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Biochemistry

Aminoglycoside-modifying enzyme of an antibiotic-producing bacterium acts as a determinant of antibiotic resistance in *Escherichia coli*

(neomycin phosphotransferase/*Bacillus circulans*/recombination *in vitro*/origin of R-plasmids)

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ABSTRACT *Bacillus circulans* NRRL B-3312, a nonpathogenic bacterium that produces the aminoglycoside antibiotic butirosin, is known to contain an aminoglycoside phosphotransferase that is similar to the neomycin phosphotransferases of clinically isolated antibiotic-resistant bacteria. Purified DNAs from *B. circulans* and the plasmid ColE1-Ap^R were digested with *Eco*RI endonuclease and the resulting fragments covalently joined with polynucleotide ligase. The recombined DNA was used to transform *E. coli* and ampicillin-neomycin resistant colonies were selected. Analysis of several clones indicated that neomycin resistance in the *E. coli* transformants was due to the presence of the *B. circulans* phosphotransferase gene. This observation is consistent with the notion that antibiotic-modifying enzymes from antibiotic-producing organisms may be the sources of antibiotic resistance in plasmid-containing bacteria.

Plasmid-determined antibiotic resistance is widespread in clinical isolates of bacteria. Most determinants of resistance (r-determinants) have no homology with the chromosomal genes of their hosts and Watanabe (1) has suggested that r-determinants exist "somewhere" in nature as a reservoir of chromosomal genes and that, under the appropriate selection pressures, they were "picked-up" by promiscuous sex factors and, directly or indirectly, transferred to their present hosts.

It has been demonstrated (2–8) that many antibiotic-producing bacteria contain antibiotic-modifying enzymes that are related to those that determine antibiotic resistance in plasmid-containing bacteria. To date, aminoglycoside *O*-phosphorylating and *N*-acetylating enzymes (2), aminocyclitol *O*-phosphorylating enzymes (3–6) and chloramphenicol acetyltransferase (EC 2.3.1.28) (7, 8) have been detected in antibiotic-producing strains; these enzymes have substrate ranges similar to the plasmid-mediated enzymes found in various Gram-positive and Gram-negative bacteria. It has been suggested, therefore, that antibiotic-producing organisms might represent the origin of r-determinants (2–3).

Support for this notion would come from the demonstration of protein or nucleic acid relationships between antibiotic-producing organisms and plasmid-containing antibiotic-resistant bacteria. One can also ask if a gene coding for antibiotic-modification in an antibiotic-producing organism could be transplanted to unrelated bacteria such as *Escherichia coli*; and whether or not this gene would determine antibiotic resistance in the latter.

Abbreviations: ColE1, colicinogenic factor E1; ColE1-Ap^R, plasmid directing production of colicin E1 and resistance to ampicillin; TnA, transposable element for ampicillin; R-plasmid, antibiotic resistance plasmid. Phenotypes include NeoB, neomycin B; Par, paromomycin; Liv A, lividomycin A; But, butirosin; Rib, ribostamycin; Kan A, kanamycin A; Ami, amikacin; Tob, tobramycin; Gen A, gentamicin A; Gen B, gentamicin B; Sm, streptomycin; Amp, ampicillin; Tet, tetracycline.

Bacillus circulans NRRL B-3312 is used for the production of butirosin, an antibiotic closely related to neomycin. This nonpathogenic strain contains a phosphotransferase that has a similar activity profile to the neomycin phosphotransferases of clinical isolates (9). Using a colicinogenic factor E1(ColE1) derivative as a vehicle, we have transferred the gene determining neomycin phosphotransferase from *B. circulans* to *E. coli*. By virtue of this phosphotransferase the hybrid plasmid confers resistance to a variety of aminoglycosides in its *E. coli* host. The resistant phenotype so obtained is typical of that associated with neomycin phosphotransferases that occur "naturally" on plasmids in organisms such as *E. coli*.

