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The Inherent DNase of Pyocin AP41 Causes Breakdown of Chromosomal DNA

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Pyocin AP41 degrades the chromosomal DNA in sensitive strains of *Pseudomonas aeruginosa* but has little effect on RNA, protein, and lipid syntheses. In vitro experiments showed that the carboxyl-terminal part of the large subunit of pyocin AP41 carries an inherent DNase that is responsible for its killing action.

Pyocin AP41 is a typical S-type pyocin, proteinase-susceptible bacteriocin, produced by certain strains of *Pseudomonas aeruginosa* (3, 14). Biochemical studies (11) and molecular analysis of the pyocin determinant (12, 13) have shown that pyocin AP41 is a complex of two protein subunits with molecular weights of 84,000 and 10,000. The large component, as well as pyocin AP41 (LS complex), kills susceptible bacteria, such as the PAO strain. The characteristic action of pyocin AP41 is that it causes production of resident pyocins, like R2 and S2, in the PAO strain and/or bacteriophages (F116 and PS17) in their lysogenic strains in a *recA*-dependent way (11). In these respects, pyocin AP41 resembles colicin E2, which is known to induce production of phage λ in lysogenic *Escherichia coli* (2) and causes degradation of chromosomal DNA (5, 6, 10). Autoinduction of colicin E2 in cells with broken-down immunity has also been reported (9). Colicin E2 is a complex of two subunits, and its large subunit (the killing or active subunit) has DNA-cleaving activity in vitro which is carried on its C-terminal part (15, 16). However, the DNase activity observed in vitro alone is not sufficiently potent to explain the single-hit killing in vivo, since DNA molecules are not considerably degraded in vitro compared with in vivo with equivalent amounts of colicin molecules (16). Our recent findings showed that the large subunits of pyocin AP41 and E2 group colicins share the homologous domain of DNase (13), suggesting that pyocin AP41 possesses DNase activity. Pyocin AP41 also might be multifunctional in its mode of killing, since the large subunit of pyocin AP41 is a rather large molecule having an extra domain compared with E2 group colicins and homologous S-type pyocins S1 and S2 (14). Here we report the killing action of pyocin AP41 in vivo, as well as its function in vitro.

In this study, we used *P. aeruginosa* PAF41 and PAO3295 (*recA*) as a producer strain and a strain sensitive to pyocin AP41, respectively (3, 11).

Effects on macromolecular metabolism in sensitive bacteria. To clarify the molecular mechanisms of the killing action of pyocin AP41, we first examined its effects on protein, nucleic acid, and lipid metabolism in sensitive bacteria. For efficient killing, cells were grown in nutrient broth no. 2 (NB; Oxoid Ltd., London, England) at 25 g/liter in L-shaped tubes with constant seesaw shaking until the cell density reached approximately 2×10^8/ml. Then the radioactive precursor was added to each culture; this was followed by treatment with or without pyocin AP41. Trichloroacetic acid-insoluble counts were measured at a given time. As shown in Fig. 1, uptake of [^{14}C]adenosine into the DNA fraction (c) was remarkably changed after addition of pyocin AP41, while protein synthesis (a), RNA synthesis (b), and lipid synthesis (d) were barely affected, at least until 60 min after treatment. Namely, incorporation into the DNA fraction continued for 30 min and then ceased completely. The slight effect of pyocin AP41 on lipid synthesis is in remarkable contrast to that of the homologous pyocin, S2 (7, 14). These results suggest that pyocin AP41 preferentially inhibits DNA synthesis and/or causes degradation of DNA. To discriminate between these possibilities, the structural stability of chromosomal DNA in sensitive cells was examined after treatment with pyocin AP41. It is evident from Fig. 1e that the chromosomal DNA was degraded within 10 min after addition of pyocin. Our previous discovery that pyocin AP41 induces production of resident pyocins and phages in sensitive bacteria, like UV irradiation or treatment with mitomycin C or nalidixic acid, is in concert with the above-described finding and may support the notion that the primary effect of pyocin AP41 is degradation of chromosomal DNA.

