A CRYPTIC PLASMID FROM *NOCARDIA ORIENTALIS* NRRL 2452, A VANCOMYCIN PRODUCER

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(Received for publication September 17, 1985)

A plasmid was found in *Nocardia orientalis* (formerly *Streptomyces orientalis*). Physical characterization of the plasmid DNA indicates a size of 33.5 kb and a single cleavage site for *Eco*R I. The presence of plasmid, and variation in its copy member, did not directly affect vancomycin resistance or production levels. The plasmid represents the first to be isolated and characterized from a glycopeptide-producing nocardia.

Glycopeptide antibiotics, produced by various actinomycetes, are clinically important cell wall inhibitors that are active against many Gram-positive bacteria[1]. These antibiotics have a similar aglycone structure, primarily derived from acetate and tyrosine, suggesting a related biosynthetic pathway[2]. In order to facilitate the study of the regulation and biosynthesis of this class antibiotics we initiated a search for plasmids that might form a basis for cloning relevant genes. A number of glycopeptide producers were examined including three strains of *Nocardia orientalis* (previously *Streptomyces orientalis* NRRL 2450, 2451 and 2452), *Actinoplanes teichomyceticus* and *Kibdelosporangium aridum*. *K. aridum* is a new genus of actinomycetes from our laboratories that produces new glycopeptide antibiotics, the aridicins[3].

Materials and Methods

Bacterial Strains

K. aridum ATCC 39323, *A. teichomyceticus* ATCC 31121 and 3 strains of *N. orientalis* NRRL 2450, 2451 and 2452 were used. In addition, an arsenate-selected strain of *N. orientalis* NRRL 2452/V33, and a cured strain *N. orientalis* NRRL 2452 NV1 were used in the characterization of the plasmid.

Fermentation Conditions

Agar slant cultures were prepared by inoculation of yeast - malt extract (YME) agar (Difco). Slants were incubated at 28°C for 10 days. The contents were suspended in 2 ml sterile water and inoculated into 50 ml seed medium in 250-ml Erlenmeyer flask. Seed medium consisted of soluble starch 15 g, sucrose 5 g, soy peptone 7.5 g, corn steep liquor 5 g, NaCl 1.5 g, mineral salt solution 5 ml, $CaCO_3$ 1.5 g, in 1 liter of tap water, pH adjusted to 7.0 with dilute NaOH solution. Mineral salt solution consisted of $ZnSO_4 \cdot 7H_2O$ 2.8 g, $Fe(NH_4)_2HC_6H_5O_7$ 2.7 g, $CuSO_4 \cdot 5H_2O$ 0.12 g, $MnSO_4 \cdot H_2O$ 1.0 g, $CoCl_2 \cdot 6H_2O$ 0.1 g, $Na_2B_4O_7 \cdot 10H_2O$ 0.09 g and $Na_2MoO_4 \cdot 2H_2O$ 0.05 g, in 1 liter of tap water. The seed cultures were incubated at 28°C for 4 days on a reciprocal shaker at 250 rpm and 5-cm throw. Five ml of seed culture was used to inoculate 250-ml Erlenmeyer flask containing 50 ml production medium. This production medium consisted of glucose 20 g, yeast extract 1 g, soy peptone 10 g, $CoCl_2$ 0.001 g and $CaCO_3$ 1 g, in 1 liter of tap water. The cultures were incubated at 28°C for 4 days on a reciprocal shaker at 250 rpm and 5-cm throw.

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Antibiotic Assays

An aliquot of fermentation broth was centrifuged, and the supernatant was treated through prewet sep-pak C_{18} cartridge (Waters Associate) with 0.1 M phosphate buffer, pH 3.2. The cartridge was then eluted with 50% acetonitrile - 0.05 M phosphate buffer, pH 3.2. Vancomycin in the eluant was determined using HPLC on a Beckman Ultrasphere ODS column (4.6×150 mm) with a gradient of acetonitrile (7~34%) in 0.1 M phosphate buffer, pH 3.2, and UV detection at 220 nm.