MATERIALS AND METHODS

Bacterial Strains. *B. circulans* NRLL B-3312 (9) which produces butirosins (But) A and B was obtained from Tom Pridham, Northern Regional Research Laboratory. *B. circulans* ATCC 4513 was purchased from the American Type Culture Collection. *E. coli* C-1a (10) was obtained from W. F. Dove. Plasmids JR67 (Iw, Sm, Neo) (11) and RP4 (P, Amp, Tet, Neo) (12) were introduced into *E. coli* C-1a by conjugation. *E. coli* C600 harboring ColE1-Ap^R (RSF 2124) (13) was obtained from S. Falkow. ColE1-Ap^R consists of a ColE1 factor which acquired TnA, a 3.2 megadalton transposable element. TnA specifies resistance to ampicillin through synthesis of the TEM-β-lactamase.

DNA Preparation. *B. circulans* NRLL B-3312 DNA was purified by phenol extraction and banding in a cesium chloride gradient. Covalently closed circular plasmid DNA from *E. coli* C600/ColE1-Ap^R and hybrid plasmid DNA from transformants isolated in this study were purified as described by two successive bandings in cesium chloride-ethidium bromide (14, 15). Bacteriophage λcb2 DNA was provided by W. Szybalski.

Enzymes. Restriction endonucleases *Eco*RI and *Sma* I were purified and used as described (14). Restriction endonuclease *Bam*H I was prepared according to Wilson and Young (16). T4-induced polynucleotide ligase [polynucleotide synthetase (ATP), poly(deoxyribonucleotide):poly(deoxyribonucleotide) ligase (AMP-forming), EC 6.5.1.1] was prepared according to Sano and Feix (17).

Construction of Hybrid ColE1-Ap^R–*B. circulans* DNA. ColE1-Ap^R DNA and *B. circulans* DNA (0.7 μg each) were separately digested to completion with *Eco*RI restriction endonuclease in 50 μl reaction mixtures containing 90 mM Tris·HCl (pH 7.4) and 10 mM $MgCl_2$. The reactions were allowed to proceed for 35 min at 37°, then heated at 68° for 10 min and chilled at 0°. The two digests were mixed and the following were added: 50 mM $MgCl_2$ (20 μl); 0.1 M dithioerythritol (20

Table 1. Minimal inhibitory concentrations (μg/ml) of various antibiotics for *B. circulans* strains ATCC 4513 and NRRL B-3312 and *E. coli* C-1a without or with different plasmids

Strain/plasmid	NeoB	Par	LivA	But	Rib	KanA	Ami	Tob	GenA	GenB	Amp	Tet
B. circulans ATCC 4513	≤0.5	4	16	2	8	2	≤0.5	1	128	1	—	—
B. circulans NRRL B-3312	≤0.5	4	16	128	32	2	≤0.5	1	16	≤0.5	—	—
E. coli C-1a	2	8	8	2	8	1	≤0.5	1	64	≤0.5	4	4
E. coli C-1a/pAT6	64	128	32	256	>2048	32	4	2	256	4	2048	4
E. coli C-1a/JR67	128	1024	8	256	>2048	1024	≤0.5	1	512	16	4	4
E. coli C-1a/RP4	64	2048	4096	2	2048	512	≤0.5	2	1024	256	4096	512

μl); 0.5 mM adenosine triphosphate (20 μl); water (40 μl) (final volume 200 μl). The reaction mixture was heated at 68° for 5 min and then immediately chilled at 0°. Digestion was monitored by agarose gel electrophoresis of a 50 μl sample. To the remaining 150 μl reaction mixture we added approximately 0.1 nU of T4 ligase (5 μl) and after 3 hr at 4°, 50 μl was removed to monitor the ligation reaction by electrophoresis as described (14); the remainder of the reaction mixture (100 μl) was incubated on ice for 17 hr.

