In vitro emergence of rifampicin resistance in Propionibacterium acnes and molecular characterization of mutations in the rpoB gene

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Objectives: Activity of rifampicin against Propionibacterium acnes biofilms was recently demonstrated, but rifampicin resistance has not yet been described in this organism. We investigated the in vitro emergence of rifampicin resistance in P. acnes and characterized its molecular background.

Methods: P. acnes ATCC 11827 was used (MIC 0.007 mg/L). The mutation rate was determined by inoculation of 10^9 cfu of P. acnes on rifampicin-containing agar plates incubated anaerobically for 7 days. Progressive emergence of resistance was studied by serial exposure to increasing concentrations of rifampicin in 72 h cycles using a low (10^6 cfu/mL) and high (10^8 cfu/mL) inoculum. The stability of resistance was determined after three subcultures of rifampicin-resistant isolates on rifampicin-free agar. For resistant mutants, the whole rpoB gene was amplified, sequenced and compared with a P. acnes reference sequence (NC006085).

Results: P. acnes growth was observed on rifampicin-containing plates with mutation rates of 2 ± 1 cfu × 10^{-9} (4096×MIC) and 12 ± 5 cfu × 10^{-9} (4×MIC). High-level rifampicin resistance emerged progressively after 4 (high inoculum) and 13 (low inoculum) cycles. In rifampicin-resistant isolates, the MIC remained >32 mg/L after three subcultures. Mutations were detected in clusters I (amino acids 418–444) and II (amino acids 471–486) of the rpoB gene after sequence alignment with a Staphylococcus aureus reference sequence (CA445512). The five following substitutions were found: His-437 → Tyr, Ser-442 → Leu, Leu-444 → Ser, Ile-483 → Val and Ser-485 → Leu.

Conclusion: The rifampicin MIC increased from highly susceptible to highly resistant values. The resistance remained stable and was associated with mutations in the rpoB gene. To our knowledge, this is the first report of the emergence of rifampicin resistance in P. acnes.

Keywords: biofilm, implant-related infections, combinations

Introduction

Propionibacterium acnes is increasingly recognized as the cause of foreign-body infections, including those involving prosthetic joints, spine hardware and ventriculo-peritoneal shunts.1–3 P. acnes is highly susceptible to a wide range of antimicrobials, including clindamycin, β-lactams and quinolones.4 However, the optimal treatment regimen of P. acnes biofilm infections has not been defined. The efficiency of rifampicin for the eradication of P. acnes biofilms has been demonstrated in vitro4 and, recently, in vivo in an animal model of foreign-body infection.5 Rifampicin acts by interacting with the β-subunit of the bacterial RNA polymerase encoded by the rpoB gene.6 Alignment of the rpoB gene sequence from different species has confirmed conserved domains among the sequences.8 Resistance to rifampicin has been described in several bacterial species, such as Staphylococcus aureus,9 Escherichia coli,10 Streptococcus pyogenes11 and Mycobacterium tuberculosis.12 Resistance is generally due to point mutations in the rpoB gene leading to a reduced binding between the antibiotic and the enzyme. Mutations particularly occur in the conserved domains of cluster I (amino acids 507–533), cluster II (amino acids 563–572) and cluster III (amino acids 684–690), according to E. coli numbering.13 The aim of this study was to investigate the emergence of rifampicin resistance in P. acnes in vitro and whether combination with an additional antimicrobial agent can prevent the emergence of rifampicin resistance. In addition, the molecular background of rifampicin resistance in P. acnes isolates was characterized.
Materials and methods

Study organism

All experiments were performed with P. acnes strain ATCC 11827. The MICs of rifampicin, clindamycin, penicillin G, daptomycin and levofloxacin were 0.007, 0.125, 0.03, 1 and 1 μg/mL, respectively.5 Bacteria were stored at −70°C by using the cryovial bead preservation system (Microbank; Pro-Lab Diagnostics, Richmond Hill, Ontario, Canada). For inoculum preparation, one bead was spread on a blood agar plate and incubated for 72 h. One distinct colony was resuspended in 10 mL of reduced brain heart infusion (BHI) and incubated at 37°C for another 72 h. All incubations were performed in anaerobic conditions using an AnaeroGen system (Oxoid, Basingstoke, Hampshire, UK) at 37°C.

