PLASMID-MEDIATED PRISTINAMYCIN RESISTANCE

PAC IIA: A NEW ENZYME WHICH MODIFIES PRISTINAMYCIN IIA

FRANÇOIS LE GOFFIC, MARIE-LOUISE CAPMAU, DIDIER BONNET, CLAUDE CERCEAU, CLAUDE SOUSSY*, ALAIN DUBLANCHET and J. DUVAL*

Centre d'Etude et de Recherche de Chimie Organique Appliquée,
Centre National de la Recherche Scientifique, 94320 Thiais, France
*Service de Bactériologie, Centre Hospitalier Henri-Mondor,
94010 Créteil, France

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A wild strain of *Staphylococcus aureus* which inactivates a wide variety of antibiotics has been found to inactivate pristinamycin IIA, an antistaphylococcal antibiotic. This phenomenon has been demonstrated to be plasmid mediated. The plasmid directs the biosynthesis of an acetyltransferase which is able to O-acetylate the drug. We propose to call the new enzyme PAC (IIA): Pristinamycin acetyltransferase.

The pristinamycins belong to the group of "mikamycins" which are a complex of synergistic compounds[2] identical in structure with ostreogrycin. These compounds are produced by *Streptomyces pristinaespiralis* and are widely used against staphylococcal infections.

Pristinamycin IIA (M. W. 526) is a macrocyclic lactone containing pyrrolidine and oxazole rings with structure as shown in Fig. 1a. It is identical with mikamycin A, ostreogrycin A, streptogramin A, vernamycin A, PA II4A$_1$ and virginiamycin M. Pristinamycin IIB (M. W. 528), ostreogrycin G or virginiamycin M$_2$ has the structure with the Δ-2, 3 bond saturated (Fig. 1b).

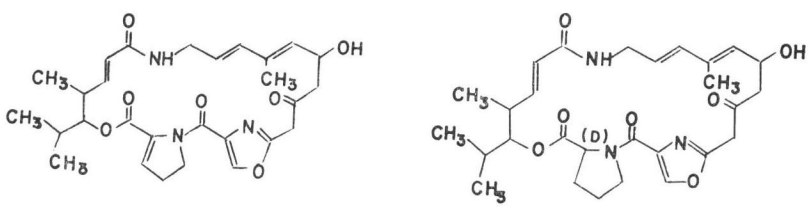
The group B antibiotic is a depsipeptide (Fig. 2) and includes pristinamycin IA, mikamycin B, streptogramin B, PA II4B and ostreogrycin B. Other derivatives of this B group are also present in the fermentation broth of the *Streptomyces pristinaespiralis* producing strain.

Until recently, only a few resistant mutants to these drugs have been reported in the literature[1] but no pristinamycin resistant strain of bacteria has been found in clinical situations.

We now report the characterization of the first plasmid-mediated pristinamycin acetyltransferase obtained from a clinical isolate of *Staphylococcus aureus*. This enzyme is able to acetylate pristinamycin IIA on the hydroxyl function of this molecule. The enzyme has not been purified, however, we have isolated inactivated pristinamycin IIA and determined its structure by UV, NMR and mass spectrometry.

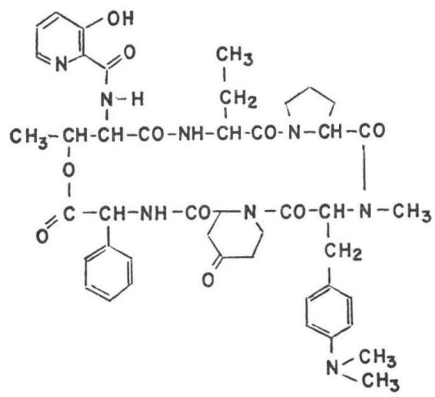
Fig. 1. The structure of the pristinamycins

(a) Pristinamycin IIA (b) Pristinamycin IIB



Recently, DE MEESTER and RONDELET[3] published an analogous mechanism of inactivation of the M factor of virginiamycin.

Fig. 2. The structure of pristinamycin IA.



Material and Methods

Bacterial strain

The resistant strain, a *S. aureus* referred as STE, is a clinical isolate from skin burns. Resistant levels and antibiotic spectra were determined by standard agar dilution method and curing experiments were performed as described earlier[6].

Analytical techniques

UV spectra were measured on a Cary 15 spectrometer in ethanol solution. Proton magnetic resonance spectra were measured on a Brucker instrument at 90 mHz in $CDCl_3$ and CD_3OD solutions and the mass spectra on a Varian MAT 311A spectrometer (field desorption).

Thin- or thick-layer chromatography were performed on silicic gel plates prepared with Kiesel-Gel HF 254 from Merck, the eluent being a mixture of chloroform - methanol (87: 13).

Chemicals and antibiotics

(^{14}C) Pristinamycin IIA (1 mCi/mm), pristinamycin IIA were provided by Rhône-Poulenc. (^{14}C) Acetyl Coenzyme A was prepared[5] from Coenzyme A. (^{14}C) Sodium acetate was obtained from Commissariat à l'Energie Atomique (France).

