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Genetic Determinant of Pyocin AP41 as an Insert in the *Pseudomonas aeruginosa* Chromosome

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The genetic determinant for pyocin AP41, a bacteriocin produced by *Pseudomonas aeruginosa* PAF, was transferred to *P. aeruginosa* PAO and analyzed. By conjugation experiments, the pyocin determinant was found to be located on the chromosome, being closely linked to *argG* at about 45 min on the genetic map. Cloning of the pyocin AP41 gene into the plasmid R68.45 was attempted in vivo by taking advantage of its linkage at *argG*. R′ *argG*$^+$ plasmids were isolated by interspecific conjugation between *P. aeruginosa* and *Escherichia coli recA argG* strains. Some of the R′ *argG*$^+$ plasmids did contain the pyocin AP41 determinant. Genetic and physical analyses of these R′ plasmids indicated that the pyocin AP41 determinant was located within a 2.9-kilobase extra segment found at a certain position of the chromosome of various pyocin AP41 producer strains.

Pyocins are bacteriocins produced by *Pseudomonas aeruginosa* and are classified into three major groups, R, F, and S (9, 10). The former two pyocins are large protein complexes with morphology similar to certain bacteriophage tails, whereas S-type pyocins are smaller, simpler, and proteinase susceptible. Recently, we have purified and characterized pyocin AP41, a typical S-type pyocin that is produced by *P. aeruginosa* strain PAF41 and that kills strain PAO (14; unpublished observation). Our results suggest that pyocin AP41 is either a 1:1 or 1:2 complex of 90,000- and 7,000-dalton polypeptides and that both the pyocin AP41 complex and its large component have the ability to kill sensitive cells. Upon treatment with pyocin AP41, the production of resident pyocins or phages (or both) is induced, whereas DNA synthesis is preferentially inhibited in the sensitive cells. When the AP41 complex is treated with trypsin, only the large component is digested, yielding a 16,000-dalton polypeptide that has no killing activity, but exhibits DNA endonuclease activity. Furthermore, the small component inhibits the endonuclease activity almost stoichiometrically. Since colicin E2 is known to have properties similar to those described above, one might imagine that both pyocin AP41 in *P. aeruginosa* and colicin E2 in *Escherichia coli* belong to the same category of bacteriocin.

In the present communication, the genetic and physical structure of the AP41 determinant was investigated so as to elucidate the relationship, if any, between pyocin AP41 and colicin E2. For this purpose, the pyocin determinant was transferred from *P. aeruginosa* strain PAF to strain PAO, the genetically best-characterized strain, and analyzed by conventional genetic methods. Our data, however, suggested that, unlike the colicin E2 gene, the determinant of pyocin AP41 was not extrachromosomal, but situated on the chromosome. A useful method of cloning genes into plasmid R68.45 in vivo has been developed by Holloway (7). Taking advantage of the close linkage of pyocin AP41 to *argG*, the pyocin gene(s) was cloned into R68.45, and the restriction map of the DNA segment around the pyocin AP41 gene(s) was constructed. The comparison of this restriction map with that of the corresponding region of the AP41-nonproducer cell together with the results of blot hybridization indicated that the pyocin AP41 determinant was located within a 2.9-kilobase (kb) extra segment found at a certain position on the chromosome of various AP41-producer strains of *P. aeruginosa*.

(A preliminary account of this work was presented at the Third Tokyo Symposium on Microbial Drug Resistance, 1981 [15].)

MATERIALS AND METHODS

Media. Minimal medium for *E. coli* was M9 (2), and that for *P. aeruginosa* was G medium (14). Amino acids were added as necessary at a concentration of 50 μg/ml each; thiamine and thymidine were added at 20 μg/ml each. Nutrient broth and agar have been described previously (14). King A agar for the detection of pyocin AP41 productivity was a product of Eiken Chemical Co. Ltd., Tokyo.

Drugs were used at the following concentrations: for *E. coli*, carbenicillin (Fujisawa Pharmaceuticals Co.) at 100 μg/ml, kanamycin (Meiji Seika Kaisha, Ltd) at 25 μg/ml, and tetracycline (Lederle Japan Ltd.) at 10 μg/ml. For *P. aeruginosa*, carbenicillin at 500 μg/ml, kanamycin at 250 μg/ml, tetracycline at 100 μg/ml, and streptomycin (Meiji Seika Kaisha, Ltd.) at 250 μg/ml.

Bacterial strains. The bacterial strains used in the present study are listed in Table 1. R68.45 is a self-transmissible, chromosome-mobilizing, broad-host-range plasmid conferring resistance to carbenicillin, kanamycin, and tetracycline and tolerance to AP41 (3). Strains carrying R68.45 are designated as PAO12(R68.45), PRD428(R68.45), and so on. Besides those shown in Table 1, the following *P. aeruginosa* strains were employed with reference to pyocin productivity: pyocin AP41 producers, strains NIHD, NIHL, SL7, and GGB; pyocin AP41 nonproducers, PAT2408, PML28, PML1516k, and NIH3 (9).