Screen for Plasmid DNA

The glycopeptide-producing cultures were grown on YME agar at 28°C for 4 days. A portion of the lawn culture on the agar surface was harvested by scraping and suspended in 1.5 ml Tris-EDTA buffer, pH 8.0. After centrifugation, 200 µl of each packed cell volume was assayed for plasmid DNA by the method of KIESER[4].

Isolation and Characterization of pYO33

A slant culture of *N. orientalis* NRRL 2452/V33, which grows as a uniform aerial mycelium culture on YME agar, was inoculated into Trypticase soy broth (TSB) and incubated overnight at 28°C on a reciprocal shaker at 250 rpm. A portion of the culture was inoculated into TSB containing 1.5% glycine, and incubated overnight. The culture was harvested by centrifugation and the plasmid DNA was isolated using the method of BIRNBOIN and DOLY[5]. Physical characterization of pYO33 was carried out using a number of restriction endonucleases listed in Table 2.

Isolation of *N. orientalis* NRRL 2452 NV1 (Plasmid Cured Strain)

The isolation of a "cured" strain was carried out in two stages. In the first stage, protoplasts of strains NRRL 2452 pYO33 were transformed with the plasmid pJW8 (a smaller thiostrepton-resistant derivative of pYO33; WIDGER, FARE and TAYLOR: unpublished results). Regenerants selected for thiostrepton resistance were found to harbor only pJW8 as the resident plasmid.

These clones, in turn, were cultivated in shaken-flask cultures in TSB containing novobiocin (50 µg/ml to 0.4 µg/ml, in two-fold dilutions). Growth which occurred at 0.4 µg/ml was used as inoculum for a second set of flasks containing TSB and novobiocin (12.5 µg/ml). This culture was spread on YME plates without thiostrepton. Ninety-five % of the cells that grew were found to be sensitive to thiostrepton. Several clones were examined by miniprep[4] and SOUTHERN[6] blotting procedures. pYO33 or pJW8 sequences could not be detected in any of the clones, indicating a loss of plasmids from the cells.

Plasmid Copy Number Estimation

DNA was prepared from each host by gently lysing cells with lysozyme (Sigma) 2 µg/ml in "P" medium[7] and 0.5% sodium dodecyl sulfate (SDS) in TE buffer (10 mM Tris pH 8.0, 1 mM EDTA, pH 8.0). The preparation was sonicated for 10 seconds (1 second burst at 15% power) with a Model 350 Bronson sonicator. The preparation was then treated with RNase (100 µg/ml, 37°C, 1 hour) and proteinase K (50 µg/ml, 37°C, 3 hours). The solution was extracted with phenol - $CHCl_3$ (4: 1) twice and then ethanol precipitated three times. The precipitate was dissolved in TE buffer.

DNA samples (12.5 µl) which were diluted serially (two-fold) in 0.4 M NaOH, then heated to 65°C for 10 minutes and cooled on ice were mixed with 125 µl of 1 M ammonium acetate. Each dilution of the series was transferred to wells over a nitrocellulose filter as previously described[8]. The dried filter was soaked in prehybridization mix for 2 hours at 42°C, and then incubated in hybridization buffer, containing nick translated[9] pYO33 DNA probe for 18 hours at 42°C.

After hybridization, the filter was washed with 0.1×saline, sodium phosphate, EDTA[6] and 0.1% SDS two times at room temp and once at 55°C for 30 minutes.

To determine the amount of probe which hybridized to the DNA on the nitrocellulose filter, the filter was exposed to X-ray film (Kodak AR-5) and areas of radioactivity were placed in scintillation fluid for counting. Labeled probe, hybridized to diluted pYO33 standards, was used to estimate the plasmid copy number.

Table 1. Examination of glycopeptide producers for the presence of plasmids.

