A PLASMID WHICH DOES NOT ENCODE THE AMINOGLYCOSIDE PHOSPHOTRANSFERASE IN THE BUTIROSIN-PRODUCING STRAIN OF *BACILLUS CIRCULANS*

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Bacillus circulans NRRL B-3312 produces the aminoglycoside antibiotic butirosin and encodes an aminoglycoside 3′-phosphotransferase. We detected a 48 kilobase plasmid, pIP850, in this strain; this was analyzed by agarose gel electrophoresis following digestion with *Eco*RI restriction endonuclease and by nucleic acid hybridization. The results obtained indicate that plasmid pIP850 does not carry the structural gene for the aminoglycoside modifying enzyme.

In antibiotic-producing organisms the role of plasmids in production[1] of and resistance[2] to antibiotics has recently been demonstrated. *Bacillus circulans* strain NRRL B-3312[3] is used for the industrial production of butirosin, an aminoglycoside closely related to neomycin[4]. This strain also produces an enzyme (APH) which phosphorylates the 3′-hydroxyl group of butirosin and structurally-related compounds[5~7].

In this paper we describe a plasmid in strain NRRL B-3312 and attempts to correlate the synthesis of the aminoglycoside-modifying enzyme with this extrachromosomal structure.

Materials and Methods

Bacterial Strains and Plasmids

B. circulans strain NRRL B-3312[3] which produces butirosins A and B was obtained from Dr. T. PRIDHAM, Northern Regional Research Laboratories, Peoria, Ill, 61604, U. S. A. *Escherichia coli* C-1a strains harboring plasmids, pAT6[7], RP4[8], ColE1:: Tn3[9], and ColE1[10] were from our laboratory collection. Hybrid plasmid pAT6 consists of ColE1:: Tn3 with a 7.5 kb *Eco*RI insert of *B. circulans* DNA encoding the APH (3′) enzyme.

Media

Brain heart infusion broth and agar (Difco) were used, except where stated. Incubation were at 30°C for *B. circulans* and at 37°C for *E. coli*.

Enzymes

Restriction endonuclease *Eco*RI was purified as described[11]. Proteinase K was from Merck; lysozyme was from Sigma; ribonuclease A (bovine pancreas) was from Calbiochem and *E. coli* K12 RNA polymerase holoenzyme was from Miles.

Chemicals

Adenosine 5′-[α-^{32}P]triphosphate triethylammonium salt ([α-^{32}P] ATP) and adenosine 5′-[γ-^{32}P]-triphosphate triethylammonium salt([γ-^{32}P] ATP) were obtained from the Radiochemical Center Amersham. The antibiotics were provided by the following laboratories: gentamicins (Gen) A, B, Schering; kanamycins (Kan) A, B, C, amikacin (Ami), and ampicillin (Ap), Bristol; neamine (NeoA), neomycin B (NeoB), Upjohn; paromomycin (Par) and butirosin (But), Parke-Davis; tobramycin (Tob), Lilly; lividomycin (Liv) A, Kowa; ribostamycin (Rib), Meiji; chloramphenicol (Cm), Roussel; tetracycline (Tc), Pfizer. *N*-Lauroyl sarcosine was from Sigma.

Tests for Antibiotic Susceptibility

Disk agar diffusion susceptibility tests[12] were done on MUELLER-HINTON agar medium.

Assay for Aminoglycoside-modifying Enzymes.

The bacterial extracts were prepared[7] and the enzymes assayed by the phosphocellulose paper binding technique[13] as described.

Preparation of DNA

Plasmid DNA from *B. circulans* was purified by a technique already described[14] and modified as follows. Cells from 200 ml of an exponential phase culture were harvested and resuspended in 20 ml of SET buffer (0.15 M NaCl, 0.1 M EDTA, 0.05 M tris-HCl (pH 8.0)) containing lysozyme (1 mg/ml) and ribonuclease A (500 μg/ml). This suspension was divided into 1 ml aliquots and incubated at 37°C for 1 hour. The resulting protoplasts were lysed with 0.2% *N*-lauroyl sarcosine. The DNA was sheared by passing the lysates five times through a 2-ml syringe, pooled, adjusted to 33 g with SET buffer, and mixed with 31 g of cesium chloride. Ethidium bromide was added to a final concentration of 150 μg/ml. The solution was centrifuged at 18°C for 19 hours at 50,000 rpm in a Beckman VTi-50 rotor. Fractions containing plasmid DNA were pooled and banded a second time at 18°C for 8 hours at 65,000 rpm in a Beckman VTi-65 rotor. Fractions containing DNA were pooled and ethidium bromide was removed by four consecutive extractions with *iso*-propanol. The DNA was dialyzed four times against 1 liter of 10 mM tris-HCl, 1 mM EDTA (pH 7.5), and stored at 4°C.