DNase activity in vitro. To determine whether the degradation of chromosomal DNA is caused by possible DNase activity associated with pyocin AP41 molecules, we directly observed the structural change of covalently closed circular (CCC) DNA incubated with pyocin AP41 and its subunits in vitro. Figure 2A shows that pyocin AP41 (lane b), as well as the large component (lane c), appeared to possess DNase activity since it converted the plasmid DNA from the CCC form to the open circle and/or the linear form, demonstrating that AP41 DNase is a DNA endonuclease. It is, however, evident that the DNase activity of the large component is much stronger than that of pyocin AP41 (LS complex).

Isolation of the trypsin fragment and its DNase activity. In the course of the experiments, we noticed that one of the preparations of purified pyocin AP41 exhibited stronger activity than did the large component after being kept at 4°C for about 2 months. Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of this particular preparation showed that the protein band corresponding to the large component disappeared and in its place a protein band of unique size with faster mobility (apparent molecular weight, 16,000; aging fragment) appeared, along with a trace amount of a few protein bands and the band corresponding to the small component. It reminded us that processing of the pyocin molecules might increase the activity of DNase associated with the large component. As it was difficult to obtain a large amount of the aging fragment reproducibly, we tried to generate a similar protein by processing pyocin AP41 with known proteases. Pyocin AP41 (LS complex) was

