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Cloning of a DNA region of *Actinoplanes teichomyceticus* conferring teicoplanin resistance

M.C. Moroni*, C. Granozzi, R. Lorenzetti, F. Parenti, M. Sosio and M. Denaro

Merrell Dow Research Institute, Lepetit Research Center, Via R. Lepetit 34, 21040 Gerenzano/VA, Italy

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Teicoplanin is a glycopeptide antibiotic, produced by *Actinoplanes teichomyceticus*, active against Gram positive bacteria and recently introduced into clinical practice. It blocks cell wall biosynthesis by inhibiting peptidoglycan polymerization. The mechanism(s) of resistance of the producer strains of this class of antibiotics have not yet been characterized. We have constructed a genomic bank of *A. teichomyceticus* in *Streptomyces lividans*. A clone from this bank, pTR168, was able to confer resistance to teicoplanin on its sensitive host. The restriction map of plasmid pTR168 and the hybridization pattern to *A. teichomyceticus* DNA were determined; we have also studied the mechanism of this resistance which seems correlated with a reduced binding of the antibiotic to the cell wall.

Teicoplanin; A/40926; Vancomycin; Gene cloning; Glycopeptide; Antibiotics resistance; (*Actinoplanes teichomyceticus*)

1. INTRODUCTION

Antibiotic-producing microorganisms have developed a variety of mechanisms to resist to their own products; many of these mechanisms have been studied and fully characterized [1]. For the glycopeptide antibiotics no specific mechanisms of resistance have been studied, so far, in producing strains. However clinical isolates resistant to these antibiotics are emerging [2] and some transferable resistances have already been described [3,4], although their mechanism of action has not yet been elucidated.

We have investigated the resistance of *Actinoplanes teichomyceticus*, producer of teicoplanin [5], to its own product.

Teicoplanin is a glycopeptide antibiotic, of the vancomycin class, recently introduced into clinical practice for treating serious Gram positive infections [6]. This antibiotic, like vancomycin, blocks cell wall biosynthesis by binding to amino-acyl-D-alanyl-D-alanine with consequent inhibition of peptidoglycan polymerization [7]. Teicoplanin is produced at the end of the logarithmic growth phase; at the same time the producing microorganism becomes resistant to the antibiotic. It was not known whether this apparent resistance was merely a reflection of the growth phase or involved genetic determinants specific to the producer strain. Against pathogenic bacteria, glycopeptide antibiotics, like other cell wall inhibitors, are bactericidal only against actively growing cells [8].

Correspondence address: M. Denaro and/or R. Lorenzetti, Dept of Microbiology, Lepetit Research Center, MDRI, Via R. Lepetit 34, 21040 Gerenzano, Italy

* *Present address:* Institute of Microbiology, University of Kopenhagen, Copenhagen, Denmark

2. MATERIALS AND METHODS

2.1. *Strains, plasmids and antibiotics*

Actinoplanes teichomyceticus (ATCC 31121) [9], *Streptomyces lividans* 66 (John Innes Collection 1326) [10] and the plasmid pIJ702 (carrying the thiostrepton resistance gene) [10] were used.

25 mg/l of thiostrepton (Sigma, St. Louis, MO, USA) were used for selecting clones resistant to this antibiotic; maintenance was on 50 mg/l. 20 mg/l of teicoplanin were used for selection and maintenance of resistant cells.

Vancomycin was from E. Lilly & Co. (Indianapolis, IN, USA); teicoplanin and A/40926 [11] were from Merrell Dow

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Inc. (Cincinnati, OH). The MIC on *S. lividans* of the last 2 antibiotics is 3.2 mg/l.

2.2. *rDNA techniques*

Total DNA from *A. teichomyceticus* was extracted by the procedure of Hopwood et al. [10], except that 5 mg/ml of lysozyme (Sigma) followed by 2 h of incubation, to protoplast the mycelium, were used.

Restriction endonucleases were from Boehringer (Mannheim, FRG). Gibco/BRL Inc. (Gaithersburg, MD, USA) or New England Biolabs (Beverly, MA, USA). T4 DNA ligase and all other enzymes were from Boehringer. All enzymatic reactions were performed according to the supplier's instructions unless otherwise specified.

Protocols for standard rDNA techniques were as described in the literature [10,12].