Transformation and Selection. The procedure used for transformation was that described by Mandel and Higa (18). After transformation, the cells were plated on medium containing ampicillin (100 μg/ml) and neomycin (20 μg/ml). Colonies were picked and tested for colicin production by the overlay procedure of Fredericq (19) and for immunity to colicin E1 by testing growth of clones in the presence of purified colicin E1. Five transformants were tested further for the synthesis of neomycin phosphotransferase by the enzymatic assay described below. Plasmid DNA extracted from the same five transformants was tested for increased molecular weight by agarose gel electrophoresis, by using ColE1-Ap^R as an internal standard, and for the presence of foreign DNA fragments by the same technique after digestion with *Eco*RI endonuclease.

Enzymatic Assays. The extracts for assay were prepared as follows: 100 ml of an exponential culture in broth was harvested and washed once in 10 mM Tris·HCl (pH 8.0), 1 mM EDTA, and resuspended in 2 ml of 10 mM Tris·HCl (pH 7.8), 10 mM $MgCl_2$, 25 mM NH_4Cl, 0.6 mM 2-mercaptoethanol. The cells were subjected to four 60 sec bursts from a sonicator at 65 W output. The resulting suspension was centrifuged at 105,000 × *g* for 45 min and the supernatant removed. The enzyme assays were performed as described by Haas and Dowding (20).

DNA Base Composition. A determination of base composition was made after buoyant density measurements (15). Electron microscopy (15) and heteroduplex analysis (21) were performed as described. The method of Steers *et al.* (22) was used for determination of minimal inhibitory concentrations.

Biohazard Procedures. *B. circulans* and *E. coli* are representatives of microbial species which have not been shown to exchange genetic material; therefore, the genetic transplant *in vitro* reported here is a restricted experiment according to the NIH recombinant DNA guidelines. This work was completed before the institution of these guidelines. However, we employed strict microbiological procedures suitable for the containment of bacterial pathogens. The authors do not contemplate any further experiments involving this type of plasmid until the required facilities are available.

RESULTS

Construction of ColE1-Ap^R *B. circulans* Plasmids. Total DNA from *B. circulans* and ColE1-Ap^R DNA, treated with *Eco*RI endonuclease and ligated, was used to transform *E. coli* C-1a. Clones were selected for their ability to grow in the presence of ampicillin and neomycin. The transformants were tested for (*i*) inability to produce colicin E1, (*ii*) immunity to colicin E1, (*iii*) synthesis of neomycin phosphotransferase, and (*iv*) presence of extrachromosomal DNA. Plasmid DNA was isolated and examined (*i*) by agarose gel electrophoresis, by using the ColE1-Ap^R vehicle as internal molecular weight standard, and (*ii*) after digestion with *Eco*RI endonuclease, for the presence of fragments of *B. circulans* DNA. Forty out of the 67 transformants were unable to produce colicin E1 but retained immunity to colicin E1. One of the transformants was studied further and its hybrid plasmid was called pAT6.

Properties of the Transformants. *Phenotypic Properties.* The minimal inhibitory concentrations of a variety of aminoglycosides for the antibiotic-producing *B. circulans* NRRL B-3312 were determined, by using the nonproducing *B. circulans* cells (ATCC 4513) as a reference (Table 1). Except for butirosin and, at a lesser extent ribostamycin, the neomycin phosphotransferase does not confer antibiotic resistance to the antibiotic-producing strain. The minimal inhibitory concentrations of various antibiotics for *E. coli* C-1a and *E. coli* C-