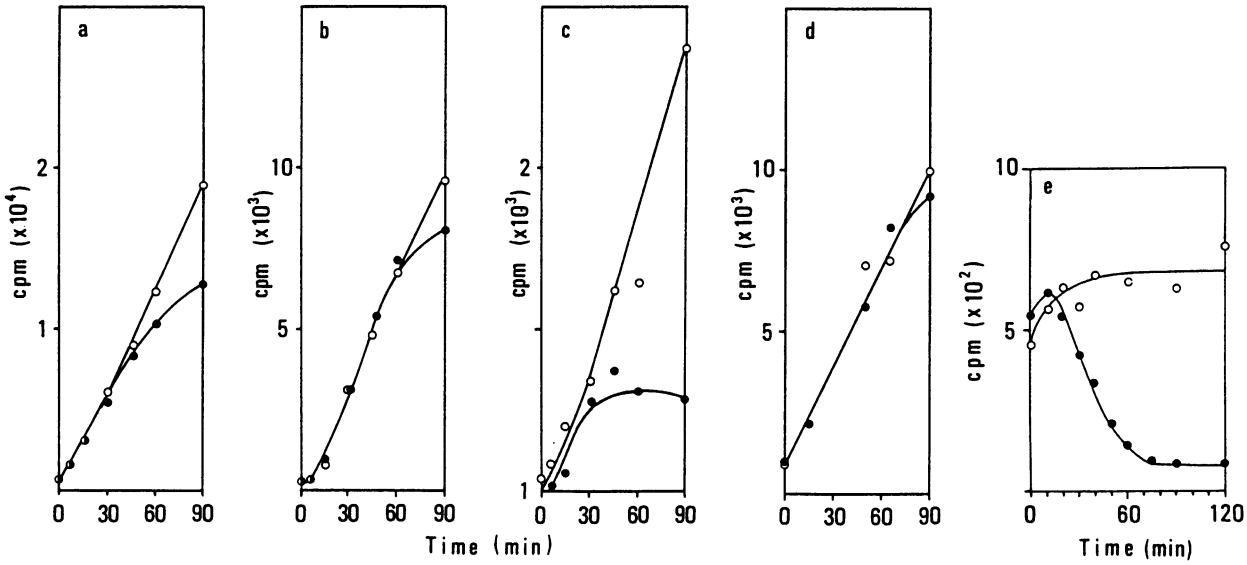

FIG. 1. Effects of pyocin AP41 on macromolecular syntheses and chromosomal stability. (a to d) Incorporation of radioactive precursors into protein (a), RNA (b), DNA (c), and lipid (d) fractions. PAO3295 was grown in NB and labeled with the following radioisotopes: a, [^{14}C]leucine (354 mCi/mmol, 1 μCi/ml; Amersham Japan Ltd., Tokyo, Japan); b and c, [^{14}C]adenosine (559 mCi/mmol, 2 μCi/ml; Amersham Japan Ltd.); d, [2-^{3}H]glycerol (11.5 Ci/mmol, 2 μCi/ml, 0.2 mM; NEN Research Products, Boston, Mass.). Since NB was used for efficient killing, the specific activity in the medium could not be estimated. Pyocin AP41 (128 U/ml; LS complex) was purified as described previously (11) and added at time zero, i.e., 2 (a to c) or 3 (d) min after addition of the radioisotope. The amount of [^{14}C]adenosine incorporated into the DNA fraction was estimated as alkali-resistant trichloroacetic acid-insoluble counts recovered by pretreatment of cell suspensions with 1 N KOH at 37°C for 1 h. Alkali-labile trichloroacetic acid-insoluble counts were assumed to be incorporation into the RNA fraction. Incorporation into the lipid fraction was estimated as described by Ohkawa et al. (7). (e) Chromosomal stability. PAO3295 was cultured in NB containing [^{14}C]adenosine (the same lot as described above; 1 μCi/ml) for about 4 generations (3.5 h) at 37°C until the logarithmic phase. Cells were collected by low-speed centrifugation and suspended in prewarmed fresh NB to a density of approximately 2×10^8/ml. Pyocin AP41 (145 U/ml) was added at time zero. The amount of [^{14}C]adenosine incorporated into the DNA fraction was measured. The viability of pyocin AP41-treated cultures at the times indicated (1.0 at time zero, before addition of pyocin) was as follows: a, 6.0×10^{-2} to 3.9×10^{-2} (120 min); b and c, 6.0×10^{-2} to 4.6×10^{-2} (120 min); d, 1.6×10^{-3} (90 min); e, 1.7×10^{-2} (65 min) and 8.9×10^{-4} (120 min). Symbols: ●, pyocin AP41 treated; ○, nontreated.

digested with several enzymes, such as trypsin, thermolysin, papain, α-chymotrypsin, and *Staphylococcus aureus* V8 protease, and analyzed by SDS-PAGE. Of the enzymes tested, only trypsin gave a satisfactory result, whereas the large component alone was almost completely digested and yielded no detectable protein band on SDS-PAGE. When the large component was preincubated with the small component, the digested products were similar to those obtained with pyocin AP41, suggesting that the 16,000-M_r fragment is protected from the trypsin by association with the small component. The 16,000-M_r fragment, designated as the trypsin fragment, was then purified as follows. Purified pyocin AP41 (LS complex; 10 mg) was incubated with 2 mg of freshly dissolved trypsin (treated with DPCC [diphenylcarbamyl chloride] [Sigma Chemical Co., St. Louis, Mo.]) in 3 ml of saline buffer (10 mM Tris-HCl [pH 7.3], 0.25 M NaCl) at 37°C for 1 h. By using a Sephacryl S200 column with the saline buffer, the TS complex (a complex of the trypsin fragment and the small component) was isolated. These two components were further separated by a Sephadex G75 column under denaturation conditions by using a saline buffer containing 6 M urea. The purified trypsin fragment did not retain the killing activity in vivo but exhibited about fourfold higher DNase activity than did the large component (Fig. 2A).

