ANTIMICROBIAL AGENTS AND CHEMOTHERAPY, May 1997, p. 1162–1165 Vol. 41, No. 5
0066-4804/97/$04.00+0

Clinical Resistance to Erythromycin and Clindamycin in Cutaneous Propionibacteria Isolated from Acne Patients Is Associated with Mutations in 23S rRNA

JEREMY I. ROSS,[1]* E. ANNE EADY,[1] JONATHAN H. COVE,[1] CHRISTOPHER E. JONES,[1] A. HAMID RATYAL,[1] YVONNE W. MILLER,[1] SANDHYA VYAKRNAM,[1] AND WILLIAM J. CUNLIFFE[2]

The Skin Research Centre, Department of Microbiology, University of Leeds, Leeds LS2 9JT,[1] and Department of Dermatology, Leeds General Infirmary, Leeds LS1 3EX,[2] United Kingdom

Received 6 December 1996/Returned for modification 27 January 1997/Accepted 9 March 1997

The genetic basis of erythromycin resistance in cutaneous propionibacteria was determined by comparing the nucleotide sequences of the peptidyl transferase region in the 23S rRNAs from 9 susceptible and 26 resistant clinical isolates as well as 4 laboratory-selected erythromycin-resistant mutants of a susceptible strain. In 13 isolates and the 4 laboratory mutants, cross-resistance to macrolides, lincosamides, and B-type streptogramins was associated with an A→G transition at a position cognate with *Escherichia coli* 23S rRNA base 2058. These strains were resistant to ≥512 μg of erythromycin per ml. Two other mutations were identified, an A→G transition at base 2059 in seven strains, associated with high-level resistance to all macrolides, and a G→A transition at base 2057 in six strains, associated with low-level resistance to erythromycin. These mutations correspond to three of four phenotypic classes previously identified by using MIC determinations.

Acne vulgaris is a multifactorial skin disease characterized by the presence of a variety of inflamed and noninflamed lesions on the face and upper trunk (6). It occurs mainly in adolescence, but persistent or late-onset acne is increasing. The skin bacterium *Propionibacterium acnes* has been implicated in the pathogenesis of inflamed lesions, and agents, including some antibiotics, which reduce the numbers of this organism in vivo are therapeutic (6). Individual courses of antibiotic therapy last from a minimum of 8 weeks for some topical formulations to several years. Despite this long-term use of a limited number of antibiotics, concern about the development of resistance in *P. acnes* has not led to the adoption of usage policies. Combined resistance to erythromycin and clindamycin in cutaneous propionibacteria was first reported in 1979 in the United States in 20% of acne patients using topical formulations of either drug (2). Since then, the isolation of erythromycin-resistant organisms from acne patients following antibiotic treatment has been documented in several countries (5, 10, 14, 15).

The most common form of acquired resistance to erythromycin (a 14-membered-ring macrolide) in bacteria involves the mono- or dimethylation of *Escherichia coli*-equivalent base A-2058 in the 23S rRNA by methyl transferases encoded by *erm* genes (for a review, see reference 26). Methylation of this base confers complete cross-resistance to macrolides (14- to 16-membered rings), lincosamides, and type B streptogramins (MLS resistance). Methyl transferase genes are widely disseminated in both gram-positive and gram-negative bacteria and can be located on plasmids or transposons. Other mechanisms of resistance to erythromycin include enzymatic inactivation (1) and active efflux (21). More recently, transition mutations in the peptidyl transferase region of the 23S rRNAs of clinical isolates of various species of mycobacteria and *Helicobacter pylori* have been associated with macrolide resistance (17, 18, 23, 24).