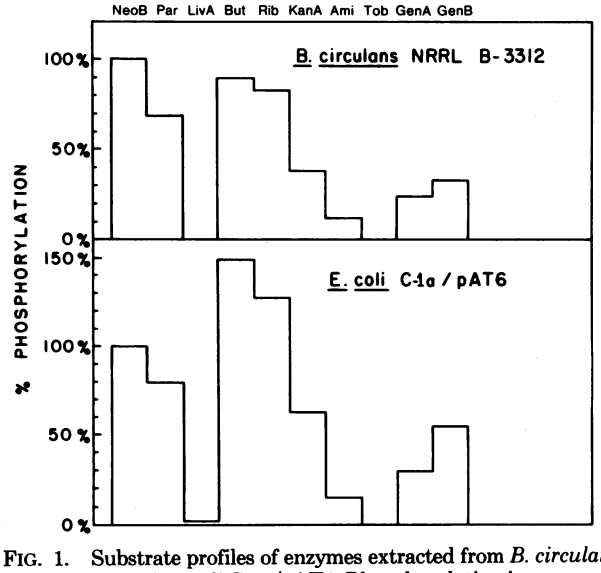

FIG. 1. Substrate profiles of enzymes extracted from *B. circulans* NRRL B-3312 and *E. coli* C-1a/pAT6. Phosphorylation is expressed relative to neomycin B as 100%.

Table 2. Physical properties of ColE1-Ap^R and pAT6 plasmids

Plasmid	Mean contour length ± SD (μm)	Number of molecules measured	Mol. wt. ± SD based on contour length (× 10^{-6})	Density in CsCl (g/cm^3)	Guanine + cytosine content (%)	Restriction endonuclease sites (*Eco*RI, *Sma* I, *Bam*HI)
ColE1-Ap^R (RSF 2124)	3.9 ± 0.2	105	7.4 ± 0.4	1.701	47	1 1 1
pAT6	6.4 ± 0.3	104	12.4 ± 0.5	1.704	50.5	2 3 2

1a/pAT6 are shown in Table 1; these are consistent with the substrate range of the *B. circulans* phosphotransferase (see below). JR67 and RP4 are plasmids from clinical isolates that encode for neomycin phosphotransferases type II and I (20) and determine aminoglycoside resistance phenotypes in *E. coli* C-1a similar to that due to pAT6.

Enzymatic Properties. The aminoglycoside substrate profiles of neomycin phosphotransferases extracted from *B. circulans* NRRL B-3312 or *E. coli* C-1a/pAT6 are very similar (Fig. 1). The fact that lividomycin A is not phosphorylated indicates that the enzymes are type II neomycin phosphotransferases; since kanamycin A is a substrate and tobramycin is not, this indicates that the 3′ hydroxyl group is the site of modification. Therefore, the two enzymes appear to be identical. The neomycin phosphotransferases encoded by the hybrid plasmid pAT6 in *E. coli* and by *B. circulans* are synthesized constitutively because they are produced in the absence of neomycin.

Properties of Plasmid DNA Purified from the Transformants. Covalently-closed circular DNA was purified from *E. coli* C-1a/pAT6 by ultracentrifugation in a cesium chloride gradient in the presence of ethidium bromide. This DNA transformed another *E. coli* strain for neomycin resistance at a high frequency (about $10^5/\mu g$); immunity to colicin E1 accompanied the resistance marker.

Agarose Gel Electrophoresis of Restriction Endonuclease Digests. ColE1-Ap^R–*B. circulans* plasmid DNA from five independent ampicillin-neomycin-resistant clones yielded two fragments on *Eco*RI endonuclease treatment as revealed by agarose gel electrophoresis. All of the plasmids tested yielded a fragment corresponding to linear ColE1-Ap^R DNA [molecular weight 7.4 × 10^6 (Table 2)]. The other fragment had a molecular weight of approximately 4.8 × 10^6. This analysis shows that only one *Eco*RI fragment of the *B. circulans* chromosome was cloned. The hybrid plasmid pAT6 was studied further with various restriction endonucleases; pAT6 DNA yielded three and two fragments respectively, after *Sma* I and *Bam*HI endonuclease treatment (Fig. 2).

