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Monooxygenase-Like Sequence of a *Rhodococcus equi* Gene Conferring Increased Resistance to Rifampin by Inactivating This Antibiotic

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A DNA clone from *Rhodococcus equi* conferring low-level rifampin resistance through the ability to inactivate this antibiotic via its decomposition was identified. The *iri* (inactivation of rifampin) gene consisted of an open reading frame of 1,437 bp encoding a 479-amino-acid sequence strongly resembling those of monooxygenases acting upon phenolic compounds or involved in polyketide antibiotic synthesis. When expressed in *Escherichia coli*, the gene conferred resistance to a >50-μg/ml concentration of the drug.

Rifampin is one of the principal chemotherapeutic agents used to combat infections by species of *Mycobacterium* (9) and *Rhodococcus* (2). Recently, it has been demonstrated that part of the response of nocardioform and closely related bacteria to challenge with this antibiotic may involve an inactivation process. In both free-living strains and clinical isolates, four mechanisms have been identified: phosphorylation (23), glucosylation (22), ribosylation (5), and decomposition (3, 22) of rifampin. The mechanisms differ between genera: ribosylation has been confined to *Mycobacterium* and the closely related *Gordona* and *Tsukamurella*, glucosylation has been confined to *Nocardia*, and phosphorylation has been confined to *Nocardia* and *Rhodococcus* (19). Decomposition, resulting in decolorization of the antibiotic, was observed in species of *Nocardia* and *Rhodococcus* as well as *Mycobacterium* (3, 19, 23). None of the other clinical strains tested demonstrated an ability to inactivate rifampin. This was true of strains of *Corynebacterium*, a genus closely related to those mentioned above, as well as *Escherichia coli*, *Staphylococcus aureus*, and *Pseudomonas aeruginosa* (6). *Bacillus* strains were the only other bacteria found to inactivate this antibiotic; most species in this genus had the ability to do so, by either phosphorylation or decomposition (6).

The incidence of resistance to rifampin is increasing in clinical isolates. In mycobacterial species, high-level resistance has been shown to be due to mutational alteration of the target molecule, the β subunit of RNA polymerase (11, 20). However some strains with low to intermediate resistance do not have mutations in this protein (14a, 15). There may be other cellular components which are able to mutate and confer a degree of resistance; genes for an inactivating enzyme are obvious candidates. This work was undertaken to identify and characterize such a gene. Whereas with phosphorylation, ribosylation, and glucosylation the inactivated derivative of rifampin could be recovered and studied, in the case of decomposition the molecule was disrupted to a degree such that no information on the nature of the enzymatic attack could be obtained (22). It was anticipated that the sequence of a gene responsible for this decomposition might provide information on this. Many strains of *Nocardia* and *Rhodococcus*, both free-living and pathogenic, inactivate the antibiotic via this mechanism (3, 19, 22). Both *Rhodococcus equi* strains tested (the type strain, ATCC 14887, and the pathogenic isolate IFM 0162 [19]) did so. This species was initially identified as a pathogen of foals (13) but may also infect humans (8, 10). ATCC 14887 (obtained from N. Ferreira) was used as source of genomic DNA for this work. The rifampin MIC for this strain was 0.3 μg/ml.

A library of chromosomal DNA from strain ATCC 14887 was made in shuttle vector pDA37, which is stably maintained in *E. coli* and in several rhodococcal bacteria (4). Subcloning was in shuttle vector pDA71 (16), which is smaller and possesses more restriction sites suitable for cloning. Chromosomal and plasmid DNAs were purified by cesium chloride gradient centrifugation as previously described (4). Both genomic DNA and that of plasmid pDA37 were digested with *Bgl*II and a library of about 13,000 clones constructed in *E. coli* MM294 (relevant characteristics: *hsdR endA1*). The average size of inserts was 4.7 kb. *E. coli* transformations were done by the $CaCl_2$ method (17). Restriction endonucleases and other enzymes used in DNA manipulations were obtained from Boehringer Mannheim, Amersham, or New England Biolabs and were used according to the manufacturer's instructions. DNA fragments were purified after electrophoresis through low-gelling-temperature agarose (SeaPlaque); after excision, the agarose was melted at 65°C, extracted twice with Tris-EDTA-saturated phenol and once with chloroform, precipitated, and dried. Sequencing of single-stranded DNA was performed with M13mp18 and M13mp19 with a Sequenase version 2.0 kit (United States Biochemical). Absorbance measurements of rifampin solutions were made with a Milton Roy Spectronic Genesys 5 UV-VIS spectrophotometer. Assays of antibiotic concentrations were done with *Bacillus subtilis* 1A3-1 or *Arthrobacter oxydans* C1S as previously described (3). Inactivation was monitored by zones of inhibition on plates on which a lawn of one of these bacteria was spread; decolorization was monitored spectrophotometrically. Rifampin resistance was found by replica spotting about 10^4 CFU on plates; the highest concentration at which there was confluent growth was taken as the resistance level.