The following DNAs were prepared according to the procedures described previously: RP4 plasmid[15], ColE1 factor and derivative plasmids[16], bacteriophage $\lambda cI857$[17], and *B. circulans*[7].

Agarose Gel Electrophoresis of Restriction Endonuclease Digests

DNA was digested with restriction endonuclease *Eco*RI in reaction mixtures of 70 μl for analytical purposes or of 350 μl for preparative gels, containing 100 mM tris-HCl (pH 7.8) and 100 mM $MgCl_2$. The reactions were allowed to proceed for 1 hour at 37°C, then heated at 68°C for 10 minutes and chilled at 0°C. The resulting DNA fragments were separated by electrophoresis in horizontal slab gels containing 0.7% or 0.8% agarose. Electrophoresis and staining were as described[18].

Hybridization

^{32}P-Labeled complementary RNA (cRNA) synthesized in reaction mixtures containing plasmid DNA, *E. coli* K12 RNA polymerase, GTP, CTP, UTP, and [α-^{32}P]ATP was separated from non incorporated radioactive material by passage through a column of Sephadex G-50 as described previously[17]. Restriction endonuclease generated DNA fragments fractionated by agarose gel electrophoresis were transferred to cellulose nitrate sheets (Sartorius) as described by SOUTHERN[19]. Hybridization of cRNA probes to the DNA immobilized on the membrane filters was as described by DENHARDT[20]. Hybridization was revealed by autoradiography using XRI-Omat Kodak films.

Testing for Resistance

Resistance of *B. circulans* strain NRRL B-3312 to butirosin and ribostamycin and susceptibility to other aminoglycoside antibiotics[7] was tested by the disk agar diffusion method[12]. The synthesis, by this strain, of an aminoglycoside 3′-phosphotransferase was monitored by phosphocellulose paper binding assays[13].

Results

Plasmid Content of *B. circulans* NRRL B-3312

Strain B-3312 was shown by ultracentrifugation in cesium chloride - ethidium bromide to contain covalently closed circular DNA. The electrophoretic mobility of the purified plasmid DNA in agarose gel is shown in Fig. 1.

Strain B-3312 was found to harbor a plasmid which was designated pIP850. It had a molecular size of approximately 48 kilobases (kb) and five *Eco*RI-generated DNA fragments which were numbered in order of decreasing size.

Comparative analysis of the *Eco*RI-generated fragment patterns of pIP850 and pAT6 plasmid DNA indicated that none of the pIP850 fragments comigrated with the 7.5 kb insert of pAT6.

Analysis of Plasmid pIP850 DNA by Hybridization

Plasmid DNA was cleaved with *Eco*RI restriction endonuclease and the resulting fragments were separated by agarose gel electrophoresis, denatured *in situ*, transferred to a nitrocellulose filter (driver) and hybridized to *in vitro* ^{32}P-labeled RNA (cRNA) probes complementary to plasmid DNA. The distribution of homologous sequences among the differently sized fragments of plasmid DNA was then determined by autoradiography. In each experiment, the homologous reaction (*i.e.*, RNA complementary to the driver as probe) was used as internal standard. The results of the hybridization experiments are shown in Fig. 2.

As expected, and already shown[21], ^{32}P-labeled pAT6 cRNA hybridized with an *Eco*RI-generated fragment of *B. circulans* total DNA having an electrophoretic mobility identical to that of the insert in pAT6. For the same reason, plasmid pIP850 cRNA probe hybridized with

Fig. 1. Analysis of *Eco*RI-generated patterns of plasmid DNA by gel electrophoresis.

Two to three μg of plasmid DNA were digested with *Eco*RI.