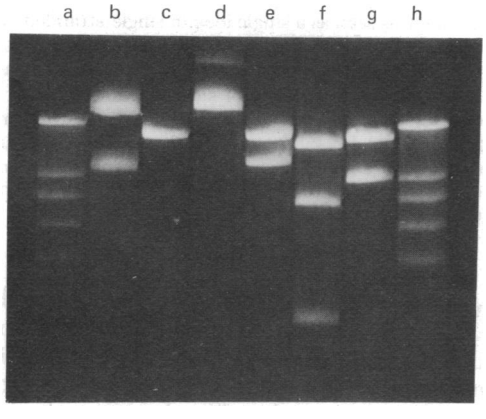

FIG. 2. Agarose gel electrophoresis of the hybrid plasmid pAT6 and reference DNA samples. Direction of migration is downward. (a) and (h) Phage λcb2 (*Eco*RI) molecular weight standards of 13.7, 4.5, 3.5, 2.9, and 2.3, each ×10^6; (b) ColE1-Ap^R; (c) ColE1-Ap^R (*Eco*RI); (d) pAT6; (e) pAT6 (*Eco*RI); (f) pAT6 (*Sma* I); (g) pAT6 (*Bam*HI). Electrophoresis was carried out at 50 V for 3 hr in 1% agarose gels.

Base Composition of the Hybrid Plasmid DNA and Its Constituents. The hybrid plasmid pAT6 DNA was analyzed by CsCl density gradient ultracentrifugation (Fig. 3, Table 2). The buoyant densities of *B. circulans* chromosomal DNA and ColE1-Ap^R DNA are 1.707 and 1.701, corresponding to guanine plus cytosine (G+C) contents of 53.5 and 47%, respectively. As expected, the buoyant density of pAT6 DNA (1.704, 50.5% G+C) is intermediate between those of ColE1-Ap^R and *B. circulans* chromosomal DNAs. If pAT6 was digested with *Eco*RI and analyzed similarly, then two peaks were seen; one with the same density as ColE1-Ap^R, the other with a density 1.708, slightly higher than that of the *B. circulans* chromosome

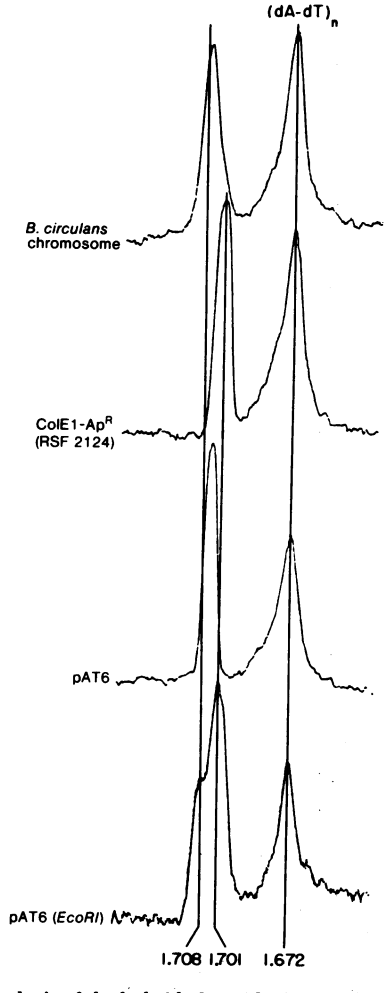

FIG. 3. Analysis of the hybrid plasmid pAT6 and its constituents by isopycnic ultracentrifugation in CsCl. $(dA\text{-}dT)_n$ (ρ = 1.672 g/cm^3) was included as internal standard in all the samples. Each gradient contains DNA, approximately 2 μg/ml.