Detection of pyocinogenicity. Pyocin productivity was investigated as follows. Colonies to be tested were transferred with toothpicks onto nutrient agar plates and incubated for a few hours until small colonies showed up. Then they were irradiated with a UV lamp (15-W Toshiba germicidal lamp) for 45 s at 50 cm to induce pyocin production. After several hours of incubation, residual growth was killed with chloroform vapor, and the plates were overlayered with a soft agar solution containing an appropriate indicator strain. After

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TABLE 1. Bacterial strains

Strain	Relevant characteristics[a]	Derivation, source, or reference
P. aeruginosa		
PAO12	*leu-38 pur-136 chl-3*	B. W. Holloway
PAO303	*argB18 chl-2*	B. W. Holloway
PAO1751	*trp-6 argF302*	B. W. Holloway
PAO1839	*argF9011 leu-9004 ami-151 hutC107*	H. Matsumoto
PAO2106	*met-9020 hex-9001 leu-9006 argF9012 ami-9001*	H. Matsumoto
PAO3012	*trp-6*	Our collection (10)
PAO3092	prototroph, PyoF3^r	Our collection (14)
PAO3238	*his-308 ilv-309 met-318 trp-6 thr-301*, PyoR2$^-$, E79^r, PyoAP^r	Our collection
PAO3243	*his-308 ilv-309 met-318 trp-6*, PyoR2$^-$, E79^r	Our collection
PAO3260	*met-9020 catA1 nar-9011 chu-9002 trpB9029 lys-9015*, PyoAP^r	Pyocin AP41-resistant derivative of PAO2196
PAO3261	*met-9020 catA1 nar-9011 chu-9002 trpB9029 lys-9015 leu-9014 argG9036*, PyoAP^r	Pyocin AP41-resistant derivative of PAO2198
PAO3262	*met-9020 hex-9001 leu-9006 argF9012 ami-9001 str-306*, PyoAP^r	Streptomycin- and pyocin AP41-resistant derivative of PAO2106
PAO3296	*met-9020 trpA325 catA1 pca-9001 nar-9011*, PyoAP^r	Pyocin AP41-resistant derivative of PAO3281
PAO3311	*met-28 trp-6 ade-312 nal-302*	Our collection
PAO3319	*met-28 trp-6 thr-319 nal-302*	Our collection
PAO3331	*met-28 trp-6 pro-312 nal-302*	Our collection
PAO3338	*met-28 pro-312 nal-302 str-302*	Our collection
PAO3339	*met-28 his-314 thr-320 nal-302 str-302*	Our collection
PAF41-2	*met thr*, PyoAP41$^+$, PyoF3$^+$	mutagenesis of PAF41
PRD428	*his-308*, PyoAP$^+$, PyoS2$^+$, PyoF3$^+$	PAF41-2(R68.45) × PAO3243
PRD445	*trpA325 catA1 nar-9011*, PyoAP$^+$	PRD428(R68.45) × PAO3296
PRD449	*trpB9029 lys-9015 leu-9014 met-9020*, PyoAP$^+$	PRD428(R68.45) × PAO3261
PRD451	*his-308*, PyoAP$^+$	PRD445(R68.45) × PAO3238
PRD471	*argF302*, PyoAP$^+$, PyoF3$^+$	PAF41-2(R68.45) × PAO1751
PRD472	*trp-6*, PyoAP$^+$	PAF41-2(R68.45) × PAO1751
PML1516d	*trp*	Our collection (10)
E. coli		
JC1553	*metB1 leu-6 his-1 argG6 str-104 recA1*	Y. Nakamura
PBTH1	*argG thyA metC str recA*	Y. Nakamura
AB1178	*thr leuB6 his argG*	A. Kikuchi
AD1	HfrH (*proA/B argF lac*)Δ *argI*Δ	A. Kikuchi
EJ11	(*proA/B argF lac*)Δ *argI his*	A. Kikuchi

[a] Symbols for genetic markers are those used in reference 8. Unless otherwise specified, PAO sublines produce pyocin R2, S2, and F2 and are sensitive to pyocin AP41, and strain PAF produces pyocins AP41 and F3. PyoR2$^-$ signifies defectiveness in pyocin R2 production; PyoAP$^+$, PyoF3$^+$, etc., signify production of pyocin AP41, pyocin F3, etc., respectively. PyoAP^r signifies insensitivity to pyocin AP41, PyoF3^r signifies resistance to pyocin F3, and E79^r signifies resistance to phage E79.

overnight incubation, clear zones were observed around pyocinogenic colonies. When pyocin productivity of the R′ plasmid in *E. coli* was tested, the incubation was done for 15 h and UV irradiation was omitted. As indicators, PAO3092 was used for pyocin AP41, PML1516d was used for pyocin S2, and PAO3012 was used for pyocin F3. For the rapid survey of productivity of pyocin AP41 with many samples, the following procedure was employed, although it was less sensitive. PAO3092 with soft agar was overlayered on a King A plate, and colonies to be tested were spotted on this lawn with toothpicks. Pyocin AP41-positive cells have small halos around their colonies after overnight incubation on this plate. Incubation was carried out at 37°C, except for the detection of pyocin F3, which was done at 30°C.

R68.45-mediated conjugation. Procedures for transfer of R68.45 and for mating mediated by this plasmid were as described by Haas and Holloway (3). The interrupted mating was carried out by the method of Haas and Holloway (4), but with the following modification. Streptomycin-sensitive donor cells (5×10^8/ml in nutrient broth) were mixed with an equal volume of streptomycin-resistant recipient cells (1×10^9/ml in nutrient broth), and 0.2-ml portions of the mixture were spread on selection plates (time zero). At appropriate intervals, 0.6-ml portions of streptomycin solution (5 mg/ml) were spread on the plates to kill the donor. Recombinants were scored after 2 days. All of the procedures were performed at 37°C.

Construction of R′68.45 *argG*$^+$. R′68.45 *argG*$^+$ plasmids were constructed by an in vivo cloning method similar to that described by Holloway (7). However, we used as recipients *E. coli recA* cells in place of *P. aeruginosa rec* cells. After donor *P. aeruginosa* and recipient *E. coli* cells were grown in nutrient broth to 2×10^8 cells per ml, 0.5 ml of the culture of the donor cells and 4.5 ml of that of the recipient cells were mixed and immediately collected onto a nitrocellulose filter (HAWP, 24 mm in diameter; Millipore Corp., Bedford, Mass.) by suction. The filter thus obtained was transferred onto a nutrient agar plate and incubated at 37°C for about 15 h. Bacteria grown on the filter were removed and suspended in 0.5 to 1.0 ml of M9 medium. *E.*

coli cells harboring R'68.45 *argG*$^+$ were selected on M9 plates with appropriate supplements as colonies appearing after 3 or 4 days of incubation.