Inactivation and extraction of pristinamycin IIA

Inactivation with the whole cells:

S. aureus STE was grown in a 1 liter culture containing 30 g Trypticase soy broth (Difco). After the early logarithmic phase of growth 30 mg of pristinamycin IIA (radioactive or not with or without ^{14}C acetate) were added and incubation was performed at 37°C for 18 hours. The cells were harvested by centrifugation and the supernatant extracted with methylene chloride (7 × 80 ml). After drying and evaporation of the solvent about 100 mg of a residue was obtained and analyzed by thin-layer chromatography. The product (X) formed in the culture medium was then purified by thick-layer chromatography (23 mg) and its structure determined by standard physicochemical methods.

Inactivation with a crude enzymatic extract:

The crude enzymatic extract was prepared as follows. The resistant strain was grown in Trypticase soy broth (Difco). The cells were collected by centrifugation, washed in a buffer containing 10 mM $MgCl_2$, 10 mM tris (hydroxymethyl) aminomethane 60 mM NH_4Cl, 6 mM β-mercaptoethanol, pH 7.5 at 4°C and disrupted by grinding in a mortar with 2.5 parts (w/w) of alumina powder. Broken cells were removed by centrifugation at 20,000 × g for 30 minutes. This crude extract was used as the source of enzyme for the inactivation experiment which was conducted as follows. Five mg (10^{-2} mmole) of pristinamycin IIA, 8.2 mg (10^{-2} mmole) of radioactive acetyl Coenzyme A (specific activity 85 μCi/mole) were incubated for 4 hours at 37°C with the crude enzymatic extract prepared previously (60 ml of the preparation added in four portions). The medium was extracted with methylene chloride (140 ml) and filtered on Kieselgühr. The organic phase was dried on anhydrous sodium sulfate.

After evaporation of the solvent a radioactive residue (10 mg) was obtained and analyzed by thin-layer chromatography. Product X was located on the plate and isolated by thick-layer chromatography.

Results

The resistance pattern of *S. aureus* STE to pristinamycins, lincomycins and macrolide antibiotics

is shown in Table 1. This strain also has an unusual resistance pattern to aminoglycoside antibiotics.

The resistances are plasmid mediated as has been shown by curing with ethidium bromide, by transduction, and by the isolation of the responsible plasmid[4] from the resistant strain.

The biochemical mechanism of the resistance to aminoglycosides has been studied previously in our laboratory and is explained by the presence of a 4′-O-nucleotidyltransferase[6].

When the resistant strain STE was grown in the presence of radioactive pristinamycin IIA and the medium extracted after late logarithmic phase of growth, the antibiotic was completely metabolized and gave rise to a new radioactive compound referred to as X (Rf 0.60) as shown by thin-layer chromatography. The same experiment has also been conducted in the presence of pristinamycin IIA and (^{14}C) acetate. Under these conditions, the same compound was found in the medium (radioactivity incorporated).

When pristinamycin IIA and (^{14}C) acetyl CoA were incubated with the crude cell extract in a suitable medium, radioactivity was incorporated into the drug. This suggests that acetylation occurred during incubation.

A control experiment performed by incubating pristinamycin IIA in culture medium alone or with the drug sensitive strain obtained by curing allows a complete recovery of starting antibiotic after extraction with methylene chloride.

Compound X was obtained in a pure form by thick-layer chromatography of the methylene chloride extract from the culture medium or from the *in vitro* enzymatic incubation.

Properties and Structure Determination of Compound X

Compound X was a single component (Rf 0.60) less polar than pristinamycin IIA (Rf 0.46) as shown by thin-layer chromatography. The fact that this compound was radioactive when (^{14}C) pristinamycin IIA was used as precursor shows that the molecule was a metabolite of pristinamycin IIA. The fact that radioactivity was incorporated in this molecule when pristinamycin IIA and (^{14}C) acetate ions were incubated with the resistant strain, or when the assay was performed with a crude enzymatic extract using (^{14}C) acetyl Coenzyme A as acetyl donor suggests that acetylation occurred during incubation.

This inactivation process was confirmed to be acetylation by the determination of the structure of X in the following manner. Compound X ($\lambda_{max.}$ 231.8, $\varepsilon = 14{,}400$) and pristinamycin IIA ($\lambda_{max.}$ 235, $\varepsilon = 16{,}800$) have the same UV spectrum. This indicates that no modification occurred on the gross structure of the drug.

The proton magnetic resonance (pmr) spectra of pristinamycin IIA and X have the general feature except in one point: there is a singlet in the spectrum of X at 1.9 ppm representing a CH_3–CO residue.

This residue is confirmed by the analysis of its mass spectrum. The molecular ion at *m/e*

Table 1. Resistance spectra of *S. aureus* STE expressed as minimal inhibitory concentrations (M. I. C. mcg/ml).

