I or S2I completely inhibited the DNase activity (Fig. 7b). By calculating on a molar basis, the inhibition was complete when twice as many molecules of S1I or S2I were added under the assay conditions. Furthermore, cross-inhibition was found with S1I and S2I in vitro, confirming the previous observation that they share immunity in vivo (26).

A peptide with stronger DNase activity was isolated by digesting pyocin S1 complex with thermolysin. As shown in Fig. 7b, 15 ng of this fragment digested covalently closed circular DNA into depolymerized forms in 60 min. On a molecular basis, however, it was about twice as active as the S1A peptide. This activity was also almost completely inhibited by 57 ng of S1I. The N-terminal sequence of this fragment, shown in Fig. 2, corresponded completely to the S1A sequence from amino acids 484 to 503. Assuming that this fragment ends at the C terminus of pyocin S1, its molecular weight should be 14,703. This fragment corresponds to the trypsin fragment of AP41 (21, 25) or to T2A of colicin E2 (20, 33). A similar fragment was isolated from pyocin S2 after digestion with *Staphylococcus aureus* V8 protease. A fraction containing a peptide with an apparent molecular weight of around 16,000 exhibited DNase activity. However, assignment of this peptide in the pyocin molecule was not done, since the fraction contained a mixture of at least two peptides of similar size (data not shown).

Inhibition of lipid synthesis. As pyocin S2 was known to inhibit lipid synthesis preceding the inhibition of protein or nucleic acid synthesis (18), we examined it for pyocin S1. Figure 8 shows that the incorporation of [2-^{3}H]glycerol into the lipid fraction of PML1516d was completely inhibited within 5 min after the addition of pyocin S1, as well as pyocin S2, under iron-limited conditions.

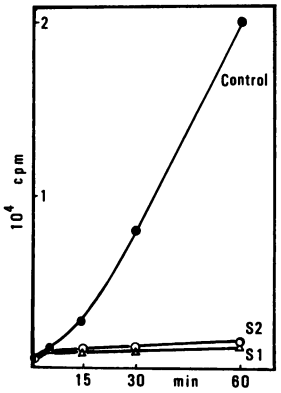

FIG. 8. Effects of pyocins S1 and S2 on lipid synthesis. PML1516d cells were cultured in G medium with 1 μM Fe at 37°C. [2-^{3}H]glycerol (2 μCi; 0.2 μmol/ml) was added to the logarithmic-phase culture 3 min before pyocin addition. At 0 min, pyocin S1 (300 U/ml) or S2 (200 U/ml) was added. Portions of 50 μl were removed at the times indicated, and the radioactivities in the acid-insoluble fraction were counted as described before. Numbers of colony formers at 60 min were 0.06% (with pyocin S1) and 0.09% (with S2) of the control.

DISCUSSION

Features of the determinants for pyocins S1 and S2. The determinants for pyocins S1 and S2 constitute an operon containing the genes for the killing protein and the immunity protein in both cases. In the 5′ upstream region, a unique sequence with four repeats of 10 to 11 nucleotides (P box) each was found. As 10.6 nucleotide residues form one turn of a DNA helix, the repeated motifs could be aligned on the same side of the DNA helix, possibly serving as a protein-binding site. Considering its location, it is quite likely that the P box is a binding site of a protein(s) which regulates the expression of the pyocin genes. The presence of the P box, instead of the SOS box in the *recA* gene of *P. aeruginosa* (24) and the genes of the SOS regulon in *E. coli* (32), suggests that the pyocin genes might be regulated by a unique regulatory system in *P. aeruginosa*, as was found recently to be the case (15).

Molecular structures of S-type pyocins. Pyocins S1, S2, and AP41 were originally distinguished as representative pyocins showing different specificities of receptor recognition (26). Sequence analyses of pyocins S1 and S2 showed that the molecular structures of these pyocins are quite similar. Furthermore, these pyocins exhibit extensive homology with pyocin AP41. The conserved regions cover nearly two-thirds of the large proteins and the entire immunity proteins. Finally, the DNase domains and the immunity proteins of these pyocins were found to be homologous to the corresponding regions of E2 group colicins, E2, E7, E8, and E9 (Fig. 4). This indicates that pyocins S1 and S2, as well as pyocin AP41, have originated from a common ancestor of the E2 group colicins.

N-terminal sequences of one-third of the large proteins, where little homology is present among three pyocins, are possibly the domains responsible for receptor binding. Although susceptibility to pyocins S1 and S2 increased in the same way under iron limitation, differences in their receptors have been shown genetically and biochemically. Resistant mutants are isolated independently from an indicator against S1 or S2 (26), and the receptor substance for each pyocin behaved differently during isolation from the cell wall (10a). The middle region, which is conserved in three pyocins but not in E2 group colicins, may be necessary for penetrating *Pseudomonas* cells. Thus, the putative domains of the pyocins, receptor binding, translocation, and DNase, are arranged differently from those in colicin E2 in which the order is translocation-receptor binding-DNase from N to C termini (20). This may be a good example of domain shuffling in molecular evolution.

Killing mechanism of pyocins S1 and S2. A biochemical study disclosed that pyocins S1 and S2 actually have potential DNases of E2 group colicins. These pyocins inhibit lipid synthesis and cause DNA breakdown of the sensitive cells. The reason that we missed DNA breakdown with pyocin S2 (18) might be because we did not employ a proper condition, iron deficiency, in the previous study. This dual effect is a peculiar feature of pyocins S1 and S2. Which is the primary effect causing cell death? In pyocins S1 and S2, inhibition of lipid synthesis is an early event, while DNA breakdown is also observed at an early phase (Fig. 6 and 8). Pyocin AP41 as well as colicin E2 show DNase activity in vitro and provoke DNA breakdown in vivo, but they do not inhibit lipid synthesis (3, 21). These findings indicate that DNA breakdown alone could kill the cells and that the two effects, DNA breakdown and inhibition of lipid synthesis, may be independent events; one may not necessarily be the cause of the other. In a previous study (18), we found a parallel relation between the extent of inhibition of lipid synthesis and that of killing. If the inhibition of lipid synthesis is solely sufficient for killing, we might be able to construct an active pyocin without DNase activity. However, we have not yet succeeded in determining a domain which is responsible for the inhibition of lipid synthesis. At present, we do not know the actual mechanism that results in cell death and the effects of a disturbance of lipid or DNA metabolism. The inhibition of lipid synthesis might have something to do with killing efficiency, since pyocins S1 and S2 show a higher efficiency of killing than pyocin AP41. Further studies will be required to determine this point.

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