The pool of clones was transformed into *Rhodococcus erythropolis* Ri8, a nocardioform mutant of *R. erythropolis* ATCC 12674 selected on the basis of its inability to inactivate rifampin (4). Ri8 is inhibited by antibiotic concentrations above 50 ng/

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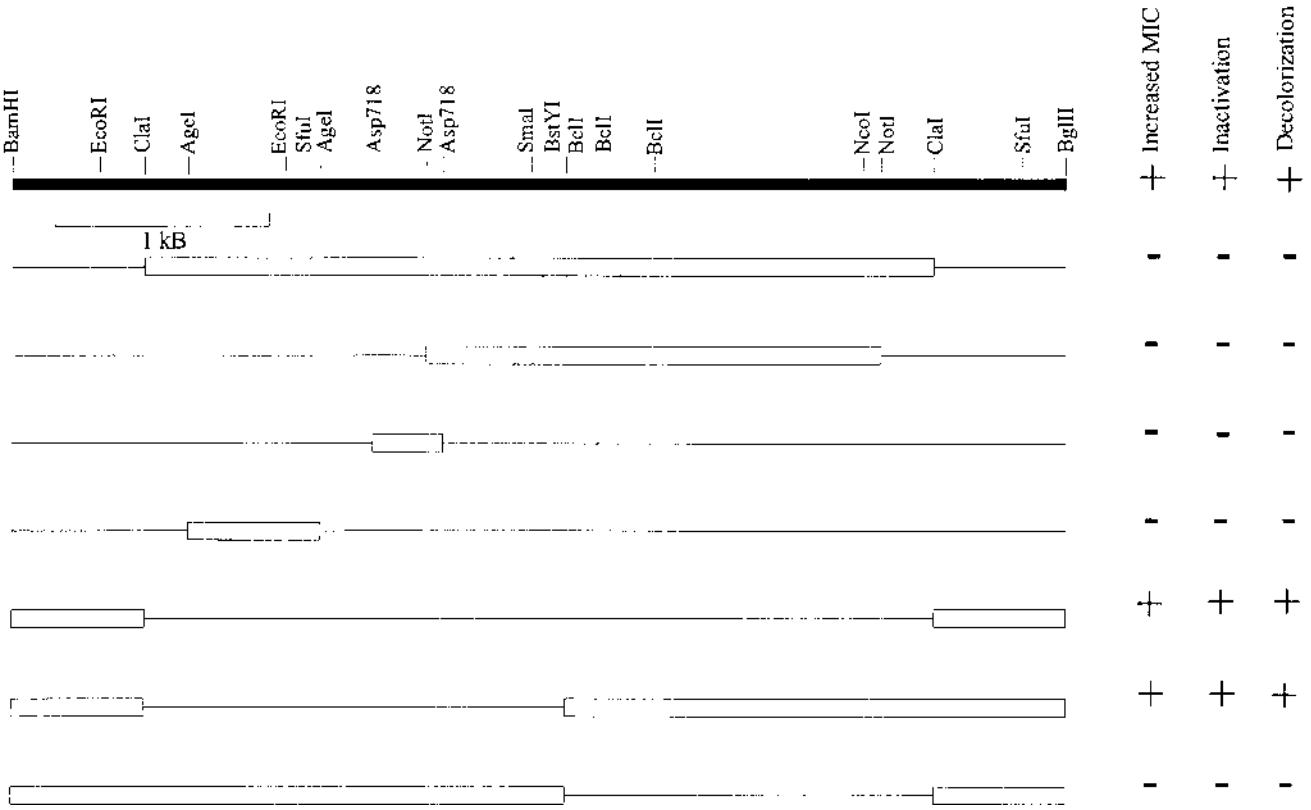