We previously classified 77 of 89 isolates of erythromycin-resistant propionibacteria collected from antibiotic-treated acne patients in Leeds between 1982 and 1988 into four phenotypic classes based on their patterns of cross-resistance to eight MLS antibiotics. The patterns of the classes were as follows: class I, constitutive MLS-type resistance (48 strains); class II, inducible MLS resistance (2 strains); class III, low-level resistance to erythromycin, with susceptibility to 16-membered-ring macrolides (15 strains); and class IV, high-level resistance to 14- and 16-membered-ring macrolides combined with low-level lincosamide resistance (12 strains). Details of MICs for resistant and susceptible isolates have been described previously (7). Subsequently, attempts have been made to elucidate the mechanisms of resistance operating in these strains. Southern blotting with *Propionibacterium* DNA, using probes of *ermA* and *ermC* from staphylococci as well as *ermB* (*Enterococcus faecalis*), *ermD* (*Bacillus licheniformis*), *ermE* (*Saccharopolyspora erythraea*), and *ermF* (*Bacteroides fragilis*), gave negative results (22). Attempts to clone, in *E. coli* and *Streptomyces lividans*, a resistance determinant from a *P. acnes* strain demonstrating class I-type resistance with plasmid vectors were unsuccessful (22). Laboratory-selected spontaneous erythromycin-resistant mutants of the fully susceptible strain *P. acnes* P37 displayed constitutive MLS resistance (data not shown). This evidence, and reports of clinical resistance to the macrolide clarithromycin by mutation in *H. pylori* and *Mycobacterium intracellulare* (17, 24), motivated us to examine the peptidyl transferase region of domain V of 23S rRNA genes.

Twenty-four resistant strains from the original survey plus nine strains isolated from antibiotic-treated acne patients during 1996 were tested (Table 1). The recent isolates were classified by the criteria previously described (7). Also tested were eight susceptible strains, including the type strains *P. acnes* NCTC 737, *Propionibacterium granulosum* NCTC 11865, and

* Corresponding author. Mailing address: Department of Microbiology, University of Leeds, Leeds LS2 9JT, United Kingdom. Phone: 0113 233 5631. Fax: 0113 233 5638. E-mail: micjir@leeds.ac.uk.

TABLE 1. Details of isolates investigated

Phenotypic class	Number of isolates of:		
	P. acnes	*P. granulosum*	*P. avidum*
I[a]	11	1	1
II[a]	0	2	0
III[a]	6	0	0
IV[a]	5	1	1
Miscellaneous[a]	4	1	0
Laboratory mutants[b]	4	0	0
Erythromycin sensitive[c]	7	1	1

[a] Phenotypic group as defined by Eady et al. (7).
[b] Laboratory-selected spontaneous mutants of strain P37.
[c] Including the type strains of each species as obtained from the National Collection of Type Cultures.

Propionibacterium avidum NCTC 11864. In addition, four spontaneous erythromycin-resistant mutants of *P. acnes* P37, selected on a medium containing 5 μg of erythromycin per ml, and the P37 wild type were also tested. Propionibacteria were isolated and maintained on 2% tryptone–1% yeast extract agar containing 0.5% glucose (TYEg) under anaerobic conditions at 34°C and identified as previously described (7). MICs were determined on TYEg by agar dilution (7). Actively growing propionibacteria were exposed to 20 μg of penicillin G per ml for 4 h to weaken their cell walls, and concentrated cells (10^{10} CFU/ml) were lysed with 10% sodium dodecyl sulfate. Following overnight incubation with proteinase K (100 μg/ml) at 55°C, total genomic DNA was extracted by two rounds of phenol-chloroform extraction and ethanol precipitation. Amplification of a 1.4-kb section of the DNA encoding the 23S rRNA, including the conserved domain V, was accomplished with the primers 5′-AGTCGGGACCTAAGGCGAG-3′ (corresponding to *E. coli* 23S rRNA bases 1342 to 1360) and (5′-TTCCCGCTTAGATGCTTTCAG-3′ (corresponding to bases 2765 to 2745) as described by Meier et al. (17). PCR amplicons were purified by using the Wizard PCR Preps DNA purification system (Promega) according to the manufacturer's instructions. Amplicons were sequenced directly as described by Goldsborough and Béranger (11), using the internal 23S rRNA primer 5′-GTAGCGAAATTCCTTGTCGG-3′ (*E. coli* bases 1930 to 1949). The 150-bp regions (*E. coli*-equivalent bases 1986 to 2136) within domain V of the 23S rRNAs of all the isolates were compared. To determine the number of copies of the rRNA operon present in propionibacteria, we performed Southern hybridization on *Bam*HI (see Fig. 1) or *Pst*I-digested total DNA with PCR-generated 23S (17) and 16S (12) rRNA fragments as probes (^{32}P labelled by using an oligonucleotide labelling kit from Pharmacia).