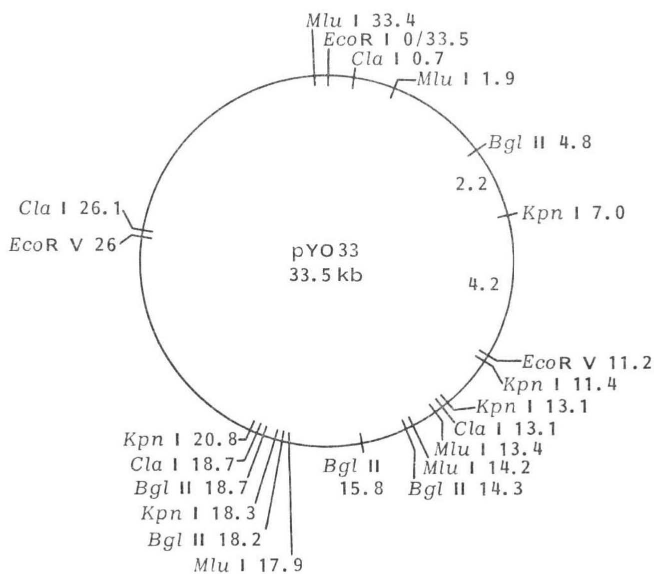
Strain	Glycopeptide produced	Presence of plasmid DNA
Kibdelosporangium aridum ATCC 39323	Aridicin	—
Actinoplanes teichomyceticus ATCC 31121	Teicoplanin	—
*Nocardia orientalis** NRRL 2450	Vancomycin	—
*N. orientalis** NRRL 2451	Vancomycin	—
*N. orientalis** NRRL 2452	Vancomycin	pSO408
*N. orientalis** NRRL 2452/V33	Vancomycin	pYO33

* Formerly *Streptomyces orientalis*.

Table 2. Cleavage sites of pYO33 with various restriction endonucleases.

Restriction endonuclease	Number of sites	Restriction endonuclease	Number of sites
*Bam*H I	17	*Nco* I	5
Bcl I	10	*Nru* I	11
Bgl II	4	*Pst* I	20
BssH II	7	*Pvu* I	9
Cla I	4	*Pvu* II	11
*Eco*R I	1	*Sac* I	10
*Eco*R V	2	*Sal* I	23
Hind III	0	*Sph* I	9
Hpa I	0	*Stu* I	9
Kpn I	5	*Xba* I	0
Mlu I	4	*Xho* I	0
Nae I	14		

Fig. 1. Restriction enzyme cleavage map of pYO33.



Results

Isolation of Plasmids

Of the five glycopeptide-producing strains only NRRL 2452 and its derivative V33 were shown to

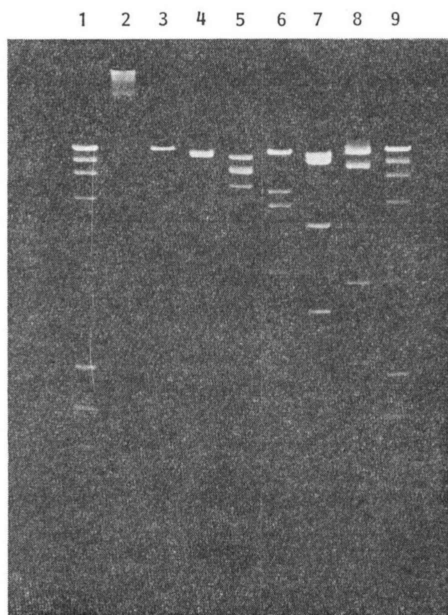
Table 3. Vancomycin production by *Nocardia orientalis* NRRL 2452 and its derivatives.

Strain	Copy number of plasmid pYO33	Vancomycin[a] resistance	Vancomycin produced[b] (μg/ml)
N. orientalis NRRL 2452	68±5	R	118±5
N. orientalis NRRL 2452/V33	115±5	R	72±5
N. orientalis NRRL 2452 NV1	<0.5	R	66±6

[a] R indicates resistance of cell growth up to 1 mg/ml vancomycin incorporated into YME agar medium.
[b] Average of three determinations.