Fragments obtained by digestion of λ *cI*857 DNA, 48 kb[31] with *Eco*RI[32] and by digestion of RP4, 54 kb[9], ColE1::Tn3 (RSF2124), 11.6 kb[10] and ColE1, 6.56 kb[11] with *Eco*RI were used as molecular size standards. Electrophoresis was carried out in a 0.8% agarose horizontal slab gel (18×13×0.4 cm) for 14 hours at 3 V/cm. The molecular size of the DNA fragments is expressed in kilobases. The values are means from four determinations in 0.7 or 0.8% agarose.

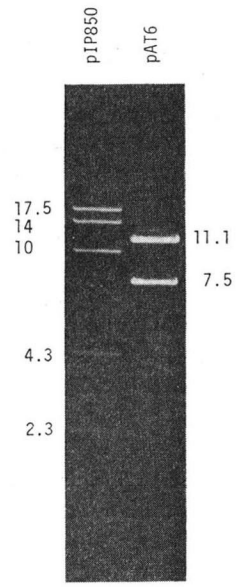
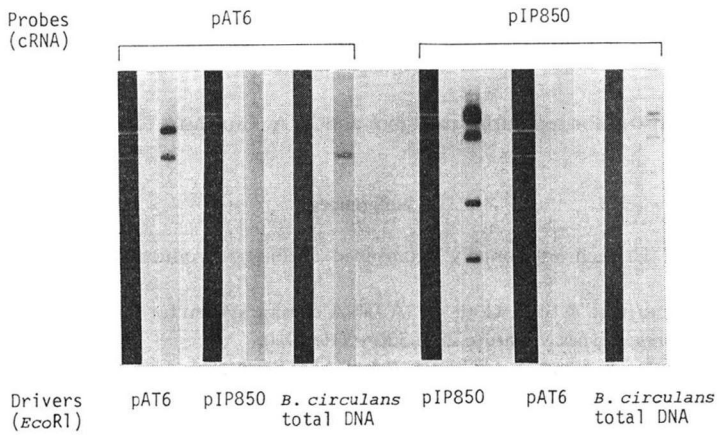


Fig. 2. Analysis of DNA by hybridization.

Five μg of the plasmid DNAs or 10 μg of *B. circulans* total DNA as indicated on the bottom line (drivers) were digested with *Eco*RI restriction endonuclease. The resulting DNA fragments were fractionated by electrophoresis through horizontal slab gels (0.7% agarose, 18×13×0.4 cm) for 14 hours at 3 V/cm, transferred to nitrocellulose sheets and hybridized to *in vitro* ^{32}P-labeled cRNA of plasmid pAT6 or pIP850 as indicated on the top line (probes). The autoradiograms were exposed for a period of 12 hours.



the five *Eco*RI-generated fragments of *B. circulans* total DNA. These fragments had migration properties similar to those of pIP850.

The probe made from pAT6 did not hybridize to plasmid pIP850 DNA. The reciprocal experiment (*i.e.*, pIP850 cRNA *versus* pAT6 DNA) was also negative. These results are consistent with that of the agarose gel electrophoresis indicating that the insert in pAT6 is different from every *Eco*RI DNA fragment of plasmid pIP850.

Discussion

It has been proposed that antibiotic-producing organisms might represent the source of R determinants for clinical isolates of pathogenic bacteria[22,23]. This suggestion relies on two kinds of evidence: many antibiotic-producing organisms code for biochemical mechanisms that are similar to those that determine antibiotic resistance in plasmid containing bacteria[22~29] and the gene encoding APH (3′) activity from *B. circulans* confers to the unrelated bacterium *E. coli* an aminoglycoside resistance phenotype similar to that encoded by "natural" R plasmids[7].

This working hypothesis implies intergeneric transfer of genetic information from antibiotic-producing organisms ultimately to pathogenic clinical isolates. Although it is not a prerequisite, the location of the structural gene for the 3′-phosphotransferase already on a plasmid in the butirosin-producing strain of *B. circulans* would constitute additional indirect evidence for the notion that clinical resistance to antibiotics determined by R plasmids originates in antibiotic-producing organisms.