Three separate mutations were identified in erythromycin-resistant strains, at *E. coli*-equivalent bases 2057, 2058, and 2059. Thirteen strains displaying class I resistance were investigated, and all were found to have a single A→G transition at base 2058. This same mutation was detected in four laboratory-derived erythromycin-resistant mutants of *P. acnes* P37. The six strains displaying class III resistance each possessed a G→A transition at base 2057. In contrast, the seven class IV strains each contained an A→G transition at base 2059 (Table 2). Two inducible MLS-resistant (class II) strains, four strains with unusual cross-resistance phenotypes, and all nine susceptible strains contained wild-type 23S rRNA in the region examined. No strains showed any evidence of heterozygosity in the 23S rRNA gene sequence.

The results showed an association between mutations in the peptidyl transferase region of the 50S ribosomal subunit and resistance to MLS antibiotics in cutaneous propionibacteria. The mutations at *E. coli*-equivalent bases 2057, 2058, and 2059 showed an absolute correlation with phenotypic classes III, I, and IV, respectively. These classes can be simply differentiated on the basis of resistance to erythromycin and josamycin (a 16-membered-ring macrolide) as shown in Table 2. *E. coli*-equivalent base 2058 is the target of ribosomal methyl transferases which confer cross-resistance to all MLS antibiotics (26). Changes at this location, specifically an A→G transition, have been linked with resistance to erythromycin in laboratory mutants (4, 16) and in clinical isolates of mycobacteria (17, 18, 25) and *H. pylori* (24). Mutations at base 2057 in propionibacteria were associated with low-level resistance to erythromycin (Table 2). This is in agreement with findings that a G→A transition at base 2057 in the cloned *rrnH* operon of *E. coli* conferred only a modest degree of resistance to erythromycin (9). In *E. coli*, mutations at base 2057 confer cross-resistance to chloramphenicol. No increase in resistance to chloramphenicol could be detected in any of the class III propionibacterium mutants (chloramphenicol MIC, ≤2 μg/ml). This mutation has not previously been reported to be associated with erythromycin resistance in clinical bacterial isolates, probably because of the low-level resistance associated with it. The mutation of A to G at base 2059 (A2059G) is the same as that associated with clarithromycin resistance in clinical isolates of *H. pylori* and the *Mycobacterium chelonae* group (24, 25) as well as with erythromycin resistance in laboratory mutants of *Mycoplasma pneumoniae* (16). Substitution of C, G, or T for the A at position 2059 has been associated with clarithromycin and azithromycin resistance in clinical isolates of *Mycobacterium avium* (18). In propionibacteria, the A2059G mutation is associated with high-level cross-resistance to 14- and 16-membered ring macrolides. This change results in only very moderate increases in the MICs of lincosamides and no resistance to type B streptogramins. The cross-resistance phenotypes associated with mutations in the peptidyl transferase region of 23S rRNAs from mycobacteria and *H. pylori* have not been reported.

It has been hypothesized that changes which disrupt the base pairing of A-2058 and U-2016 alter the binding site of MLS antibiotics, resulting in high-level erythromycin resistance (23), whereas the weaker rearrangement caused by disruption of the G-2057–C-2611 base pairing affects the binding sites of fewer antibiotics and leads to lower levels of erythromycin resistance (23). Our results are in agreement with this hypothesis.