While the AP41 DNase had been measured in the presence of 10 mM Mg^{2+}, we found that further addition of 1 mM EDTA decreased the DNase activity, suggesting that the DNase requires some divalent cation(s) other than Mg^{2+}. Of the divalent cations tested (Mg^{2+}, Ca^{2+}, Mn^{2+}, Cu^{2+}, and Zn^{2+}), the AP41 DNase exhibited the highest activity in the presence of 2 to 20 mM Mn^{2+} and little activity in the presence of Cu^{2+}. With Mn^{2+}, the activity increased by 8- to 16-fold over that observed with Mg^{2+} (Fig. 2B).

The TS complex, unlike the preparation containing the aging fragment, exhibited a very low level of DNase activity, if any, suggesting that the small component functions as a DNase inhibitor. It is clear from Fig. 2C and D that DNA degradation by the trypsin fragment was inhibited by preincubation with the small component. When the small component was added in an equivalent molar ratio, inhibition was nearly complete and no further effect was observed when excess amounts of the small component were added.

The in vitro activity associated with the large component is not sufficiently potent to explain the in vivo action, because sensitive bacteria are killed by a single pyocin molecule (single-hit killing) (11). There are several possible reasons for this discrepancy. (i) Killing occurs in cooperation with some cellular DNase(s). (ii) Assay conditions do not reflect the in vivo circumstances. Pyocin AP41 might recognize a structure that might specifically appear in replicating chromosomes, since efficient killing of sensitive cells was observed in the logarithmic phase (11). (iii) The enzyme requires some other cofactor(s) for optimal activity. (iv) Pyocin molecules are processed in vivo so as to exhibit potent DNA-cleaving activity. The first and second possibilities are difficult to verify by experiment, especially to exclude any participation by a cellular enzyme(s). It is

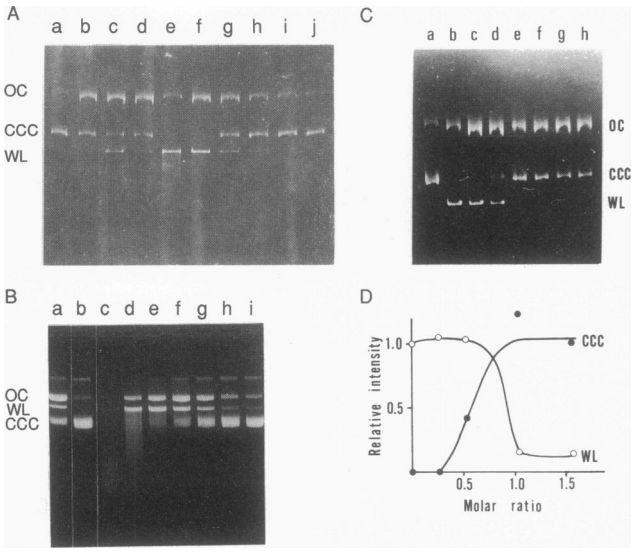

FIG. 2. (A) Structural changes in plasmid DNA caused by pyocin AP41 and its derivative proteins. Each reaction mixture (50 μl) contained 0.55 μg of M13 replicative form I DNA and the proteins indicated below in 100 mM Tris-HCl (pH 8.0)–10 mM $MgCl_2$. The reaction mixture was incubated at 37°C for 1 h and analyzed by agarose gel electrophoresis. Lanes: a, no addition; b, pyocin AP41 (6.2 μg, 66 pmol); c, large component (5.9 μg, 70 pmol); d, TS complex (1.8 μg, 78 pmol); e to j, trypsin fragment (e, 1.0 μg, 79 pmol; f, 0.5 μg; g, 0.26 μg; h, 0.13 μg; i, 0.06 μg; j, 0.03 μg). (B) Effect of Mn^{2+} on DNase activity. Each reaction mixture (10 μl) contained 0.5 μg of pBR322 DNA and the following amount (in nanograms) of the trypsin fragment in 20 mM Tris-HCl (pH 7.5)–20 mM NaCl–5 mM $MgCl_2$ (a) or 5 mM $MnCl_2$ (b to i): a, 64; b, no addition; c, 64; d, 32; e, 16; f, 8; g, 4; h, 2; i, 1. (C and D) Inhibitory action of the small component on the DNase activity associated with the trypsin fragment. The trypsin fragment (0.32 μg) was incubated with various amounts of the small component at 37°C for 30 min prior to the DNase assay. Each reaction mixture (20 μl) contained 0.275 μg of M13 replicative form I DNA and 10 μl of the preincubated mixture. Other conditions were the same as for panel A. (C) Agarose gel electrophoresis. Lanes: a, no addition; b, without the small component; c to g, 0.065 (c), 0.13 (d), 0.26 (e), 0.39 (f), or 0.52 (g) μg of the small component; h, the same as g but without the trypsin fragment. (D) Relative amounts of CCC and whole linear (WL) DNAs. The amounts of each band (CCC or WL) were measured with a densitometer, and relative intensity was calculated by assuming that the intensity of the whole linear band in lane b and that of the CCC band in lane f was 1.0. OC, open circle.