We have detected a plasmid, pIP850, in *B. circulans* strain NRRL B-3312 and demonstrated that it does not carry the gene for the aminoglycoside-modifying activity. The role of this plasmid remains unknown; it may be involved in the synthesis, regulation of production, or resistance to butirosin.

The *B. circulans* 3′-phosphotransferase determines low-level resistance to butirosin and ribostamycin when present in its original host and high-level resistance to a much wider range of aminoglycosides when present in *E. coli*[7]. This observed difference is probably due to a gene dosage effect. The gene for the phosphotransferase is associated with a relaxed replicating plasmid in *E. coli* and is unlikely to be present in multicopy in *B. circulans*: the probe made from pAT6 hybridized to a single *Eco*RI-generated fragment of *B. circulans* total DNA and a low molecular weight multicopy plasmid would have been readily detected by our technique[14]. Gene dosage effect has already been reported in *E. coli* for the same type of enzyme originating in *Staphylococcus aureus*[17].

Of course, we cannot rule out the possibility that the structural gene for the phosphotransferase is located on a large, low copy, plasmid which could have escaped the technique used in this study. This is indeed a possibility especially since DAVIES (personal communication) and DOWDING[30] have also detected extrachromosomal DNA in this *B. circulans* strain using different techniques. It would be of interest to compare the three covalently closed circular DNAs independently obtained.

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References

1) HOPWOOD, D. A.: Extrachromosomally determined antibiotic production. Ann. Rev. Microbiol. 32: 373~392, 1978
2) BIBB, M. J.; J. L. SCHOTTEL & S. N. COHEN: A DNA cloning system for interspecies gene transfer in antibiotic producing *Streptomyces*. Nature 284: 526~531, 1980
3) HOWELLS, J. D.; L. E. ANDERSON, G. L. COFFEY, G. D. SENOS, M. A. UNDERHILL, D. L. VOGLER & J. EHRLICH: Butirosin, a new aminoglycosidic antibiotic complex: bacterial origin and some microbiological studies. Antimicrob. Agents & Chemother. 2: 79~83, 1972

