Rifampicin Inactivation by *Bacillus* species

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The ability of strains of *Bacillus*, *Staphylococcus*, *Pseudomonas*, and *Escherichia coli* to inactivate rifampicin was tested. Most *Bacillus* strains were found to inactivate rifampicin. Two modes of inactivation were identified; one was phosphorylation and the other involved decolorization. Presence or absence of either mechanism appeared unrelated to the phylogenetic relatedness of strains. None of the other organisms could inactivate this antibiotic.

Rifampicin (Fig. 1) is a semisynthetic derivative of the rifamycin group of antibiotics[1,2]. These have as their mode of action the inhibition of prokaryotic DNA dependent RNA polymerase[3] and the classical mode of resistance has been shown to be mutational alteration of the target moiety, specifically, the β subunit of this enzyme[4].

Rifampicin is one of the principal agents used to combat infections by nocardioform bacteria of the genera *Mycobacterium*, *Nocardia*, and *Rhodococcus*[5~7]. Our studies in which these organisms were challenged by this compound revealed an additional aspect of the interaction, *viz.* inactivation of the antibiotic. An unexpected variety of mechanisms was found: glucosylation in *N. brasiliensis*[7], phosphorylation in *N. otitidiscaviarum*[8], ribosylation in *Mycobacterium* strains (Fig. 1)[9], and one involving decomposition in *N. asteroides*[8] and *Rhodococcus* strains[10,11].

As these were the first reports of rifampicin inactivation we wished to determine if bacteria other than no-cardioforms also possessed a similar enzymatic activity. In this paper, we describe the response of strains from several other genera to challenge by this antibiotic.

Materials and Methods

Organisms
10 strains of *Pseudomonas aeruginosa*, 8 strains of *Escherichia coli*, 4 strains of methicillin-sensitive *Staphylococcus aureus* (MSSA), and 6 strains of methicillin-resistant *S. aureus* (MRSA) were tested. These were clinical isolates and so represented opportunistic pathogens. In addition about 20 strains of *Bacillus* were studied (Table 1).

Growth Conditions and Antibiotic Challenge
Bacillus strains were grown overnight in 5 ml of Luria Bertani medium (LB: 1% Tryptone, 0.5% Yeast Extract, 0.5% NaCl), then 100 μl was pipetted into 10 ml of fresh LB. After shaking for 90 minutes, rifampicin was added (from a 100 × stock in methanol) to a final concentration of 20 μg/ml. 37°C was the incubation temperature for all

organisms except *B. stearothermophilus*, which was grown at 55°C. Aliquots removed at intervals were assayed for residual antibiotic concentration based on zones of inhibition on a lawn of *B. subtilis* PCI 219 in

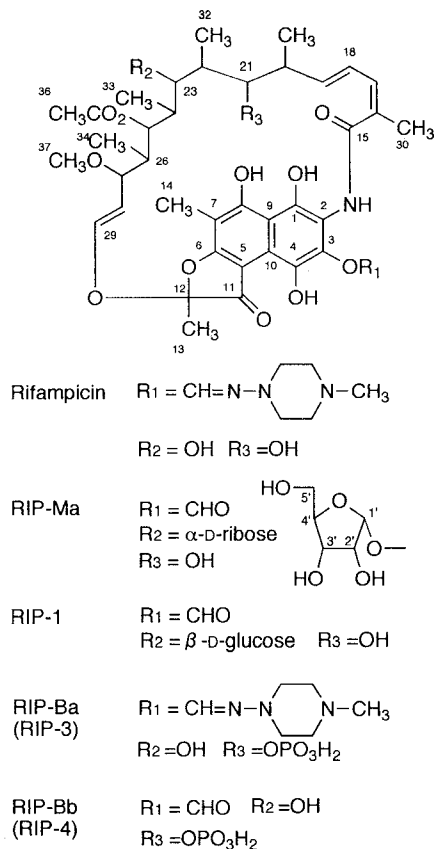Fig. 1. Structures of various inactivated compounds of rifampicin.

Table 1. Rifampicin susceptibility and inactivation ability of *Bacillus* strains.