2.3. *Teicoplanin binding assays*

2.3.1. Method A

Streptomyces lividans, carrying pIJ702 or the recombinant plasmid pTR168, conferring teicoplanin resistance, were grown at 30°C into late log phase. Two aliquots of each culture were centrifuged; the supernatants and the resuspended pellets were used to make the following mixtures: (A) *S. lividans* (pIJ702) – its own supernatant; (B) *S. lividans* (pIJ702) – supernatant from *S. lividans* (pTR168); (C) *S. lividans* (pTR168) – its own supernatant; (D) *S. lividans* (pTR168) – supernatant from *S. lividans* (pIJ702). To each mixture 10 mg/l of ^{3}H-labelled teicoplanin (37 kBq) were added followed by incubation at 30°C for 20 min with agitation. Total input radioactivity was determined at the time of addition of the antibiotic. The incubated cultures were then centrifuged and the radioactivity of the supernatants (i.e. not bound to the cells) determined. The pellets were washed with an equal volume of 0.9% NaCl and the radioactivity of the washing solution was determined along with that remaining bound to the cells.

2.3.2. Method B

The receptor-antibody binding assay (RASA) was performed according to Corti et al. [13]. Cultures of *S. lividans* harboring pIJ702 or pTR168 or uninoculated broth were incubated for 1 h at 30°C in the presence of 0.15 to 4.8 mg/l of teicoplanin. After centrifugation the amount of teicoplanin remaining in the supernatants was determined.

2.4. *Other methods*

For high-performance liquid chromatography (HPLC) analysis, teicoplanin was recovered by filtration of broths through a D-alanyl-D-alanine agarose affinity column according to E. Riva et al. [14].

Sensitivity to other antibiotics was determined by depositing standard 6 mm antibiogram discs (BBL, Cockeysville, MD, USA) onto a layer of spores of the strain under investigation, and measuring the inhibition zones after 2 days of incubation at 28°C.

3. RESULTS

A. teichomyceticus genomic DNA was partially digested with the restriction endonuclease *Bam*HI and size fractionated. The 2.5 to 10 kb fragments were ligated to *Bgl*II linearized and phosphatase-treated pIJ702. This ligation mixture was used to transform *S. lividans* 66 protoplasts; about 11000 transformants containing recombinant plasmids were obtained after selection with thiostrepton.

Spore suspensions of these transformants were plated at low density to select for teicoplanin resistance; one clone was selected for the ability to grow in the presence of 20 mg/l of the antibiotic. The plasmid present in this clone was named pTR168.

Plasmid pTR168 was extracted from the cells and utilised to retransform *S. lividans* 66 protoplasts. More than 95% of the thiostrepton-resistant transformants were also resistant to teicoplanin, demonstrating that teicoplanin resistance was linked to pTR168.

The restriction map of the plasmid revealed an insert about 2600 base pairs long (fig.1a). The restriction map also shows the internal (*Sst*I, *Kpn*I) fragment, of about 1500 base pairs, used as a probe in a Southern blot hybridization with genomic DNA of *A. teichometicus* digested with various restriction enzymes (fig.1b). The results obtained are consistent with the hypothesis that the insert in pTR168 is present as a unique sequence in the *A. teicomyceticus* genome.

In table 1 we compare the sensitivity, to a number of antibiotics, of *S. lividans* 66, harboring pIJ702 or pTR168. As expected, the only difference, between the two strains, was found for teicoplanin which did not inhibit pTR168 containing cells; this strain also appeared to be slightly less sensitive to rifampicin. Both strains were also tested for their resistance to various glycopeptide antibiotics: vancomycin, teicoplanin and A/40926.

S. lividans seems to be intrinsically resistant to vancomycin as it grew even on plates containing 100 mg/l of this antibiotic (data not shown). pTR168-containing cells grew with 100% plating efficiency in the presence of 100 mg/l of teicoplanin or 50 mg/l of A/40926, while the strain harboring pIJ702 was inhibited by 3.2 mg/l of either antibiotic. These data suggest that the resistance conferred by pTR168 is specific for glycopeptide antibiotics but not for teicoplanin alone.