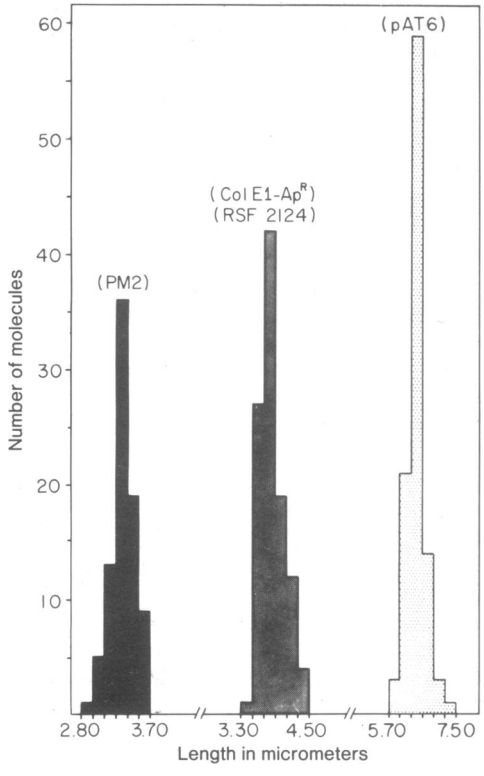

FIG. 4. Contour length distribution of ColE1-Ap^R (RSF 2124) and pAT6. Bacteriophage PM2 DNA [6.4 × 10^6 molecular weight (24)] was used as an internal standard for pAT6 length measurement. For each DNA species, the histogram is plotted in increments equal to 5% of the mean contour length of the given species.

(1.707). Thus, the *B. circulans* fragment has a G+C content (54.5%) which is slightly higher than that of the *B. circulans* chromosome (53.5%); the latter value represents an average for the whole genome.

Size Distribution of Plasmid DNAs. Analysis by electron microscopy was performed on the DNAs of the two plasmids ColE1-Ap^R and pAT6. Contour length measurements of molecules from each preparation are shown in Fig. 4. ColE1-Ap^R exists as a cluster of molecules with mean contour length of 3.9 μm (SD: 0.2) and pAT6 as a cluster of molecules with an average contour length of 6.4 μm (SD: 0.3). Numerous catenated dimers were observed in both preparations. The respective molecular weights of the pAT6 and ColE1-Ap^R plasmids estimated from the length measurements are 12.4 × 10^6 (SD: 0.5) and 7.4 × 10^6 (SD: 0.4) (Table 2). This latter value is in close agreement with a previous report (13). The molecular weight of the *B. circulans* fragment is therefore 5 × 10^6 (SD: 0.6); this agrees with the value based on agarose gel electrophoresis (4.8 × 10^6).

Analysis of DNA Heteroduplexes by Electron Microscopy. The hybrid plasmid pAT6 was characterized by heteroduplex analysis and compared with ColE1-Ap^R (Fig. 5*A*) and ColE1 (Fig. 5*B*). As already described (23), the ampicillin transposable element TnA exhibits a short stem of approximately 140 base pairs (Fig. 5*B*). By contrast, examination of a large number of single-stranded molecules failed to show the presence of an inverted repeat on the *B. circulans* fragment. Contour length measurements of such heteroduplexes are shown in Fig. 5*C*. The values obtained for the molecular weight of TnA (3.4 × 10^6, SD: 0.4) and the position of its site of insertion compared to the *Eco*RI endonuclease recognition sequence are in good agreement with those reported earlier (13, 23).