Enzymes and radioisotopes. Restriction enzymes, T4 DNA ligase, and RNase-free T4 polynucleotide kinase were purchased from Takara Shuzo Co., Ltd. (Kyoto, Japan). *E. coli* DNA polymerase I and its large fragment (Klenow enzyme) were the products of Boeringer (Mannheim, West Germany). [α-^{32}P]dATP (3,000 Ci/mmol) and [γ-^{32}P]ATP (5,000 Ci/mmol) were purchased from Amersham International Ltd. (Amersham, England).

Purification of DNA and RNA. (i) Plasmid DNA. R' plasmid DNA was purified by the alkaline extraction method (1) followed by CsCl-ethidium bromide density gradient centrifugation.

(ii) Chromosomal DNA. Chromosomal DNA was extracted from *P. aeruginosa* as described by Willetts et al. (17).

(iii) RNA. RNA was prepared by a hot phenol method (13) from *P. aeruginosa* PAF41-2 cells treated with mitomycin C (14) or untreated. In particular, in the former case, RNA was extracted from 10-ml cultures at 15, 30, 45, 60, and 90 min after induction, mixed together, and used as a probe after labeling.

Digestion with restriction enzymes. DNA was digested partially or completely by various enzymes in the buffers recommended by suppliers at 37°C.

Agarose gel electrophoresis. Electrophoresis was carried out on 0.4% agarose gel at 0.9 V/cm or 0.8 to 1.0% agarose gel at 1.8 V/cm with Tris-acetate buffer (6) containing 0.5 μg of ethidium bromide per ml.

Preparation of radioactive probes and blot hybridization. DNA probes were prepared by nick translation (11) with [α-^{32}P]dATP, whereas probe RNA was prepared by an alkaline treatment followed by labeling with kinase and [γ-^{32}P]ATP. DNA was digested with restriction enzymes, sized on agarose gel by electrophoresis, transferred to nitrocellulose filters (Shleicher & Schuell Co., Keene, N.H.; BA83) and hybridized essentially by the method of Southern (16). Autoradiograms were taken with Kodak XRP1 films with intensifying screens.

RESULTS

Transfer of the pyocin AP41 determinant from strain PAF to strain PAO. PAF41-2 carrying R68.45 was mated with PAO3243, and the recombinants for *his*$^+$, *ilv*$^+$, *met*$^+$, or *trp*$^+$ were selected and analyzed for pyocinogenicity. Most of them were PyoAP$^-$, but one was obtained which showed the following characteristics: *his-308*, PyoAP$^+$, PyoS2$^+$, and PyoF3$^+$. This strain, designated as PRD428, was mainly used for the subsequent experiments. In the case of the cross of PAF41-2 (R68.45) with PAO1751, 1 of 40 *trp-6*$^+$ recombinants was PyoAP$^+$, whereas 6 of 40 *argF*$^+$ recombinants were PyoAP$^+$. The former (PRD471) and one of the latter (PRD472) were also employed for further mating experiments. These recombinant derivatives gave adequate number of recombinants with PAO strains to enable linkage analysis to be carried out.

Linkage analysis of pyoAP with various auxotrophic markers. With the above-described PRD strains as donors, R68.45-mediated conjugation experiments were carried out with PAO recipients. Selections were made with various markers, and coinheritance of the pyocin productivity was investigated (Table 2). The recombination frequency varied with markers, ranging from 10^{-6} to 10^{-4}. The frequency of transfer of R68.45 itself was over 10^{-4}. No cotransfer of the pyocin productivity with the plasmid markers was observed. The results were essentially the same with any donors and the highest linkage was found with *lys-9015*, and *argG9036* markers which are located at about 45 min on the chromosome (Table 2). The linkage value decreased as distance between *argG* and the selected marker increased. These results suggest that the pyocin AP41 determinant is not on a plasmid, but on the chromosome close to *argG*. In the above crosses, PyoAP$^+$ donors were mated with Pyo$^-$ recipients. To confirm the chromosomal nature of the pyocin determinant, a reciprocal cross was carried out. When PAO303 (R68.45) was mated with PRD449 (*trpB leu-9014 lys-9015 met-9020*, PyoAP$^+$), 51 of 71 *lys*$^+$ recombinants were PyoAP$^-$, whereas no PyoAP$^-$ recombinants were obtained with *trp*$^+$ or *leu*$^+$ alone. Thus the close linkage of the pyocin determinant with *lys-9015* was reconfirmed.

TABLE 2. Coinheritance of pyocin AP41 productivity with chromosomal markers by R68.45-mediated conjugation[a]

Donor	Coinheritance with the following marker:												Recipient
	his-314 (13)[b]	*trpA325* (28)	*trpB9029* (28)	*met-28* (30)	*trp-6* (35)	*pro-312* (40)	*ade-312* (42)	*thr-319* or *thr-320* (42)	*leu-9006* (43)	*lys-9015* (43)	*argF9012* (45)	*argG9036* or *argG9011* (45)	*met-9020* (55)	
PRD428			0/82							85/85				PAO3260
PRD428									44/44	44/44		88/88		PAO3261
PRD428		0/77											6/40	PAO3296
PRD428				0/70	0/32		17/85							PAO3311
PRD428					0/38			33/88						PAO3319
PRD428					0/11	4/49								PAO3331
PRD471			0/88							0/7				PAO3260
PRD471				0/88		4/88								PAO3338
PRD471	0/88			0/88				10/88						PAO3339
PRD472											21/44			PAO1839
PRD472											30/44		14/19	PAO2106
PRD472	0/44							42/88						PAO3339

[a] PRD428, PRD471, and PRD422 were mated with an appropriate recipient shown in the last column. Recombinants were selected for indicated markers, and their pyocin AP41 productivity was investigated. Numbers of PyoAP$^+$ colonies over recombinants surveyed are shown.