No chemical modification of teicoplanin was detected by HPLC analysis of supernatants of

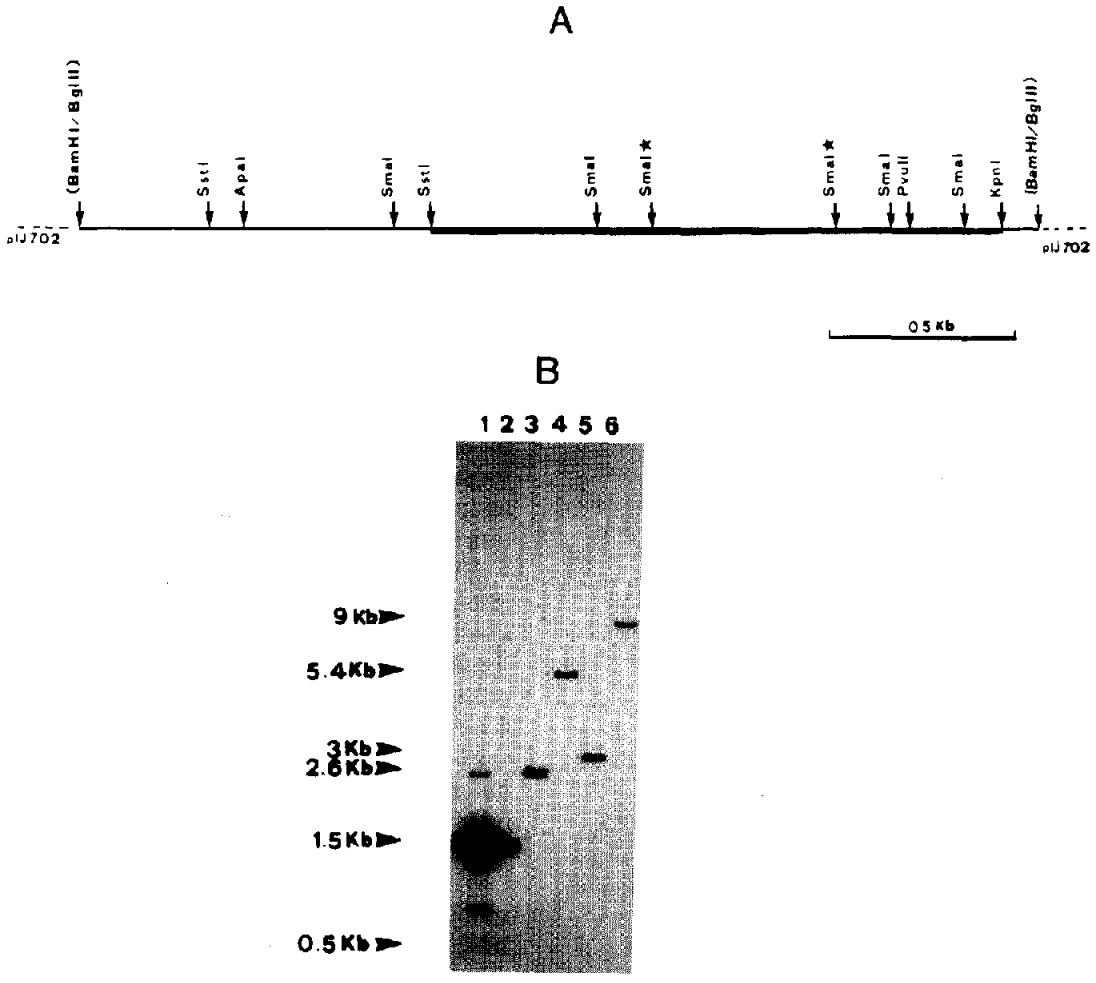

Fig.1. (A) Restriction map of pTR168. At both ends of the insert two *Xho*II sites were present, they arise from the joining of *Bgl*II/*Bam*HI sticky ends. No other *Xho*II sites are present in the insert. One *Sma*I site has not yet been precisely mapped; the two possible positions are indicated by symbols with stars. Recognition sites for the following restriction enzymes are not present: *Bam*HI, *Bcl*I, *Bgl*II, *Cla*I, *Eco*RI, *Eco*RV, *Pst*I, *Sph*I, *Xho*I. Enzymes not mapped or not reported in the above list were not assayed. The thicker line between the inner *Sst*I and the *Kpn*I recognition sites shows the fragment used as probe for the Southern hybridization. (B) Hybridization with *A. teichomyceticus* genomic DNA. Genomic DNA was subjected to restriction enzyme digestions, followed by electrophoretic separation of the restricted DNA, and then transferred onto a nylon membrane. The ^{32}P-labelled probe was hybridized with genomic DNA from *A. teichomyceticus* at 65°C for 16 h. Excess probe was washed away with 15 mM NaCl and 1.5 mM sodium citrate at the same temperature. Lane 1 contained the probe, as a positive control. The restricted genomic DNA was loaded in the other lanes, as follows: 2, *Kpn*I–*Sst*I; 3, *Bam*HI; 4, *Pst*I; 5, *Pvu*II; 6, *Bgl*II. The lengths of the fragments revealed by the probe are indicated on the left; the smaller *Pvu*II fragment was more visible in a longer exposure.