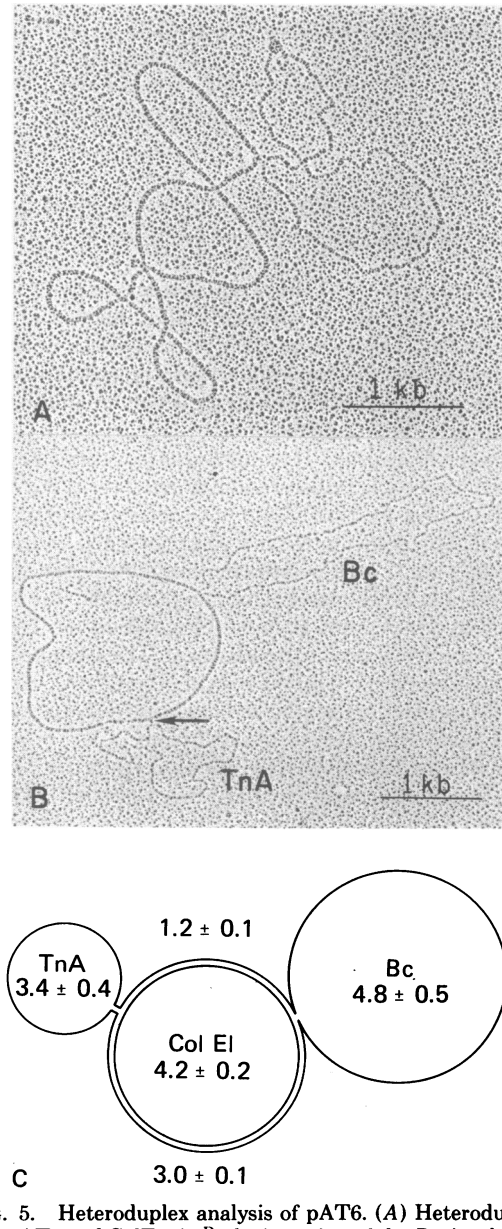

FIG. 5. Heteroduplex analysis of pAT6. (*A*) Heteroduplex between pAT6 and ColE1-Ap^R; the insertion of the *B. circulans* chromosome fragment is seen as a single loop of single-stranded DNA. (*B*) Heteroduplex between pAT6 and ColE1; the insertions of the *B. circulans* chromosome fragment (Bc) and the ampicillin transposable element (TnA) are seen as loops of single-stranded DNA. The inverted, repeated sequences of TnA have reannealed to form a short duplex region (arrow). kb, kilobase. (*C*) Contour length measurements (in megadaltons ± SD) of pAT6/ColE1 heteroduplexes (22 molecules measured). ColE1 homoduplexes (4.2 × 10^6; SD, 0.2; 9 molecules measured) were used as internal standards.

DISCUSSION

We have demonstrated that the gene for neomycin phosphotransferase of the butirosin-producing *B. circulans* can be introduced into *E. coli* where it is expressed as a determinant of resistance to aminoglycoside antibiotics. This finding is consistent with the hypothesis that clinical resistance to antibiotics determined by R-plasmids could have originated in antibiotic-producing organisms (2–3).

In Gram-negative bacteria such as *E. coli*, plasmid-directed aminoglycoside-aminocyclitol modifying enzymes confer resistance not by gross inactivation of the antibiotic in the culture medium but by modification of a small amount of the drug that

presumably prevents further uptake of the antibiotic by the cell, by an unknown mechanism (25). It is all the more surprising and further support for our hypothesis that the *B. circulans* neomycin phosphotransferase establishes resistance in *E. coli* by the same mechanism: when *E. coli* C-1a/pAT6 was grown in the presence of neomycin, no detectable change in antibiotic concentration was found.

More generally, the problem addressed in this study concerns the source of genes for biosynthesis and/or modification of secondary metabolites. Although the origins of primary metabolite synthesis probably go back to the most primitive organisms, the origins of secondary metabolite biosynthesis or modification need not necessarily have followed the same route and could conceivably have been dispersed in a lateral or even retrograde direction. The determinants of antibiotic biosynthesis and associated antibiotic modification constitute a pertinent example.

The finding of the synthesis of such similar "unlikely" molecules as neomycin and butirosin in such diverse species as *Streptomyces fradiae* and *B. circulans*, respectively, coupled with the presence of two neomycin phosphotransferases of similar substrate spectra in both species (2, 9) suggests the possibility that the antibiotic-producing organisms may be the progenitors of resistance-associated enzymes. At present, the roles of the modifying enzymes associated with antibiotic production are unknown. In any case, both chromosomal and plasmid genes for antibiotic modification from Gram-positive organisms are effectively expressed in Gram-negative bacteria. This suggests (but by no means proves) that relatively facile interspecies dispersal of genes for antibiotic biosynthesis and modification (resistance) may occur in nature.

The *B. circulans* neomycin phosphotransferase, when present in *B. circulans*, determines resistance to butirosin and low levels of ribostamycin, although it catalyzes efficient phosphorylation of a number of aminoglycosides including neomycin. In contrast, when present in *E. coli*, this same determinant specifies a level of resistance to these antibiotics and neomycin an order of magnitude higher. This is consistent with the expression of other plasmid-coded neomycin phosphotransferases such as those of JR67 and RP4 (Table 1). The fact that the butirosin-producing strain is not resistant to high levels of aminoglycosides may be a function of the localization of the enzyme in the cell and its association with transport systems for these antibiotics. Regulation of enzyme synthesis may also contribute to the observed quantitative differences; the *B. circulans* neomycin phosphotransferase is associated with a multicopy plasmid in *E. coli* (13).