[b] Positions of the markers on the chromosomes are shown by the time of entry (minutes) from the origin of transfer by FP2 (Holloway and Crockett [8], and unpublished observation by Kageyama).

TABLE 3. Characteristics of R′ plasmids

R′ plasmid	Donor (*P. aeruginosa*)	Recipient (*E. coli*)	Frequency of isolation (per donor cell)	Phenotype			Plasmid size[c] (kb)
				AP41	ArgF[a]	ArgG[b]	
pNM331	PAF41-2(R68.45)	JC1553	ND[d]	−	+	+	
pNM332, pNM333, pNM338	PAF41-2(R68.45)	JC1553	ND	+	+	+	
pNM401	PRD445(R68.45)	JC1553	10^{-10}	+	+	+	180
pNM451	PRD445(R68.45)	PBTH1	10^{-10}	+	+	+	
pNM501 through pNM514	PAO12(R68.45)	JC1553	10^{-8}	−	+	+	135 (pNM501) 190 (pNM502)
pNM551 through pNM557	PAO12(R68.45)	PBTH1	10^{-8}	−	+	+	170 (pNM555)

[a] R′ plasmids were transferred to *E. coli argF* strains AD1 and EJ11 by mating on selection plates.
[b] R′ plasmids were transferred to *E. coli argG* strains PBTH1 (or JC1553) when JC1553 (or PBTH1) was used as the recipient for R′ selection and to AB1178 in both cases by mating on selection plate.
[c] Plasmid sizes were calculated from the data in Fig. 2.
[d] ND, Not determined.

Interrupted mating experiment with R68.45. To determine the order of the genetic loci of pyocin AP41 and neighboring markers more precisely, the time course of gene transfer was measured by an interrupted mating technique. PRD451(R68.45) as a donor and PAO3262 as a recipient were employed since this pair gave a high number of recombinants (Fig. 1). Although the time course may suggest an oriented transfer from *met* to *arg* and *leu*, the linkage analyses suggested multiple origins of transfer as reported by Haas and Holloway (4). Linkage values among the selected and unselected markers were investigated, and the most probable gene order was deduced from the results (Fig. 1B).

Construction of R′68.45 argG$^+$. As the close linkage of *argG* and pyocin AP41 gene(s) was indicated by the above experiments, in vivo cloning of the *argG* gene was attempted in the expectation that the pyocin gene(s) might be picked up along with *argG*. *P. aeruginosa* AP41 producer strains (PAF41-2, PRD445) and a nonproducer strain (PAO12), all carrying both the chromosomal marker *argG*$^+$ and the R factor R68.45, were mated with *E. coli recA argG* cells (JC1553 or PBTH1). Although the frequencies were as low as 10^{-8} to 10^{-10} per donor cell, some *argG*$^+$ exconjugants were successfully recovered in every case (Table 3). These exconjugants could transfer not only *argG*$^+$, but also *argF*$^+$, characters at very high frequencies to other *E. coli recA* strains (data not shown), indicating that both *argG* and *argF* genes were present on the R′ plasmids and that the *P. aeruginosa* genes could be expressed in *E. coli* to some extent. Furthermore, some of the R′ plasmids did carry the ability to produce the pyocin AP41 (Table 3). The pyocin activity could be expressed in *E. coli* cells, although at a very low level. Such cells exhibited faint turbid halos around their colonies on a lawn of a *P. aeruginosa* strain sensitive to pyocin AP41 (PAO3092). These halos were not observed by the addition of anti-pyocin AP41 serum in the plate or with a strain tolerant to the pyocin, PAO3092(R68.45). Finally, the productivity of pyocin AP41 could be transferred from such *E. coli* cells back to *P. aeruginosa* strains (PAO), giving rise to colonies with clear inhibitory zones against the indicator strain.

Arrangement of pyoAP41, *argF*$^+$, and *argG*$^+$ genes. To determine the location of each gene on the R′ plasmids, the physical and deletion mappings were done. First, the physical maps of two R′ plasmids derived from an AP41 producer (pNM401, pNM451) and three of those derived from a nonproducer (pNM501, 555, 502) were constructed by single and double digestion of restriction enzymes, *Bam*HI and

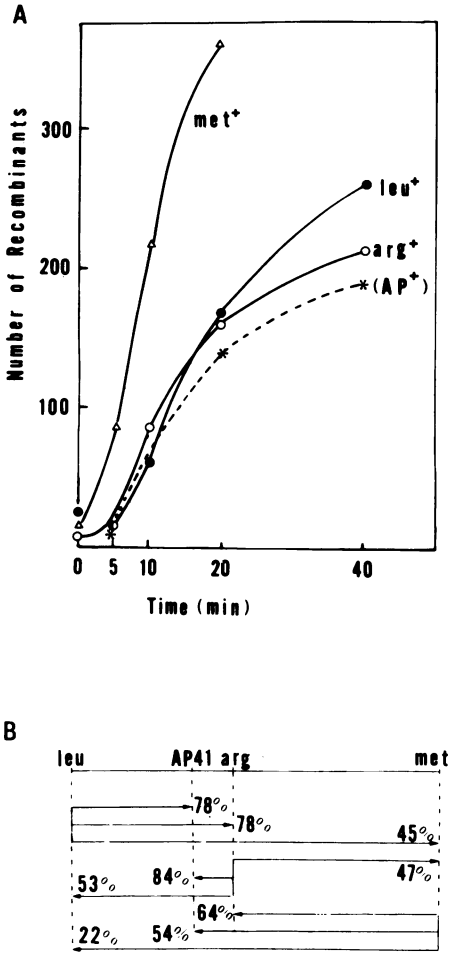

FIG. 1. Interrupted mating experiment. PRD451(R68.45) (*his-308*, PyoAP$^+$) and PAO3262 (*met-9020 leu-9006 argF9012 str-306*) were mated on selection plates at 37°C, and streptomycin (3 mg) was added to each plate to interrupt conjugation at the time indicated (see the text). (A) Numbers of recombinants which appeared on each selection plate are shown. (AP$^+$) shows the number of pyocin-producing colonies among *arg*$^+$ recombinants. (B) Recombinants for *met*$^+$, *leu*$^+$, or *arg*$^+$ obtained at 5, 10, and 20 min were investigated for distribution of unselected markers. Coinheritance of unselected markers with each selected marker is shown in percent. The arrowheads indicate unselected markers.