Antibiotics	*S. aureus* STE	*S. aureus* STE "cured"
Pristinamycin	32	1
Pristinamycin IIA	64	2
Pristinamycin IA	64	>128
Erythromycin	>128	>128
Spiramycin	>128	>128
Lincomycin	>128	>128
Clindamycin	>128	>128
Streptomycin	>128	>128
Gentamicin	0.50	0.50
Kanamycin	128	4
Amikacin	8	2
Tobramycin	64	0.50
Lividomycin	16	4
Neomycin	32	1

567 is consistent with a monoacetylpristinamycin IIA. There is also a peak at *m/e* 568 which represents the ion $M+H^+$. The spectrum of pristinamycin IIA is also characterized by two peaks at respectively *m/e* 526 and *m/e* 525 which represent respectively the ion $M+H^+$ and the molecular ion.

Both spectra have common peaks at *m/e* 508 and *m/e* 507 which represent respectively

Fig. 3. The structure of X.
The hydroxyl function of pristinamycin IIA is acetylated (acetyl residue in rectangle).

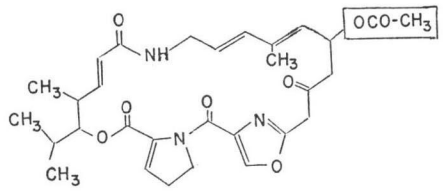


Fig. 4. Nuclear magnetic resonance spectra of pristinamycin IIA (A) and its O-acetyl derivative (B).

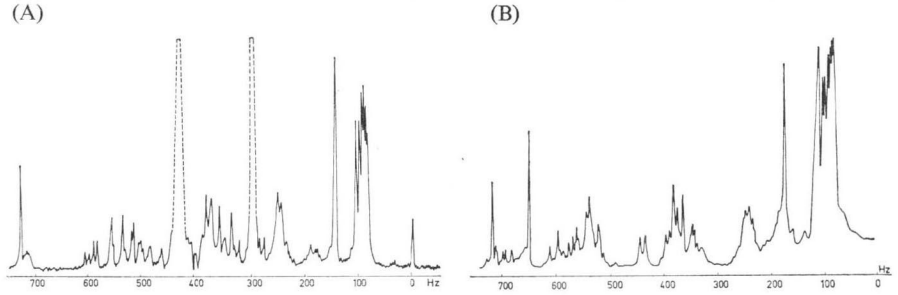
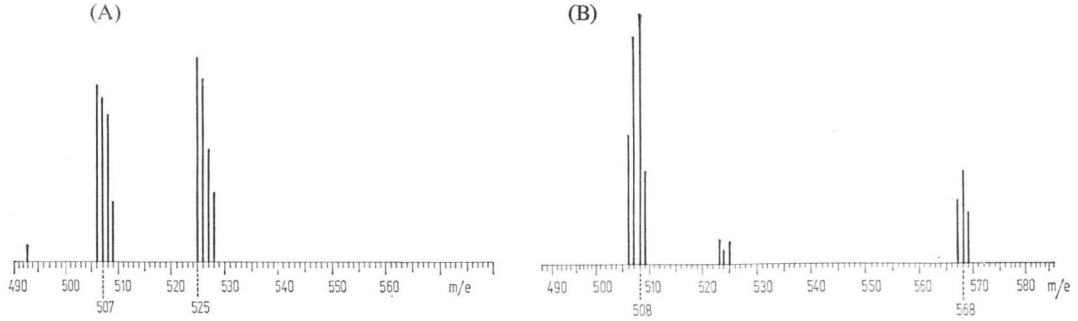


Fig. 5. Mass spectra of pristinamycin IIA (A) and its O-acetyl derivative (B).



compound X having lost respectively a molecule of acetic acid and dehydrated pristinamycin IIA. These results show clearly that X has the structure shown in Fig. 3.

This compound showed no antibacterial activity (>54 mcg/ml) against a wild-type strain of *S. aureus* as compared to the activity found for pristinamycin IIA (2 mcg/ml).

Discussion

We have described the isolation and the characterization of acetylpristinamycin IIA, whose biosynthesis is performed by a resistant strain of *S. aureus*. This microorganism has been isolated in a clinical situation and is resistant not only to the pristinamycins but also to a wide variety of antibiotics including the deoxystreptamine aminoglycoside antibiotics, streptomycin, erythromycin, spiramycin, lincomycin and clindamycin.

The resistance to pristinamycin IIA and to the deoxystreptamine antibiotics seems to be associated and directed by the same plasmid because of the simultaneous loss of this resistance profile by curing experiments. This plasmid carries at least two resistance determinant genes: the former directing the

biosynthesis of a nucleotidyl transferase responsible for the enzymatic modification of the aminoglycoside antibiotics[6], the second one carrying this genetic information for the synthesis of a pristinamycin acetyltransferase that we propose to call PAC IIA.

The reason why the resistance to streptomycin, erythromycin, spiramycin, lincomycin and clindamycin is not lost by the treatment with ethidium bromide is not known. It is possible that the cured strain of *S. aureus* still carries another plasmid responsible for these resistance.

In conclusion we can explain the resistance mechanism to pristinamycin IIA by the biosynthesis of a pristinamycin IIA acetyltransferase that produces acetylpristinamycin IIA which has completely lost its antibiotic properties.

Acknowledgments

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