Bacillus sp.		MIC (μg/ml)	Pattern*	Phylogeny#
amyloliquifaciens	HSCC186	0.0125	-	I
amylolyticus	HSCC169	0.1	+(D)	III
aneurinolyticus	HSCC145	0.025	-	U
brevis	26A1	0.2	+(P)	IV
cereus	6A1	0.05	+(D)	I
circulans	16A1	0.1	+(P)	I
fusiformis	HSCC289	0.0125	-	II
globigii	11A1	0.0125	-	ND
laterosporus	HSCC185	<0.00625	-	IV
licheniformis	5A2	0.1	+(P)	I
marcorans	HSCC175	0.2	+(P)	I
megaterium	7A1	0.1	+(P)	I
polymyxa	25A1	0.8	+(P)	III
pumilus	8A1	0.1	+(D)	ND
sphaericus	HSCC368	0.2	-	II
stearothermophilus	9A5	0.025	+(P)	U
subtilis	1A3	0.1	+(P)	I
	1A3 iri⁻	0.025	-	I
	1A4	0.1	+(P)	I
	1A5	0.39	+(P)	I
	1A9	0.39	+(P)	I
	PCI219	0.1	+(D)	I
niger	12A1	0.05	+(D)	ND
thiaminolyticus	HSCC144	0.025	-	ND

* Inactivation pattern D: Degradation, P: Phosphorylation.

Data were taken from ref. 12. Number indicates phylogeny group. U: ungrouped, ND: no description.

comparison with a dilution series of known rifampicin concentrations. For other organisms, between 10 and 30 μg/ml was used in challenge experiments, and incubation at 37°C was for 72 hours. For all organisms, rifampicin and its inactivated derivatives were extracted from 2 ml of culture using 1 ml EtOAc after adjusting to pH 2 with 1 drop 6 N HCl. 0.5 ml of EtOAc extract was vacuum dried then dissolved in 20 μl acetone. 5 μl was spotted on TLC plates. Some *Bacillus* strains decolorized rifampicin and concomitantly the antibiotic activity disappeared. Therefore, for these strains, the term "decolorization" was used.

Mutagenesis

Mutants of strain 1A3 were obtained after 15 minutes exposure of washed cells to 100 μg/ml *N*-methyl-*N'*-nitro-*N*-nitrosoguanidine in Tris-maleate buffer, pH 4.8 at 37°C. After outgrowth, clones were screened on plates for susceptibility to 30 ng/ml rifampicin and those which were unable to grow were tested for inactivating ability. Among such mutants, designated as 1A3 iri⁻ was used in the present studies.

Growth Monitoring by an Automatic Growth Measurement System

Approximately 10^4 CFU/ml of *B. subtilis* 1A3 or its iri⁻ mutant were added to duplicate wells (containing 50 μl Spizizen medium with 1% casamino acids and 0.1% yeast extract) of a microplate containing different concentrations of rifampicin in the same medium. The plate was placed in a Bioscreen C spectrometer (Labsystems, Tokyo, Japan). Growth of cells was monitored by turbidity measurements every 20 minutes. Data were recorded by a computer interfaced with analyzer and growth curves generated for each suspension.

Large-scale Preparation of Inactivation Products

Inactivated rifampicin was obtained after challenge of 1 liter of *Bacillus* IA3 strain's culture with 100 μg/ml antibiotic. After 72 hours incubation at 37°C cells were removed by centrifugation at 6,000 rpm and the supernatant adjusted to pH 2.0 with 2 N HCl then extracted three times with an equal volume of ethyl acetate. The extracted layer was washed with distilled water. The EtOAc layer was dehydrated with Na_2SO_4 and concentrated *in vacuo* to dryness. Dried material was purified by silica gel column chromatography using Wakogel C-200 (Wako Pure Chemicals, Japan) and a mixture of $CHCl_3$ and MeOH (95:5) as solvent. Preparative silica gel TLC was made using $CHCl_3$-MeOH, 4:1 for RIP-Ba and 3:1 for RIP-Bb. Sephadex LH-20 column chromatography with MeOH as solvent was employed in further purification of each compound. Throughout the procedure, inactivated compounds were monitored by TLC. A final product of 10.2 mg of RIP-Ba and 11.1 mg of RIP-Bb was obtained from 100 mg of rifampicin.

Physico-chemical Characteristics

Nuclear magnetic resonance (NMR) spectra were measured using a JEOL ALPHA 500 NMR spectrometer. Fast atom bombardment mass spectra (FAB-MS) and high-resolution FAB-MS (HRFAB-MS) were measured with a JEOL JMS-HX110 instrument. The structures of RIP-Ba and RIP-Bb were determined in comparison with the data of rifampicin, RIP-3 and RIP-4[7,8].

Results

Inactivation Experiments

Clinical isolates of Pseudomonas aeruginosa, Escherichia coli, and Staphylococcus aureus (see Materials and Methods) were tested for inactivation activity against rifampicin. Antibiotic potency, as determined by zones of inhibition on a lawn of assay organism, was not affected by any of the strains of these bacteria which were tested. TLCs of rifampicin obtained after exposure to these isolates did not reveal the presence of any modified compound. Therefore none of these strains could be shown to inactivate rifampicin.