*Hin*dIII (Fig. 2). Although an extra *Hin*dIII site was found in pNM401 and pNM451, all R′ plasmids appeared to share an about 60-kb-long segment originated from the *P. aeruginosa* chromosome, suggesting that both *argF*$^+$ and *argG*$^+$ genes are located within this common region. To confirm this and to localize further the AP41 gene(s), in vitro deletion mapping was carried out with pNM501 and pNM401 as the starting R′ plasmids. Each R′ DNA was digested with a restriction enzyme *Hin*dIII, ligated with T4 DNA ligase, and used for the transformation of *E. coli* JC1553. Notice that the vector R68.45 contains only one *Hin*dIII site near the junction of the vector and the cloned chromosomal DNA as reported by Riess et al. (12). Various types of deletion mutants were obtained (Fig. 3). By comparing the distribution of *Hin*dIII segments in deleted R′ plasmids and the phenotypes of the corresponding transformants, it is deduced that (i) *argF*$^+$ and *argG*$^+$ genes are located, respectively, on the 13.4- and 12.0-kb *Hin*dIII segments of pNM401, and (ii) the AP41 determinant is situated within the 41-kb *Hin*dIII segment of pNM401 which corresponds to the 38-kb segment of pNM501.

By digestion with *Bam*HI, the 41-kb *Hin*dIII segment of pNM401 is divided into three portions, two of which are identical in length (13.9 and 7.1 kb) to the counterparts of the 38-kb *Hin*dIII segment of pNM501 (Fig. 2). The remaining 20-kb *Bam*HI-*Hin*dIII segment is longer by approximately 3 kb than the corresponding segment of pNM501. The comparison of the structure of these 20- and 17-kb *Bam*HI-*Hin*dIII segments in more detail was done by digestion with *Pst*I, *Sal*I, and *Eco*RI (Fig. 4A). The restriction patterns were essentially the same between them, except for one *Pst*I-*Sal*I fragment. pNM501 (PyoAP$^-$) had a 1.0-kb *Pst*I-*Sal*I fragment, whereas pNM401 (PyoAP$^+$) had at the corresponding region a 3.9-kb fragment with two additional *Eco*RI sites. This difference of 2.9 kb is probably responsible for the production of pyocin AP41, and this extra segment might exist as an insert at a position marked by an arrow in Fig. 4A. To know more precisely the location of the AP41 determinant, the structures of the three R′ plasmids, pNM331, pNM451, and pNM4512, all of which appear to lack the left-hand portions of the 20-kb *Bam*HI-*Hin*dIII segment from the AP41 producer, were examined. pNM4512 is a derivative of pNM451, which was obtained by the same procedure used for the isolation of deletion mutants of pNM401 and pNM501 as described above. pNM451 and pNM4512 carried the pyocin productivity, whereas pNM331 did not. By blot hybridization with the 20-kb *Bam*HI-*Hin*dIII segment of pNM401 as a probe, partial restriction maps of these R′ plasmids were constructed (Fig. 4B). Comparing three restriction maps and considering the size of the pyocin protein, it is deduced that the coding region(s) for the AP41 gene(s) are probably confined to a narrow region labeled X in the lower margin of the Fig. 4B.

Correlation between AP41 productivity and the 2.9-kb insert. Our unpublished observations have shown that many *P. aeruginosa* strains other than PAF (most of them are those for typing purposes) produce a pyocin serologically indistinguishable from pyocin AP41. To examine whether this PyoAP$^+$ phenotype is associated with the presence of the 2.9-kb extra segment, hybridization experiments were carried out with six pyocin AP41 producers and five nonproducers. DNAs prepared from these strains were digested with restriction enzymes *Eco*RI, *Pst*I, and *Sal*I, electrophoresed on agarose gels, transferred to nitrocellulose filters, and