In vitro-generated mutations in *E. coli*-equivalent base C-2611 (23), deletions within domain II which lead to the production of a pentapeptide encoded by nucleotides 1235 to 1268 (3), and mutations in a number of ribosomal proteins (26) have all been associated with erythromycin resistance. It is

TABLE 2. Sequence alignments of part of the peptidyl transferase region of domain V of 23S rRNA alleles

Propionibacterium 23S rRNA alleles[a]	Phenotypic class	Mutation	MIC (μg/ml)[b]	
			Er	Jm
AGACGGAAAGACCCCGGGA	S	None (wild type)	≤0.06	≤0.06
AGACGG**G**AAGACCCCGGGA	I	A2058G	≥512	1–16
AGACGGAAAGACCCCGGGA	II	None (wild type)	1[c]	1–2[c]
AGACG**A**AAAGACCCCGGGA	III	G2057A	1–2	≤0.25
AGACGGA**G**AGACCCCGGGA	IV	A2059G	≥256	≥256

[a] The five sequences indicate the alleles found in clinical isolates of propionibacteria. Boldfaced, underlined nucleotides indicate the sites of mutations.
[b] Er, erythromycin; Jm, josamycin.
[c] Uninduced MIC; when induced, MIC ≥512 μg/ml.

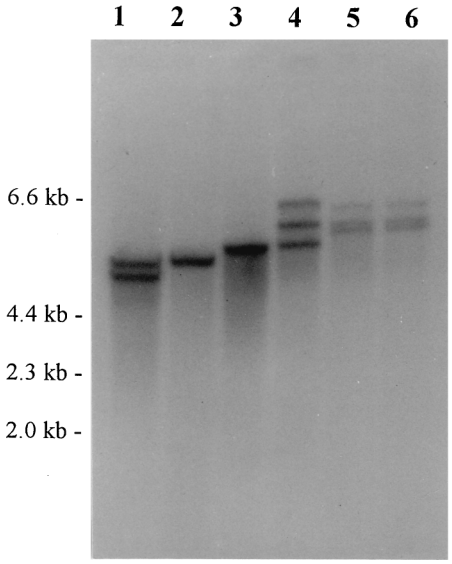

FIG. 1. Southern blot analysis to determine the number of rRNA operons. *Propionibacterium* genomic DNA was restricted to completion with *Bam*HI and hybridized with a 23S rRNA probe under stringent conditions. Lanes: 1, *P. granulosum* NCTC 11865 (susceptible); 2, *P. avidum* NCTC 11864 (susceptible); 3, *P. avidum* PB108 (resistant); 4, *P. acnes* P37 (susceptible); 5, *P. acnes* NCTC 737 (susceptible); 6, *P. acnes* P9 (resistant). The DNA in lanes 5 and 6 was diluted fourfold to allow better resolution of the lower bands.

possible that the four *Propionibacterium* isolates with unusual cross-resistance phenotypes have alterations in one of these regions; alternatively, they may have acquired unusual resistance genes from external sources. The two class II inducible strains have probably acquired *erm* genes from an as-yet-unidentified donor.

To date, mutation-associated clinical resistance to macrolides has been described in mycobacterial species with one copy of the rRNA operon per chromosome (17, 18, 25) and in *H. pylori*, which contains two copies. In the latter case, evidence of heterozygosity in the 23S rRNA gene was detected in 1 of 12 resistant strains (24). The number of copies of the rRNA operon differs among the different *Propionibacterium* species. *P. avidum* contains only a single copy, *P. granulosum* contains two copies, and *P. acnes* has three copies (Fig. 1). As no evidence of heterozygosity in the 23S rRNA gene sequence was detected in any *P. acnes* or *P. granulosum* strain, it is possible that the mutations are recessive in the presence of wild-type copies of the 23S rRNA operon. Strains carrying a mixture of mutant and wild-type ribosomes would be classified phenotypically as susceptible. The only example of a single mutated copy among multiple rRNA operons giving rise to erythromycin resistance is in *Streptomyces ambofaciens*, the spiramycin producer (20); one of four rRNA operons carrying an A2058G mutation confers an apparent 40-fold increase in the erythromycin MIC. However, the concomitant presence of four macrolide resistance determinants may be a complicating factor. It is difficult to envisage how triple mutations in the same base could arise by chance even with selection, especially as mutations in three different bases all confer erythromycin resistance. Gene conversion (13, 19) is a possible mechanism by which bacteria may replace a wild-type allele with a mutant allele by using the mutant allele as a template. This may explain the presence of an identical mutation in each of three rRNA copies in *P. acnes*.