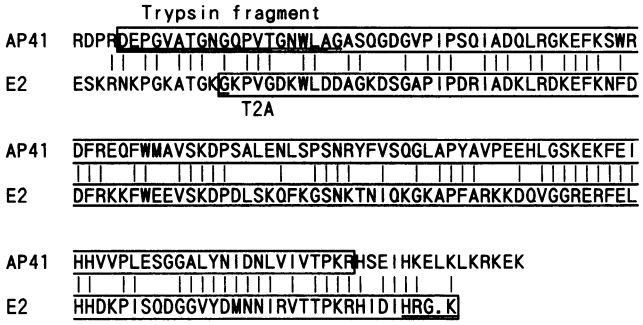

FIG. 3. Amino acid sequence of the trypsin fragment aligned with that of T2A of colicin E2. The primary sequences deduced from the nucleotide sequences of the regions conserved between pyocin AP41 and colicin E2 are shown. The underlined sequences were determined by using purified proteins. The trypsin fragment (Asp-642 to Lys-761 or Arg-762) and T2A (Gly-462 to Lys-581) are boxed. See the text for references.

possible that the assay conditions employed are not optimal. At any rate, addition of Mn^{2+} instead of Mg^{2+} produced apparently higher activity. The mode of the Mn^{2+} effect is not clear; however, it might be possible to induce structural changes in the substrate as well as activation of the enzyme. The last possibility, activation of DNase in vivo, may be plausible because the naturally occurring aging fragment shows higher DNA-cleaving activity. Processing by trypsin increases the activity of the large component, although the activity seems to be still lower than that of the aging fragment (data not shown). The trypsin fragment just caused a nick on a DNA molecule with an equivalent molar ratio of a protein and a substrate DNA, even under our optimal in vitro conditions (Fig. 2B, lane h). Since the trypsin fragment was obtained as a single protein band on SDS-PAGE, we could determine 20 amino acids from the amino terminus of the purified trypsin fragment. Together with the amino acid composition of the fragment and the deduced amino acid sequence, the trypsin fragment is considered to comprise 120 or 121 amino acids, including the carboxyl-terminal part of the large component (13). Figure 3 shows the sequences of the trypsin fragment and T2A, a similar protein of colicin E2, in which processing with trypsin does not increase DNase activity (1, 4, 8, 16). Compared with the regions conserved between the two, the trypsin fragment of pyocin AP41 lacks the 15 or 16 residues from the carboxyl terminus of the large subunit while T2A lacks the 10 residues of the amino-terminal end. Such a minor difference might affect the activity of the DNase. Although we could not characterize the molecular nature of the aging fragment well, more efficient processing might have taken place in the sensitive bacteria treated with pyocin AP41. As the pyocin AP41 genes have been cloned, it is possible to obtain a series of protein fragments similar to the trypsin fragment or the aging fragment by using recombinant DNA techniques. Polypeptides exhibiting sufficient DNase activity to explain the in vivo activity might be obtained in this way.

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