In contrast, most strains of Bacillus which we tested were able to inactivate this antibiotic. Low concentrations ($<2 \mu g/ml$) were completely inactivated in <4 hours; by selection of rifampicin resistant strains to eliminate inhibitory effects of this compound it was found that $100 \mu g/ml$ was inactivated in $8 \sim 10$ hours. Representative inactivation curves for B. subtilis strains 1A3 and PCI 219 are shown in Fig. 2A. Also included is a result with a mutant of 1A3 that had been selected on the basis of loss of rifampicin inactivating ability

(termed iri⁻). The parental strain completely inactivated $20 \mu g/ml$ in about 5 hours whereas the mutant did not detectably lower this concentration of antibiotic in 12 hours (Fig. 2A). As shown in Table 1, the rifampicin MIC of the mutant was four fold lower due to the absence of this ability. We monitored growth of 1A3 and its mutant in the presence of rifampicin ($0.015 \mu g/ml$ and $0.030 \mu g/ml$) using the Bioscreen automatic growth measurement system. The mutant showed a lag of about 26 to 28 hours in comparison with the parent (Fig. 3).

Cultures of about eighteen other Bacillus species were examined and eleven of these could inactivate rifampicin at rates similar to B. subtilis (e.g. B. cereus and B. pumilus, Fig. 2B). The remainder showed no evidence of inactivation even after prolonged incubation (see e.g. B. globigii, Fig. 2B). We measured MIC for these organisms and found that except for the thermophile B. stearothermophilus all strains with values of $0.025 \mu g/ml$ or below were unable to inactivate rifampicin whilst except for B. sphaericus all organisms with values above 0.025 $\mu g/ml$ did inactivate (Table 1).

Nature of Inactivation

TLCs of extracts from rifampicin-challenged Bacillus cultures revealed three patterns of behavior (Fig. 4). For the seven species which did not inactivate the antibiotic there was no change in this compound. The same was true for the non-inactivating mutant of B. subtilis 1A3. Most other B. subtilis strains modified the antibiotic such that Rf was close to zero, similar to what was seen with phosphorylative inactivation by Nocardia otitidis-
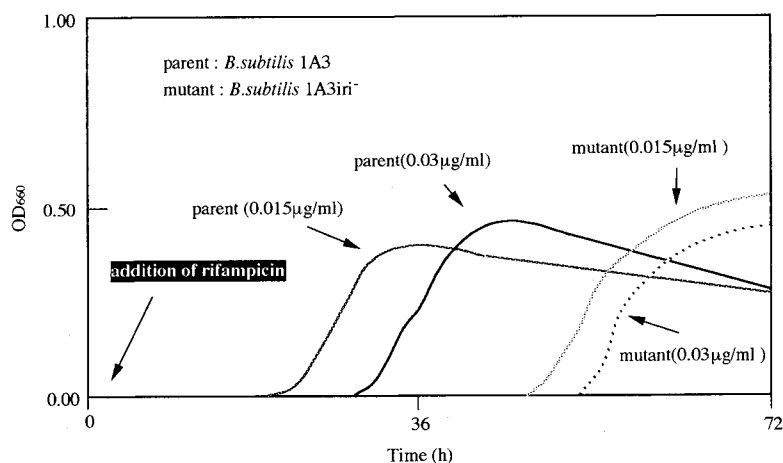
Fig. 2. Rifampicin inactivation by Rif-R (rifampicin resistant) Bacillus strains.

A: B. subtilis 1A3 (□), 1A3 iri⁻ (◇), PCI 219 (○), B: B. cereus 6A1 (■), B. pumilus 8A1 (◆), and B. globigii 11A-1 (●).
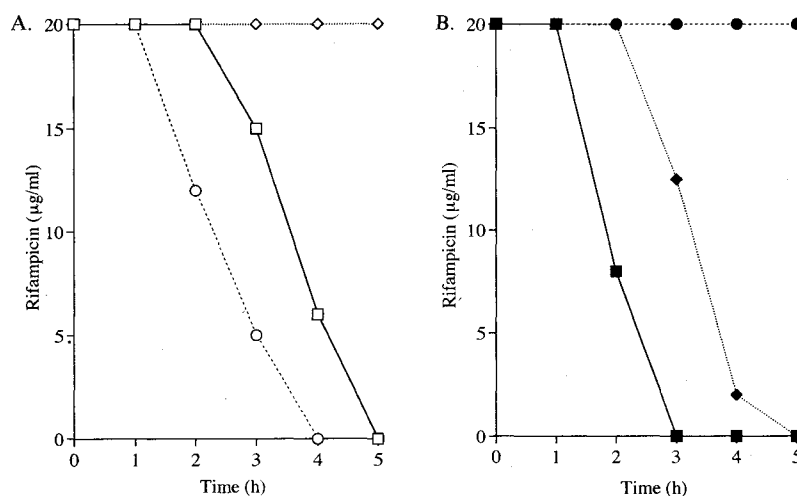
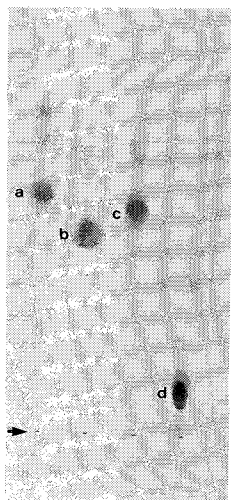Rifampicin was added to a concentration of $20 \mu g/ml$ at $t = 0$.