FIG. 1. Restriction map of 5-kb *Bam*HI-*Bgl*II fragment carrying the *iri* gene and deletion analysis of this DNA (white bars indicate deletions). Rifampin inactivation was tested by zones of inhibition on lawns of *B. subtilis*, and decolorization was monitored spectrophotometrically.

ml; clones conferring increased resistance were recovered by spreading dilutions of the pool on plates supplemented with 300 ng of rifampin per ml on the assumption that the ability to inactivate this compound would confer a phenotype of increased resistance. Rhodococcal transformations were performed with polyethylene glycol-generated protoplasts (4) or by electroporation. In the latter case, a Bio-Rad Gene Pulser I electroporator was used with cuvettes having a space of 0.2 cm at a voltage of 2 kV, a capacitance of 25 μF, a resistance of 400 Ω, and a time constant of 6.5 to 8.5 ms. After addition of 1 ml of broth and 1 h of incubation at 28°C, cells were spread on plates, which were incubated at the same temperature for 4 h. Plates (25-ml volume) were then underlaid with 250 μl of a 4-mg/ml stock solution of chloramphenicol dissolved in ethanol.

Clones growing on rifampin-containing selective plates all possessed an insert of about 8 kb, and retransformation into strain Ri8 demonstrated that this segment of DNA increased the rifampin MIC about 25-fold and conferred the ability to inactivate the antibiotic: rifampin at a concentration of 20 μg/ml was completely inactivated in about 6 h (as monitored by zones of inhibition on plates spread with a tester strain). Spectrophotometric measurements showed that, concomitant with inactivation, the antibiotic was decolorized, indicating that the mechanism was decomposition, as had been the case for strain ATCC 14887 (from which this clone was derived). A single *Bam*HI site was present in the 8-kb segment, and subcloning the *Bgl*II-*Bam*HI fragments into pDA71 demonstrated that the larger of the two conferred rifampin-inactivating ability. Figure 1 shows a restriction map of this 5 kb of DNA together with the deletion analysis, which demonstrated that a 2-kb *Bcl*I-*Cla*I fragment within this DNA segment carried the *iri* gene, conferring increased resistance to the drug and the ability to inactivate and decolorize it.

The DNA of the *Bcl*I-*Cla*I fragment was subcloned into M13mp18 or M13mp19, and the sequence was determined. It was 2,042 bp in length, with a G+C content of 63%. Codon usage analysis with the FRAME program (1) identified an open reading frame with the ATG start codon at bp 280 and the stop codon TGA at bp 1716. Thus, the open reading frame was 1,418 bp long and encoded a polypeptide of 479 amino acids (Fig. 2). In streptomycetes, a conserved Shine-Dalgarno sequence, (a/g)GGAGG, has been identified, and in the sequence shown here there was an AGGAGG sequence extending between −8 and −13. Possible promoter sequences were identified: TTGCGG and CACACT (Fig. 2). No comparative study of rhodococcal promoters has been made. Analysis of the sequence downstream from the putative stop codon revealed two possible stem-loop structures, one beginning at bp 42 and the other 128 bp away. Homology searches were conducted with BLAST programs. Both on the basis of DNA and on the basis of amino acid sequences, there was striking similarity to monooxygenases (hydroxylases) involved in the synthesis of polyketide antibiotics (7, 18, 21) or acting upon phenolic compounds (e.g., see reference 12), consistent with the presence of a naphthalenyl moiety in the rifampin molecule (14).

The original 8-kb clone and all subclones with the intact *iri* gene conferred similar 25-fold increases in rifampin resistance in rhodococcal strain Ri8, but increased resistance in *E. coli* transformants was not found except when the *Bcl*I-*Cla*I fragment was cloned into the *Bgl*II-*Sfu*I sites of shuttle vector pDA71, which resulted in a 10-fold increase in MIC in *E. coli*

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1       tcacgctctgcacaccgttggcggagtcggaggatctgagcggcggagtgccgttttctctgacgctgggtgttctcggggccatgctggtgccgacggt

101     gatcgagagttggcggctcccacgcgatacaggtgctgtggtgttggtgcgccgataatgaagcgtcttccgccaccttcatcataacgcgggacccggg

201     gcttgcggcaaggccccggtgatggtgcacactcgtctgctgcgagcaagcaaaagccttgcccgaaggaggggatcgcatgagtgacgtcatcattgtc
                                                                                    M  S  D  V  I  I  V