These results are consistent with the notion that antibiotic-producing organisms are the source of genes for resistance-plasmids. More convincing proof of a direct relationship might come from studies of nucleic acid and protein homologies between representative strains. There remains, also, the problem of how these genes have undergone natural "genetic engineering" and interspecies transfer from an antibiotic-producing Gram-positive bacterium ultimately to Gram-negative clinical isolates.

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1. Watanabe, T. (1971) *Ann. N.Y. Acad. Sci.* **182,** 126–140.
2. Benveniste, R. & Davies, J. (1973) *Proc. Natl. Acad. Sci. USA* **70,** 2276–2280.
3. Walker, M. S. & Walker, J. B. (1970) *J. Biol. Chem.* **245,** 6683–6689.
4. Walker, J. B. & Skorvaga, M. (1973) *J. Biol. Chem.* **248,** 2435–2440.
5. Nimi, O., Ito, G., Sueda, S. & Nomi, R. (1971) *Agr. Biol. Chem.* **35,** 848–855.
6. Nimi, O., Ito, G., Ohata, Y., Funayama, S. & Nomi, R. (1971) *Agr. Biol. Chem.* **35,** 856–861.
7. Argoudelis, A. D. & Coats, J. H. (1971) *J. Antibiot.* **24,** 206–208.
8. Shaw, W. V. & Hopwood, D. A. (1976) *J. Gen. Microbiol.* **94,** 159–166.
9. Dowding, J. & Davies, J. (1975) in *Microbiology-1974*, ed. Schlessinger, D. (American Society for Microbiology, Washington, D.C.), pp. 179–186.
10. Sasaki, I. & Bertani, G. (1975) *J. Gen. Microbiol.* **40,** 365–376.
11. Berg, D. E., Davies, J., Allet, B. & Rochaix, J.-D. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 3628–3632.
12. Datta, N., Hedges, R. W., Shaw, E. J., Sykes, R. B. & Richmond, M. H. (1971) *J. Bacteriol.* **108,** 1244–1249.
13. So, M., Gill, R. & Falkow, S. (1975) *Mol. Gen. Genet.* **142,** 239–249.
14. Tanaka, T. & Weisblum, B. (1975) *J. Bacteriol.* **121,** 354–362.
15. Courvalin, P. M., Carlier, C., Croissant, O. & Blangy, D. (1974) *Mol. Gen. Genet.* **132,** 181–192.
16. Wilson, G. A. & Young, F. E. (1975) *J. Mol. Biol.* **97,** 123–125.
17. Sano, H. & Feix, G. (1974) *Biochemistry* **13,** 5110–5115.
18. Mandel, M. & Higa, A. (1970) *J. Mol. Biol.* **53,** 159–162.
19. Fredericq, P. (1972) in *Uptake of Informative Molecules by Living Cells*, ed. Ledoux, L. (American Elsevier Publ. Co., New York), p. 83.
20. Haas, M. & Dowding, J. E. (1975) in *Methods in Enzymology*, ed. Hash, J. H. (Academic Press, New York), Vol. 43, pp. 611–628.
21. Westmoreland, B. C., Szybalski, W. & Ris, H. (1969). *Science* **163,** 1343–1348.
22. Steers, E., Foltz, E. L., Graves, B. S. & Riden, J. (1959) *Antibiot. Chemother.* **9,** 307–311.
23. Heffron, F., Rubens, C. & Falkow, S. (1975) *Proc. Natl. Acad. Sci. USA* **72,** 3623–3627.
24. Franklin, R. M. (1974) *Curr. Top. Microbiol. Immunol.* **68,** 107–160.
25. Davies, J. E. & Benveniste, R. E. (1974) *Ann. N.Y. Acad. Sci.* **235,** 130–136.