Fig. 3.   Growth curves of *B. subtilis* 1A3 and its mutant strain (1A3 iri⁻) in the presence of 0.015 μg and 0.030 μg/ml of rifampicin.

Rifampicin was added at 2 hours after the start of growth at 30°C. Detail see Materials and Methods.

Fig. 4.   TLC patterns of inactivated compounds of rifampicin in comparison with that of rifampicin. (Developing solvent is CHCl₃ and MeOH (4:10))

a: Rifampicin, b: ribosylated compound (RIP-Ma), c: glucosylated compound (RIP-1) and phosphorylated compound (RIP-Bb [RIP-4]). → shows origin of sample applied.

caviarum[8]. An exception was strain PCI 219, which decolorized the antibiotic in a fashion resembling that seen with many *Rhodococcus* strains[8,10,11] as well as *N. asteroides*[8]. For *Bacillus* species apart from *B. subtilis*, eight displayed the phosphorylative pattern of inactivation and four the decolorization (Table 1).

The nature of the rifampicin modification was determined after purification of compounds from broth in which *B. subtilis* 1A3 had been challenged by the antibiotic. Structures of inactivated compounds RIP-Ba and RIP-Bb were deduced from the following mass spectrometry data. Molecular weights and molecular

formulae of RIP-Ba ($C_{43}H_{59}N_4O_{15}P$) and RIP-Bb ($C_{38}H_{48}NO_{16}P$) were determined by positive and negative FAB-MS and HRFAB-MS. These molecular formulae suggested that RIP-Ba and RIP-Bb are mono-phosphorylated-rifampicin and -3-formyl-rifamycin SV, respectively. TLC patterns of RIP-Ba and RIB-Bb using different solvent systems including CHCl₃ - MeOH (2:1) showed similarity to RIP-3 and RIP-4 in each pattern. Therefore, ¹H NMR spectral data of RIP-Ba and RIP-Bb are compared with those of RIP-3 and RIP-4, whose 21-OH groups are phosphorylated[7,8]. All the signals of RIP-Ba and RIP-Bb completely coincided with those of RIP-3 and RIP-4, respectively. Finally the structures of RIP-Ba (RIP-3) and RIP-Bb (RIP-4) were determined as 21-(*O*-phosphoryl) rifampicin and 3-formyl-21-(*O*-phosphoryl) rifamycin SV, respectively (Fig. 1).

Discussion

Our previous work has demonstrated that nocardioform bacteria of the genera *Nocardia*, *Rhodococcus*, and *Mycobacterium* inactivate rifampicin in a variety of ways: by glucosylation[7], ribosylation[9], phosphorylation[8], or decolorization[8,10,11]. In contrast, we have shown here that a number of strains of *Pseudomonas*, *Escherichia*, and *Staphylococcus* do not act upon this antibiotic. However, about two-thirds of *Bacillus* species tested did modify rifampicin such that it no longer possessed bacteriostatic ability. A phylogeny of *Bacillus* has been constructed based upon small-subunit ribosomal RNA sequences[12]. In contrast to what was observed in nocardioforms, no correlation could be seen between inactivation pattern and putative evolutionary relationships within this genus: all three patterns were found in group 1, phosphorylation or no activity in group 2 and

in group 4, and decolorization or phosphorylation in group 5. Even within the single species *B. subtilis* (Table 1) both phosphorylation and decolorization mechanisms were present.

Although we could isolate two inactivated compounds RIP-Ba and RIP-Bb, the process on the release of piperazine moiety from rifampicin or its inactivated compound (RIP-Ba) is not clear. Concerning the decolorization of rifampicin, we tried to isolate the possible inactivated compound(s) by various extraction methods using various organic solvents, however, hitherto we have not succeeded. This may be partially due to the lack of appropriate detection methods for such decolorized compounds. Alternatively, the inactivated antibiotic may be degradated into compounds to small to be obtained by usual extraction method. Further detail studies are necessary.

Rhodococcal and mycobacterial DNAs responsible for inactivation mediated by decolorization and ribosylation respectively have both been cloned[11] (unpublished). It remains to be determined if *Bacillus*, *Nocardia*, and *Rhodococcus* decolorizations are due to a common mechanism.

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