It is likely that prolonged exposure to antibiotics is required for the mutant strains to arise and colonize the patient. In our experience, resistant propionibacteria begin to appear on the skin of antibiotic-treated acne patients 12 to 24 weeks after the start of therapy (8). Studies are required to compare the fitness, in vivo and in vitro, of wild-type strains and mutant derivatives. In the absence of selective pressure, it is possible that mutants may remain indefinitely as part of the resident cutaneous flora.

We thank Dermik Laboratories Inc. and the Leeds Foundation for Dermatological Research for their generous support.

REFERENCES

1. **Arthur, M., A. Andremont, and P. Courvalin.** 1987. Distribution of erythromycin esterase and rRNA methylase genes in members of the family *Enterobacteriaceae* highly resistant to erythromycin. Antimicrob. Agents Chemother. **31:**404–409.
2. **Crawford, W. W., I. P. Crawford, R. B. Stoughton, and R. C. Cornell.** 1979. Laboratory induction and clinical occurrence of combined clindamycin and erythromycin resistance in *Corynebacterium acnes*. J. Invest. Dermatol. **72:** 187–190.
3. **Dam, M., S. Douthwaite, T. Tenson, and A. S. Mankin.** 1996. Mutations in domain II of 23S rRNA facilitate translation of a 23S rRNA-encoded pentapeptide conferring erythromycin resistance. J. Mol. Biol. **259:**1–6.
4. **Douthwaite, S., and C. Aagaard.** 1993. Erythromycin binding is reduced in ribosomes with conformational alterations in the 23S rRNA peptidyl transferase loop. J. Mol. Biol. **232:**725–731.
5. **Eady, E. A., J. H. Cove, K. T. Holland, and W. J. Cunliffe.** 1989. Erythromycin resistant propionibacteria in antibiotic treated acne patients: association with therapeutic failure. Br. J. Dermatol. **121:**51–57.
6. **Eady, E. A., and E. Ingham.** 1994. *Propionibacterium acnes*—friend or foe? Rev. Med. Microbiol. **5:**163–173.
7. **Eady, E. A., J. I. Ross, J. H. Cove, K. T. Holland, and W. J. Cunliffe.** 1989. Macrolide-lincosamide-streptogramin B (MLS) resistance in cutaneous propionibacteria: definition of phenotypes. J. Antimicrob. Chemother. **23:**493–502.
8. **Eady, E. A., J. I. Ross, J. H. Cove, K. T. Holland, and W. J. Cunliffe.** 1989. The effects of oral erythromycin therapy for acne on the development of resistance in cutaneous staphylococci and propionibacteria, p. 265–270. *In* R. Marks and G. Plewig (ed.), Acne and related disorders. Martin Dunitz, London, United Kingdom.
9. **Ettayebi, M., S. M. Prasad, and E. A. Morgan.** 1985. Chloramphenicol-erythromycin resistance mutations in a 23S rRNA gene of *Escherichia coli*. J. Bacteriol. **162:**551–557.
10. **Forssman, T.** 1993. Antibiotikaresistenzen beachten. Therapiewoche **43:** 2248–2252.
11. **Goldsborough, A. S., and F. Béranger.** 1995. Ligation- and PCR-based method for sequencing plasmid inserts. BioTechniques **19:**910–912.
12. **Greisen, K., M. Loeffelholz, A. Purohit, and D. Leong.** 1994. PCR primers and probes for the 16S rRNA gene of most species of pathogenic bacteria, including bacteria found in cerebrospinal fluid. J. Clin. Microbiol. **32:**335–351.
13. **Kobayashi, I.** 1992. Mechanisms for gene conversion and homologous recombination: the double-strand break repair model and the successive half crossing over model. Adv. Biophys. **28:**81–133.
14. **Kurokawa, I., S. Nishijima, and Y. Asada.** 1988. The antibiotic sensitivity of *Propionibacterium acnes*: a 15-year bacteriological study and retrospective evaluation. J. Dermatol. **15:**149–154.
15. **Leyden, J. J., K. J. McGinley, S. Cavalieri, G. F. Webster, O. H. Mills, and A. M. Kligman.** 1983. *Propionibacterium acnes* resistance to antibiotics in acne patients. J. Am. Acad. Dermatol. **8:**41–45.
16. **Lucier, T. S., K. Heitzman, S.-K. Liu, and P.-C. Hu.** 1995. Transition mutations in the 23S rRNA of erythromycin-resistant isolates of *Mycoplasma pneumoniae*. Antimicrob. Agents Chemother. **39:**2770–2773.
17. **Meier, A., P. Kirschner, B. Springer, V. A. Steingrube, B. A. Brown, R. J. Wallace, Jr., and E. C. Böttger.** 1994. Identification of mutations in 23S rRNA gene of clarithromycin-resistant *Mycobacterium intracellulare*. Antimicrob. Agents Chemother. **38:**381–384.
18. **Nash, K. A., and C. B. Inderlied.** 1995. Genetic basis of macrolide resistance in *Mycobacterium avium* isolated from patients with disseminated disease. Antimicrob. Agents Chemother. **39:**2625–2630.
19. **Ninio, J.** 1996. Gene conversion as a focusing mechanism for correlated mutations: a hypothesis. Mol. Gen. Genet. **251:**503–508.
20. **Pernodet, J. L., F. Boccard, M.-T. Alegre, M.-H. Blondelet-Rouault, and M. Guérineau.** 1988. Resistance to macrolides, lincosamides and streptogramin type B antibiotics due to a mutation in an rRNA operon of *Streptomyces ambofaciens*. EMBO J. **7:**277–282.
21. **Sutcliffe, J., T. Grebe, A. Tait-Kamradt, and L. Wondrack.** 1996. Detection of erythromycin-resistant determinants by PCR. Antimicrob. Agents Chemother. **40:**2562–2566.