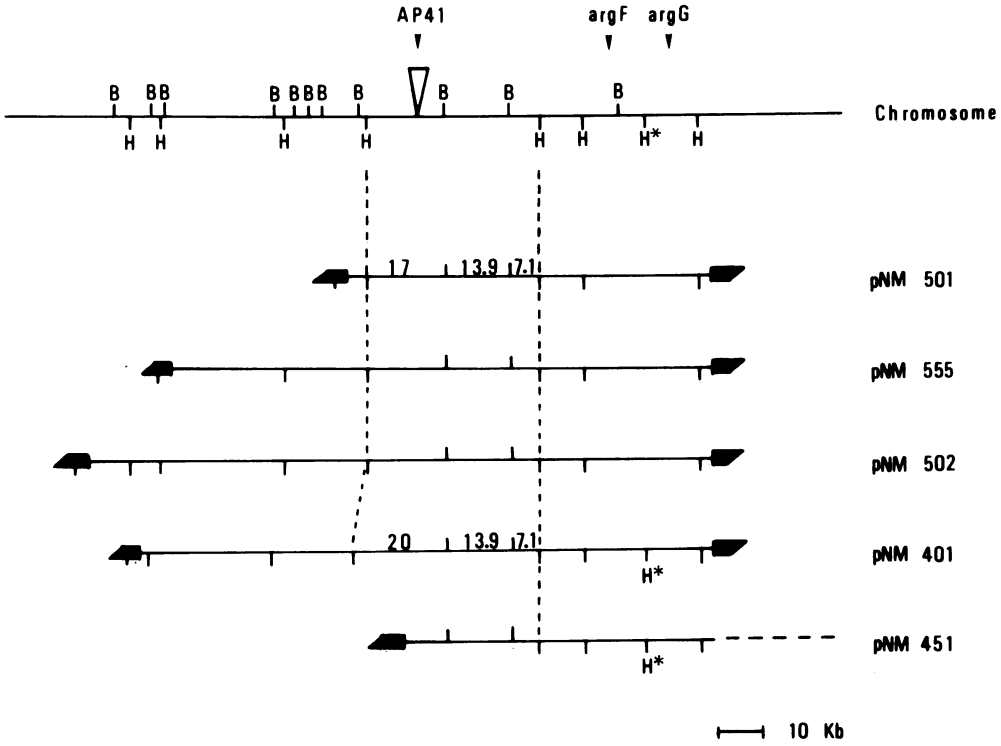

FIG. 2. Restriction maps of R′ plasmids. In the upper margin, the final results about the restriction enzyme cleavage sites and the genetic loci of the chromosome region covered by the R prime plasmids are shown. The heavy black line indicates the vector R68.45. The distance of the region surrounded by the broken lines is shown in kb. Abbreviations: B, *Bam*HI; H, *Hin*dIII; H*, an *Hin*dIII site that is present in the PRD strain, but not in the PAO strain.

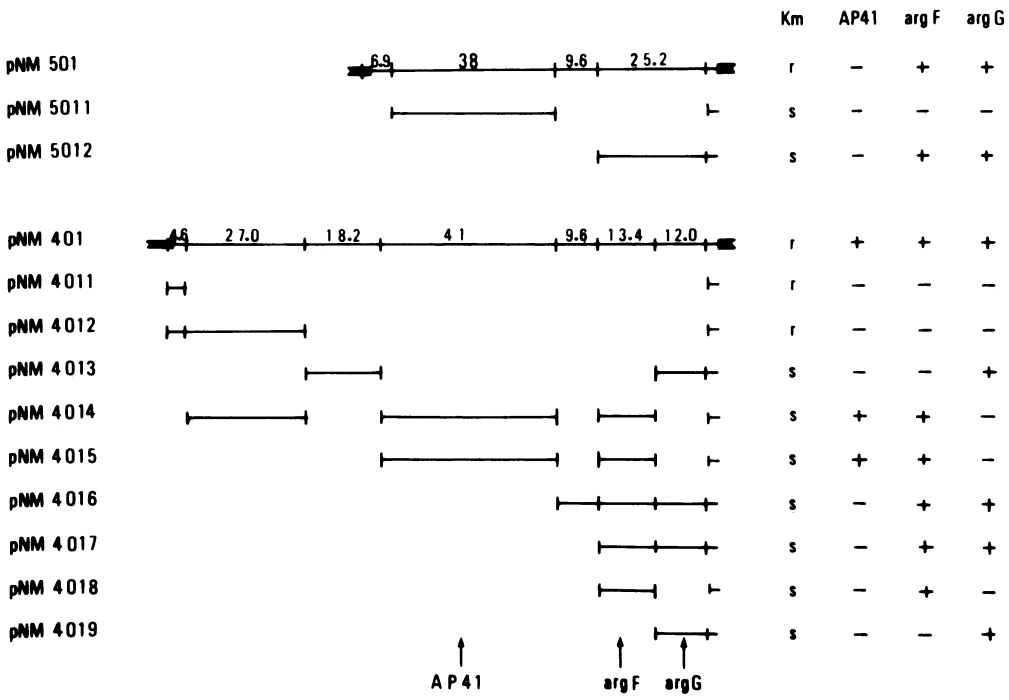

FIG. 3. Arrangement of pyocin AP41 gene(s) and neighboring genes. The *Hin*dIII segments of the chromosomal region on the parental (pNM501 and pNM401) and deleted plasmids are shown at the left, and the phenotypes carried by these plasmids are shown at the right. The heavy black line shows R68.45, and the segment sizes are shown in kb.