301     ggtgctggaccaactggattgatgctggcaggtgagctccggctacagggcgtcgatgtcgtcgtcgtggacaaggacgaggagccgactcagttcgtcc
        G  A  G  P  T  G  L  M  L  A  G  E  L  R  L  Q  G  V  D  V  V  V  V  D  K  D  E  E  P  T  Q  F  V  R

401     gtgccctcggcatccatgtgcgcagcatcgaaatcatggagcagcgcgggttgctggacaagttcctcgcgcacggccgcaagtatccgctcggtggatt
          A  L  G  I  H  V  R  S  I  E  I  M  E  Q  R  G  L  L  D  K  F  L  A  H  G  R  K  Y  P  L  G  G  F

501     cttcgcggggatcagcaaaccggcacccgcgcacctcgatactgcgcacgggtacgtcctgggcatacctcagcccgagatcgacaggattcttgccgaa
          F  A  G  I  S  K  P  A  P  A  H  L  D  T  A  H  G  Y  V  L  G  I  P  Q  P  E  I  D  R  I  L  A  E

601     catgccaccgaagtcggcgcggacattcagcgagggaagcgcgtcgtcgcgatccgtcaagataccgacaacgtcgcagcggaattgtccgacggcacaa
        H  A  T  E  V  G  A  D  I  Q  R  G  K  R  V  V  A  I  R  Q  D  T  D  N  V  A  A  E  L  S  D  G  T  T

701     cacttcacgcgcggtaccttgtaggctgcgacggcggccgcagcactgttcggaagctgagatcgacgtcggtattcccggcgagccgtacgagcgccga
          L  H  A  R  Y  L  V  G  C  D  G  G  R  S  T  V  R  K  L  R  S  T  S  V  F  P  A  S  R  T  S  A  D

801     cacgttgatcggcgaaatggacgtgaccatgcctgctgatgaactggccgccgttgtcgccgaaatccgggaaacgcacaaacgattcggagtcggtccc
        T  L  I  G  E  M  D  V  T  M  P  A  D  E  L  A  A  V  V  A  E  I  R  E  T  H  K  R  F  G  V  G  P

901     gccggcaacggtgcttttcgtgtcgtggtccctgcggccgaagttgccgacggtcgcgcaacaccgaccaccctcgacgacatcaaacaacagctactgg
        A  G  N  G  A  F  R  V  V  V  P  A  A  E  V  A  D  G  R  A  T  P  T  T  L  D  D  I  K  Q  Q  L  L  A

1001    ccattgccggtaccgacttcggtgtgcactcgccgcggtggctctcgcgcttcggcgacgccactcgtctggcggacgactaccggcgcgaccgggtgtt
          I  A  G  T  D  F  G  V  H  S  P  R  W  L  S  R  F  G  D  A  T  R  L  A  D  D  Y  R  R  D  R  V  F

1101    tctcgccggcgacgccgcacacatccacccaccgcgatgggcggtcaaggtctcaatctcggtgtgcaggacgccttcaacctcggctggaagctcgccgcc
          L  A  G  D  A  A  H  I  H  P  P  M  G  G  Q  G  L  N  L  G  V  Q  D  A  F  N  L  G  W  K  L  A  A

1201    gagatcaacggctgggcaccggtgggcctgctcgacacgtacgaatcggaacggcgtccggtggctgccgacgtgctggacaacacgcgcgcccaggccg
        E  I  N  G  W  A  P  V  G  L  L  D  T  Y  E  S  E  R  R  P  V  A  A  D  V  L  D  N  T  R  A  Q  A  E

1301    agttgatctccaccgctgccggaccacaagcggtgcggcgcttgatctccgagctgatggaattcgaagacgtcaagcgctatttgaccgagaagatcac
          L  I  S  T  A  A  G  P  Q  A  V  R  R  L  I  S  E  L  M  E  F  E  D  V  K  R  Y  L  T  E  K  I  T

1401    tgcgatctcgattcgctacgatttcggcgaaggcgacgacctactcggtcggaggctgcggaacatcgcgttgacgcgcggcaacctgtacgacctgatg
          A  I  S  I  R  Y  D  F  G  E  G  D  D  L  L  G  R  R  L  R  N  I  A  L  T  R  G  N  L  Y  D  L  M