22. **Tipper, J. L.** 1994. Erythromycin resistance in cutaneous propionibacteria and staphylococci. Ph.D. thesis, University of Leeds, Leeds, United Kingdom.
23. **Vannuffel, P., M. Di Giambattista, E. A. Morgan, and C. Cocito.** 1992. Identification of a single base change in ribosomal RNA leading to erythromycin resistance. J. Biol. Chem. **267:**8377–8382.
24. **Versalovic, J., D. Shortridge, K. Kibler, M. V. Griffy, J. Beyer, R. K. Flamm, S. K. Tanaka, D. Y. Graham, and M. F. Go.** 1996. Mutations in 23S rRNA are associated with clarithromycin resistance in *Helicobacter pylori*. Antimicrob. Agents Chemother. **40:**477–480.
25. **Wallace, R. J., Jr., A. Meier, B. A. Brown, Y. Zhang, P. Sander, G. O. Onyi, and E. C. Böttger.** 1996. Genetic basis for clarithromycin resistance among isolates of *Mycobacterium chelonae* and *Mycobacterium abscessus*. Antimicrob. Agents Chemother. **40:**1676–1681.
26. **Weisblum, B.** 1995. Erythromycin resistance by ribosome modification. Antimicrob. Agents Chemother. **39:**577–585.