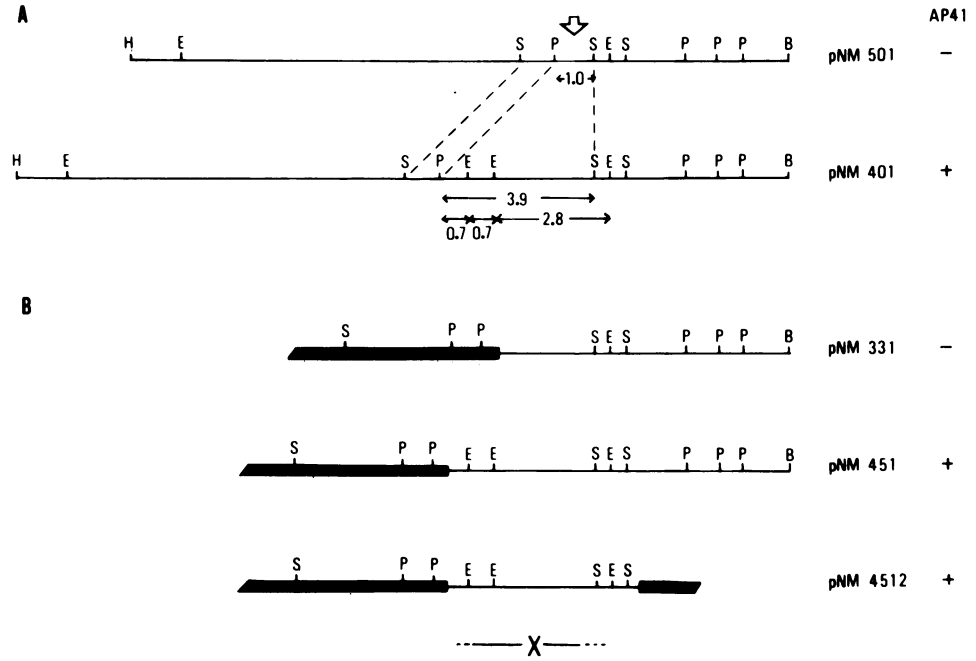

FIG. 4. (A) Restriction maps of the *Bam*HI-*Hin*dIII segments from pNM501 (17 kb) and pNM401 (20 kb). Each plasmid was first digested with *Bam*HI, and a fragment of 19 kb (pNM502) or 22 kb (pNM401) was isolated. Each fragment was then labeled its 3′ end with ^{32}P and separated on agarose gel after digestion with *Hin*dIII. The resulting labeled segment (17 or 20 kb) was recovered from gel by electroelution, partially digested with the indicated enzymes, and analyzed on agarose gels with the labeled molecular weight standard. The distance from the *Bam*HI site was deduced from the autoradiogram. Several *Sal*I and *Pst*I sites on the left half are not shown because their precise positions have not been determined. Abbreviations: B, *Bam*HI; P, *Pst*I; S, *Sal*I; E, *Eco*RI; H, *Hin*dIII. (B) Partial restriction maps of pNM331, pNM451, and pNM4512. The maps were constructed by blot hybridization with the 20-kb *Bam*HI-*Hin*dIII segment as a probe. The black bar shows R68.45.

hybridized with either the 2.8- or 0.7-kb *Eco*RI segment of pNM401, which covers most of the extra segment (Fig. 4A). The hybridization patterns of all six AP41 producers were identical not only to one another, but also to those of pNM401, whereas the DNA extracted from the nonproducers showed little homology to the probes (Fig. 5). The sizes of the hybridized bands coincided well to those expected from the restriction map (Fig. 4A). Figure 6 shows the pattern of the blot hybridization of *Sal*I-digested samples, when the 1.0-kb *Pst*I-*Sal*I segment of pNM501 (indicated by an arrow in Fig. 4A), which encompasses the neighboring region upon the insertion site of the 2.9-kb segment, was used as a probe. All strains lacking the ability to produce pyocin AP41 exhibited a 2.0-kb band, whereas all AP41 producers showed a 4.9-kb band, which is longer by 2.9 kb. These sizes are again coincided well with the values shown in Fig. 4A. These results suggest that all the pyocin producers tested share the 2.9-kb segment inserted at the same restriction fragment of the chromosome, and that the extra segment is generally responsible for the production of pyocin AP41.

Specific hybridization of the 2.9-kb segment by mitomycin C-induced RNA from the AP41-producer cells. We have already shown that the production of AP41 increases extensively upon treatment with mitomycin C (14), thus suggesting that the AP41 RNA is much enriched in the induced AP41-producer cells. RNA was extracted from PAF41-2 cells treated with mitomycin C or untreated, labeled with ^{32}P, and used as probes for blot hybridization of *Eco*RI-

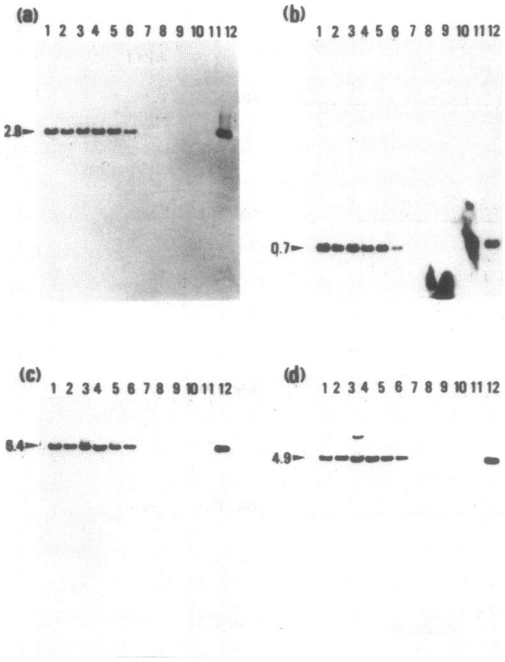

FIG. 5. Presence of the 2.9-kb extra segment on the chromosome of AP41 producer strains. Bacterial DNA (lanes 1 through 11) and DNA of pNM401 (lane 12) were digested with *Eco*RI (a and b), *Pst*I (c), or *Sal*I (d), electrophoresed on agarose gel, and transferred to nitrocellulose filters. The filters were hybridized with the nick-translated probes, the 2.8-kb *Eco*RI fragment (a, c, and d), and the 0.7-kb *Eco*RI fragment (b). The bacterial strains are as follows: 1, NIHL; 2, SL7; 3, PAF41; 4, GGB, 5, NIHD; 6, PRD445; 7, PAO12; 8, PAT2408; 9, PML28; 10, PML1516k; 11, NIH3.

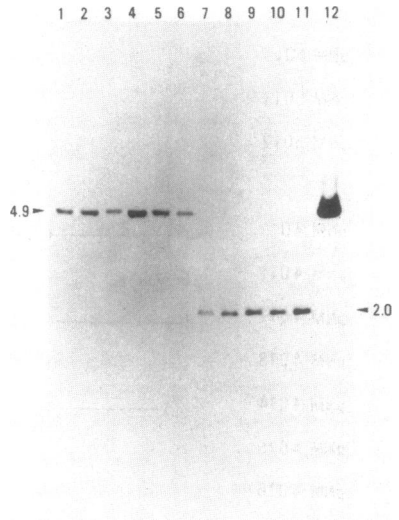

FIG. 6. Comparison of the chromosome region covering the insertion site of the 2.9-kb segment between AP41 producers and nonproducers. Bacterial DNA (lanes 1 through 11) and DNA of pNM401 were digested with *Sal*I and hybridized with the nick-translated 1.0-kb *Pst*I-*Sal*I segment of pNM501 as shown in Fig. 4A. The bacterial strains (lane 1 through 11) are as described in the legend to Fig. 5.