resistant (pTR168) or sensitive (pIJ702) cultures after up to 18 h of contact with the antibiotic (data not shown). However, less teicoplanin was recovered from the culture of sensitive cells; as if these had bound more antibiotic than the resistant ones. Two possible mechanisms by which a cell could avoid binding this class of antibiotics are: (i) the production of a molecule able to sequester the antibiotic from the culture medium; (ii) the production of a molecule able to mask the binding sites.

We have studied the binding of teicoplanin to *S. lividans* carrying either the parental or the recombinant plasmid.

Table 1

Comparison of the activity of known antibiotics on *S. lividans* harboring the parental plasmid pIJ702 or the recombinant plasmid pTR168

Antibiotic	mcg or (IU)[a] per disc	Inhibition zone (mm)	
		pIJ702	pTR168
Neomycin	30	13.5	12.5
Paromomycin	30	15.5	14.5
Kanamycin	30	22.5	21.5
Gentamicin	10	12.5	12.5
Tobramycin	10	14.0	13.5
Amikacin	30	21.5	20.5
Erythromycin	15	16.0	15.0
Novobiocin	30	22.5	22.5
Rifampicin	30	19.0	16.0
Bacitracin	(10)	21.0	21.0
Teicoplanin	30	24.5	0

[a] IU, international units

Cultures of the two strains were prepared and cells reconstituted with homologous or heterologous supernatants as described in section 2.3. As shown in table 2, the sensitive cells bound about half of the teicoplanin present in the medium. Regardless of the source of the supernatant, very little antibiotic was removed by washing. The resistant cells, in either supernatant, did not bind significant amounts of antibiotic.

We also evaluated the amount of free and bound teicoplanin by an immunological method (RASA). Again, sensitive cells bound the antibiotic more efficiently than the resistant ones. For all the concentrations tested, more than 85% of the teicoplanin remained in the supernatants of cells harboring pTR168 while only 25% remained when cells bearing pIJ702 were used. The differences in the binding values, obtained with pIJ702-containing cells, between the two different methods might be due to the longer incubation time used in the RASA assay.

Table 2

Teicoplanin binding assay

	A	B	C	D
Mycelium:	(pIJ702)	(pIJ702)	(pTR168)	(pTR168)
Supernatant:	(pIJ702)	(pTR168)	(pTR168)	(pIJ702)
Broth	46.5%	42.7%	85.0%	83.1%
Washing	5.5%	5.6%	7.2%	6.7%
Cells	47.9%	51.6%	7.6%	10.0%

Values are expressed as per cent of the total input radioactivity; the sum of each column is not exactly 100% because of the variability in the sampling and counting

4. DISCUSSION

Antibiotic-producing microorganisms are commonly resistant to their own products [1] and consequently have been used as sources for isolating antibiotic-resistance genes [15]. Various forms of self-defense have already been described; this is the first study of this type for glycopeptide producers. Our findings strongly suggest the presence of at least one gene involved in the resistance of *A. teichomyceticus* to its own product. Work is in progress to further analyse the structure of the DNA region present in pTR168 and to explore the possibility that more than one gene is involved in resistance of the producer strain.

Recently, transferable resistance to these glycopeptide antibiotics was described in clinical isolates. Two different plasmids conferring resistance to glycopeptides were found in strains of *E. faecium* [4] and another transferable resistance has been associated with the appearance of a 39 kDa protein in *E. faecalis* [3]. However the biochemical mechanisms of these resistances remain unknown. Since the target of the antibiotic is in the cell wall, resistance due to an exclusion mechanism would seem unlikely [4,16]. Our data show that the resistance, induced by the presence of pTR168, is correlated with a reduced binding of teicoplanin to the cells. The protein(s) involved and possible changes in the cell wall structure are currently under investigation; the possible involvement of a S-layer [17] is also taken into consideration. Furthermore, it is interesting to note that the mechanism of resistance present in the pathogenic strains of *E. faecium* seems to be similar with that of the parental producer strain (Bernareggi, A. et al., unpublished).

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