4) UMEZAWA, H.: The chemistry and conformation of aminoglycoside antibiotics. *In*: Drug Action and Drug Resistance in Bacteria. Vol. 2. Aminoglycoside Antibiotics (S. MITSUHASHI, *ed.*), University of Tokyo Press, Tokyo, 1975
5) DOWDING, J. E. & J. DAVIES: Mechanisms and origins of plasmid-determined antibiotic resistance. In Microbiology-1974, pp. 179~186. *Ed.* D. SCHLESSINGER. Amer. Soc. Microbiol., Washington, 1975
6) DAVIES, J. & D. I. SMITH: Plasmid-determined resistance to antimicrobial agents. Annu. Rev. Microbiol. 32: 469~518, 1978
7) COURVALIN, P.; B. WEISBLUM & J. DAVIES: Aminoglycoside-modifying enzyme of an antibiotic-producing bacterium acts as a determinant of antibiotic resistance in *Escherichia coli*. Proc. Natl. Acad. Sci. U. S. A. 74: 999~1003, 1977
8) BARTH, P. T. & N. J. GRINTER: Map of plasmid RP4 derived by insertion of transposon C. J. Mol. Biol. 113: 455~474, 1977
9) SO, M.; R. GILL & S. FALKOW: The generation of a ColEl-Ap cloning vehicle which allows detection of insert DNA. Mol. Gen. Genet. 142: 239~249, 1975
10) BAZARAL, M. & D. R. HELINSKI: Characterization of multiple circular DNA forms of colicinogenic factor E1 from *Proteus mirabilis*. Biochemistry 7: 3513~3519, 1972
11) SUMEGI, J.; D. BREEDVELD, P. HOSSENLOPP & P. CHAMBON: A rapid procedure for purification of *Eco*RI endonuclease. Biochem. Biophys. Res. Commun. 76: 78~85, 1977
12) MORLEY, D. C.: A simple method of testing the sensitivity of wound bacteria to penicillin and sulfathiazole by use of impregnated blotting paper discs. J. Path. Bact. 57: 379~382, 1945
13) HAAS, M. & J. E. DOWDING: Aminoglycoside-modifying enzymes. *In*: Methods in Enzymology, Vol. 43. HASH, J. H. (*ed.*), p. 611, Acad. Press, New York, 1975
14) COURVALIN, P.; C. CARLIER & E. COLLATZ: Plasmid-mediated resistance to aminocyclitol antibiotics in group D streptococci. J. Bacteriol. 143: 541~551, 1980
15) LABIGNE-ROUSSEL, A.; G. GERBAUD & P. COURVALIN: Translocation of sequences encoding antibiotic resistance from the chromosome to a receptor plasmid in *Salmonella ordonez*. Mol. Gen. Genet. 182: 390~408, 1981
16) TANAKA, T. & B. WEISBLUM: Construction of a colicin E1-R factor composite plasmid *in vitro*: means for amplification of deoxyribonucleic acid. J. Bacteriol. 121: 354~362, 1975
17) COURVALIN, P. & M. FIANDT: Aminoglycoside-modifying enzymes of *Staphylococcus aureus*: expression in *Escherichia coli*. Gene 9: 247~269, 1980
18) SHINNICK, T. M.; E. LUND, O. SMITHIES & F. R. BLATTNER: Hybridization of labeled RNA to DNA in agarose gels. Nucleic Acids Res. 2: 1911~1929, 1975
19) SOUTHERN, E. M.: Detection of specific sequences among DNA fragments separated by gel electrophoresis. J. Mol. Biol. 98: 503~517, 1975
20) DENHARDT, D. T.: A membrane-filter technique for the detection of complementary DNA. Biochem. Biophys. Res. Commun. 23: 641~646, 1966
21) COURVALIN, P.; M. FIANDT & J. DAVIES: DNA relationships between genes coding for aminoglycoside-modifying enzymes from antibiotic-producing bacteria and R plasmids, p. 262~266. *In*: Microbiology-1978, D. SCHLESSINGER (*ed.*), Amer. Soc. Microbiol., Washington, D.C., 1978
22) WALKER, M. S. & J. B. WALKER: Streptomycin biosynthesis and metabolism. J. Biol. Chem. 245: 6683~6689, 1970
23) BENVENISTE, R. & J. DAVIES: Aminoglycoside antibiotic-inactivating enzymes in actinomycetes similar to those present in clinical isolates of antibiotic-resistant bacteria. Proc. Natl. Acad. Sci. U.S.A. 70: 2276~2280, 1973
24) NIMI, O.; C. ITO, Y. OHATA, S. FUNAYAMA & R. NOMI: Streptomycin-phosphorylating enzyme produced by *Streptomyces griseus*. Agric. Biol. Chem. 35: 856~861, 1971
25) NIMI, O.; G. ITO, S. SUEDA & R. NOMI: Phosphorylation of streptomycin at C_6-OH of streptidine moiety by an intracellular enzyme of *Streptomyces griseus*. Agric. Biol. Chem. 35: 848~855, 1971
26) ARGOUDELIS, A. D. & J. H. COATS: Microbial transformation of antibiotics. VI. Acylation of chloramphenicol by *Streptomyces coelicolor*. J. Antibiotics 24: 206~208, 1971
27) WALKER, J. B. & M. SKORVAGA: Phosphorylation of streptomycin and dihydrostreptomycin by *Streptomyces*. J. Biol. Chem. 248: 2435~2440, 1973
28) SHAW, W. V. & D. A. HOPWOOD: Chloramphenicol acetylation in *Streptomyces*. J. Gen. Microbiol. 94: 159~166, 1976
29) GRAHAM, M. Y. & B. WEISBLUM: 23S Ribosomal ribonucleic acid of macrolide-producing *Streptomyces* contains methylated adenine. J. Bacteriol. 137: 1464~1467, 1979

30) DOWDING, J. E.: Susceptibility of butirosin and neomycin B in *Bacillus circulans* the butirosin-producing organism. J. Gen. Microbiol. 115: 385~389, 1979
31) FIANDT, M.; A. HONIGMAN, E. C. ROSENSOLD & W. SZYBALSKI: Precise measurement of the *b*2 deletion in coliphage lambda. Gene 2: 289~293, 1977
32) THOMAS, M. & R. W. DAVIS: Studies on the cleavage of bacteriophage lambda DNA with *Eco*RI restriction endonuclease. J. Mol. Biol. 91: 315~328, 1975