*Bam*HI-*Hin*dIII triple digests of pNM4015 (PyoAP$^+$) and pNM502 (PyoAP$^-$) (Fig. 7). In case of pNM4015 digests, a distinct band corresponding to the 2.8-kb *Eco*RI segment was detected as the one hybridizable with the RNA extracted from the induced cells, whereas no appreciable signal was detected in the case of pNM502 digests, irrespective of the probe RNA used. Although it is not clear in Fig. 7, a separate experiment (data not shown) revealed that the 0.7-kb *Eco*RI segment of the 2.9-kb insert also hybridized with the RNA extracted from mitomycin C-induced AP41-producer cells. In other words, the 2.9-kb extra segment was transcribed preferentially under the condition of induced pyocin synthesis.

DISCUSSION

P. aeruginosa strain PAF produces two pyocins, pyocin AP41 and pyocin F3, whereas strain PAO is sensitive to pyocin AP41 and produces pyocins R2, F2, and S2. Since genetic analyses have been done with strain PAO (8), but not with PAF, mapping of the pyocin AP41 determinant was carried out after its transfer to PAO sublines. Conjugation experiments revealed that the pyocin gene(s) was incorporated in the chrosomose close to *argG*. The gene arrangement deduced from the results of linkage analyses as well as those obtained from deletion plasmids is follows: early *leu-9006 lys-9015 pyoAP41 argF argG met-9020* late.

The association of the pyocin gene(s) on the chromosome close to *argF* and *G* is specific not only for the PRD strain used, but also for PAF, since independently isolated PRD strains (PAF × PAO) showed essentially the same results; more conclusively, the pyocin gene(s) was cloned in vivo into R68.45 along with *argG* from PAF as well as PRD.

R′ plasmids carrying *argG*$^+$ were isolated by using *E. coli recA* strains as recipients. All of them carried *argF* too, and some carried PyoAP$^+$ as expected from conjugation experiments. The extent of expression of *P. aeruginosa* genes in *E. coli* seemed to be low. In case of *argF*, the expression of the

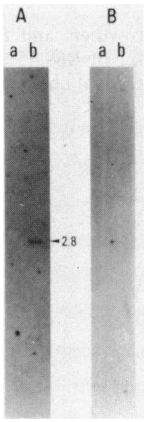

FIG. 7. Specific hybridization of the extra segment by mitomycin C-induced RNA. DNAs from pNM502 (lane a) and pNM4015 (lane b) were digested with *Hin*dIII, *Bam*HI, and *Eco*RI, electrophoresed on agarose gels, and transferred to nitrocellulose filters. The filters were hybridized with RNA extracted from the mitomycin C-induced (A) and noninduced (B) PAF41-2 cells. About 11 μg of RNA was used in each experiment after being labeled with ^{32}P.

gene was only 2 to 3% in *E. coli* as compared with *P. aeruginosa* prototrophic strains, as determined by measuring the specific activity of ornithine transcarbamylase (the product of *argF*) of the cells cultivated without arginine (data not shown). With these R′ plasmids, restriction and deletion mappings were performed (Fig. 2). The pyocin AP41 determinant was located on a 3.9-kb *Pst*I-*Sal*I fragment of a 41-kb *Hin*dIII fragment. Since the corresponding *Pst*I-*Sal*I fragment of non-pyocinogenic plasmid was 1 kb long, the difference of 2.9 kb may be responsible for the pyocin productivity. Several lines of evidence support the view that the 2.9-kb segment may cover the pyocin gene(s). A plasmid lacking a portion of this segment can not produce active AP41; RNA extracted from AP41-producing cells specifically hybridizes to 2.8- and 0.7-kb *Eco*RI fragments which cover most of this segment. The size of this segment, 2.9 kb, has the capacity for coding for at least the larger, 90,000-dalton subunit protein. Furthermore, we have recently isolated a recombinant clone containing only the 3.9-kb *Pst*I-*Sal*I fragment covering the 2.9-kg segment, and this clone exhibited a PyoAP$^+$ phenotype (manuscript in preparation). Thus it is quite likely that a major portion of the insert was occupied by the AP41 determinant. (We will hereafter refer to the 2.9-kb segment found in the AP41 producers as the AP41 extra segment).

The existence of the AP41 extra segment in other AP41 producer strains was also verified. The representative six producer strains did not contain any plasmid having the sequence hybridizable to the AP41 extra segment (data not shown). The AP41 extra segment was shared by all six AP41-producer strains and is exclusively localized on the same restriction fragment of the chromosome, whereas all AP41-nonproducer strains contained a common fragment lacking the AP41 extra segment. Thus, the site-specific insertion (or deletion) of the AP41 extra segment seems to be responsible for generating the AP41 producers (or nonproducers) in wild strains of *P. aeruginosa*, although whether the insertion (or deletion) of the AP41 extra segment resulted from a single event or serial multiple events remains to be determined. The relation, if any, of the presence of an extra *Hin*dIII site (asterisk in Fig. 2) in the region containing both *argF* and *argG* genes and the presence of the AP41 extra segment was examined by blot hybridization with the 13.4- and 12.0-kb *Hin*dIII fragments as probes (data not shown). Regardless of pyocin productivity, 10 strains except PAO (Fig. 5) had a *Hin*dIII site between *argF* and *argG* which is indistinguishable from that of PRD. Therefore, this event seems to be independent from the insertion (or deletion) of the AP41 extra segment. The above result also suggests that PRD, a recombinant of PAF and PAO strains, contains a chromosomal segment derived from PAF covering not only the AP41 determinant but also the *argF* and *argG* region.

The genetic determinants of similar bacteriocins so far known (such as colicins E1, E2, E3, I, and V) in *E. coli* (5) and in *Enterobacter cloacae* (cloacin DF13) (5) have been shown to be located on extrachromosomal elements. In this respect, pyocin AP41 is unique. Sequencing analysis of the AP41 extra segment and its surrounding region is now in progress, and the results will give a useful clue to how this extra segment originated on the *P. aeruginosa* chromosome.

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