Fig. 2. Electrophoretic mobility of digested and undigested pYO33 on agarose gel.

Lane 1 Molecular-weight standards λ (*Hin*d III)+ φX174 (*Hae* III), lane 2 pYO33 (undigested), lane 3 pYO33 (*Eco*R I), lane 4 pYO33 (*Eco*R V), lane 5 pYO33 (*Cla* I), lane 6 pYO33 (*Kpn* I), lane 7 pYO33 (*Mlu* I), lane 8 pYO33 (*Bgl* II), lane 9 molecular-weight standards λ (*Hin*d III)+φX174 (*Hae* III).



contain a plasmid (Table 1). The plasmid from strain *N. orientalis* NRRL 2452/V33 was designated pYO33 (copy number ~100). Examination of the parent strain of NRRL 2452/V33, *N. orientalis* NRRL 2452, revealed an identical plasmid designated pSO408 with a copy number of ~70 per cell.

Enzymatic Characterization of pYO33

A restriction enzyme cleavage map of pYO33 DNA was obtained using 23 restriction endonucleases (Table 2). A circular cleavage map of the plasmid was constructed (Fig. 1) using a computer program developed by Intelligenetics Inc. The plasmid size of 33.5 kb was obtained by summing the sizes of restriction fragments obtained from enzyme digestions. The plasmid has a single site for *Eco*R I, two sites for *Eco*R V, five sites for *Kpn* I and five sites for *Mlu* I (Fig. 2). The number of cleavage sites for other enzymes used are shown in Table 2. Electrophoretic mobilities of digested and undigested pYO33 DNA on 0.8% agarose gel are shown in Fig. 2. *Eco*R I digestion shows a single fragment (lane 3); *Eco*R V, two fragments close together (lane 4) and other enzyme digestions, multifragments (Fig. 2). The pattern for restriction endonuclease digestion of pSO408 was found to be identical with that of pYO33.

Cryptic Nature of pYO33 on Vancomycin Production and Resistance

The physiological relationship of pYO33 to vancomycin production and resistance was investigated using *N. orientalis* NRRL 2452 and its derivatives. They were all resistant up to 1 mg/ml vancomycin and produced 66~117 mg vancomycin per ml, regardless of the presence of plasmids (Table 3). Although the high copy plasmid pYO33 was isolated from an arsenate-selected host NRRL 2452/V33, we have no evidence to indicate plasmid involvement in resistance to arsenate.

Discussion

N. orientalis NRRL 2452 produces a clinically important antibiotic, vancomycin, that is particularly effective against methicillin-resistant staphylococci[1]. During studies of glycopeptide-producing cultures a plasmid, pYO33, was found in a strain of *N. orientalis* NRRL 2452/V33 that produces vancomycin. Two other vancomycin producers, *N. orientalis* NRRL 2450 and NRRL 2451, did not contain plasmid DNA. The plasmid is large (33.5 kb) and has unique restriction sites which differentiate it from known plasmids found thus far in actinomycetes.

N. orientalis NRRL 2452, NRRL 2452/V33 and NRRL 2452 NV1 are equally sensitive and resistant to a number of antibiotics including vancomycin, indicating that the plasmid does not have any role in vancomycin resistance. Furthermore, they produce vancomycin in liquid culture, irrespective of the presence or absence of a high copy number of plasmid DNA indicating that the biosynthetic genes for vancomycin production are not on the plasmid and are probably located in the chromosomal DNA. However, the plasmid offers a potential vehicle for cloning individual or multiple genes in the biosynthetic pathway of glycopeptide antibiotics.

Earlier studies reported genetic evidence of a conjugative plasmid in a *Nocardia* sp.[10] and of a miniplasmid in *Nocardia corallina*[9]. However, this is the first report of the physical isolation and characterization of a plasmid from a glycopeptide producing nocardia.

Acknowledgment

We thank R. POLACHEK and J. DINGERDISSEN for HPLC analyses and R. GERBER for fermentation studies.

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