1501    cgatccggccgcggacttcttctcgaccagggtggccaactgtccgtcgatggttggagcgatcgcgccgaccatatcgttgacacaagcactgaattgg
        R  S  G  R  G  L  L  L  D  Q  G  G  Q  L  S  V  D  G  W  S  D  R  A  D  H  I  V  D  T  S  T  E  L  E

1601    aagctccggctgtcctgcttcggccggacggtcatgtggcatggatcggggatgcgcaggcggagttggatactcagctgtccacatggttcggccggtc
          A  P  A  V  L  L  R  P  D  G  H  V  A  W  I  G  D  A  Q  A  E  L  D  T  Q  L  S  T  W  F  G  R  S

1701    ggcgagggaccgcgcgtgacgcttcgatttcggtaaccgagcaccgtgacacctcgggtgcactaccgttgccgtttatgggacaaggattggggcagat
          A  R  D  R  A  *

1801    cgcgcccgcggccttgatcgccgtcggagtgctgatactcacgggctgcacgaccaatgtcgatgtgccgaagacatttgctgccacattgacggtgacc

1901    ggtcaggcagagcaagtcgaggccggctctggagagtgcgtgatcgactcgatccgtgtcgctccgaacgaccagattcaaatttttcgggggcagtggcg

2001    ctgcgtccgtcaggtcgacactcgaggtcgaggcaatcga
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FIG. 2. Sequence of the *iri* gene and flanking regions. The putative Shine-Dalgarno sequence is double underlined, and the −35 and −10 sequences are underlined. The predicted amino acid sequence is shown in capital letters.

(Fig. 3b). This effect, presumably due to transcription from the λ P_R upstream of the *Bgl*II site in this plasmid, was observed at temperatures of <30°C but not above. Since there are no reports of this λ promoter not transcribing at 30°C or above, it may be that the *iri* gene product is less thermostable in *E. coli*. In contrast, the increase in antibiotic MIC in *Rhodococcus* did not vary significantly over the range of temperatures at which this bacterium grew, up to its maximum of 37°C (Fig. 3a). In either a gram-positive or gram-negative background, introduction of the *iri* gene did not reduce susceptibility to any of a range of other antibiotics; tetracycline, oxytetracycline, erythromycin, chloramphenicol, nalidixic acid, kanamycin, streptomycin, and paromomycin were tested.

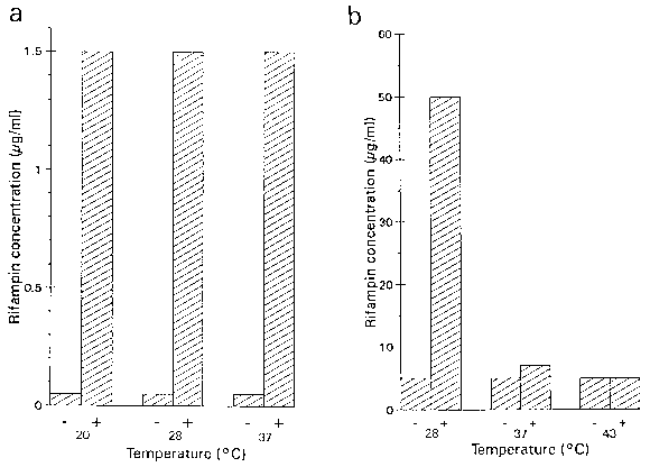

FIG. 3. Effect of temperature on MIC of rifampin in *Rhodococcus* strain Ri8 (a) and *E. coli* MM294 (b) transformed with either vector pDA71 only (−) or vector pDA71 carrying the *Bcl*I-*Cla*I fragment (+).

We conclude that the inactivation gene cloned from the *R. equi* type strain, ATCC 14887, can confer a 10-fold increase in resistance to rifampin in *E. coli* as well as a 25-fold increase in *Rhodococcus*. The sequence similarity to monooxygenases acting upon phenolic compounds suggests that this enzyme has as its target the naphthalenyl moiety of rifampin, so alteration of this portion of the molecule might increase the efficacy of rifamycin derivatives against organisms possessing such an inactivation mechanism if it plays a role in the bacterial response to challenge by these antibiotics. In addition, identification of the *iri* gene will facilitate investigation of whether it can confer increased rifampin resistance by mutational alteration.

Nucleotide sequence accession number. The sequence presented in Fig. 2 has been assigned GenBank accession number U56415.

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