Antimicrobial agents

Rifampicin powder (prepared in sterile water, 60 mg/mL; Sandoz AG, Steinhausen, Switzerland), clindamycin powder (prepared in sterile water 200 mg/mL; Sigma), levofloxacin solution (5 mg/mL; Sanofi Aventis Pharma AG, Zurich, Switzerland) and penicillin G (25 mg/mL; Grünenthal Pharma AG, Mitlödi, Switzerland) were purchased from the respective manufacturers. Daptomycin powder was supplied by Novartis Pharma AG (Bern, Switzerland). A stock solution of 50 mg/mL was prepared in sterile 0.9% saline.

Rifampicin resistance studies

For the analysis of spontaneous rifampicin resistance, agar-based single-step mutation studies were performed, as previously described.14 Brucella agar supplemented with vitamin K, haemin and horse blood prepared according to CLSI M11-A7 guidelines was used.15 Briefly, a large inoculum (10⁷ cfu) of the bacteria was spread on Brucella agar plates containing rifampicin concentrations of 4x and 4096x MIC, corresponding to 0.03 and 32 mg/L, respectively. The lower concentration corresponded to an increased MIC that is still in the susceptible range (0.03 mg/L) and the higher concentration corresponded to full resistance (32 mg/L). The plates were incubated for 5−7 days before the colonies were enumerated. The spontaneous resistance rate was calculated from the number of colonies that grew on plates containing drug versus the number of colonies that grew on drug-free agar.

Detection of mutations in the rpoB gene in rifampicin-resistant P. acnes isolates

Total DNA from P. acnes ATCC 11827 and isogenic resistant isolates was extracted using the InstaGene Matrix method (Bio-Rad Laboratories, Hercules, CA, USA). The procedure was performed according to the manufacturer’s instructions. After centrifugation, the supernatant was used as DNA template for PCR analysis.

The whole rpoB gene, including the rifampicin-resistance-determining region in the rpoB gene of E. coli10 and S. aureus18 was amplified by PCR. Different sets of primers were designed according to the sequence alignment of four strains (GenBank accession numbers CP002815, CP002409, NC006085 and CP001977) and are presented in Table 1. Six overlapping regions of the rpoB gene from P. acnes were amplified: a 571 bp fragment from nucleotide positions +12 to +582; a 708 bp fragment from nucleotide positions +484 to +1191; a 693 bp fragment from nucleotide positions +1113 to +1805; a 636 bp fragment from nucleotide positions +1751 to +2386; a 656 bp fragment from nucleotide positions +2320 to +2975; and a 561 bp fragment from nucleotide positions +2912 to +3472, corresponding to the whole genome (P. acnes coordinates using P. acnes KPA171202; GenBank accession number NC006085).19 PCR was performed in a final volume of 50 μL containing 10 mM Tris-HCl (pH 8.3), 50 mM KCl, 2.5 mM MgCl2, 200 μM of each nucleotide, 0.5 μM of each primer and 2.5 U of Taq DNA polymerase (Phusion High-Fidelity DNA Polymerase; Finnzymes, Ilkirch, France). The PCR conditions were as follows: a 90 s first step of denaturation at 94°C, 30 cycles with 60 s of denaturation at 94°C, 60 s of hybridization at 55°C and 60 s of extension at 72°C, with a final extension step of 7 min at 72°C.

The PCR fragments were purified and sequenced using the ABI PRISM BigDye Terminator v1.1 Cycle Sequencing Ready Reaction Kit (Applied Biosystems, Courtaboeuf, France). Sequence analysis was performed on a 3130XL Genetic Analyzer DNA sequencer (Applied Biosystems, Courtaboeuf, France). The sequence of the rpoB gene was compared with that of the rpoB gene of the P. acnes reference strain (GenBank accession number NC006085) using different free software available on the

Table 1. Primers designed to amplify and sequence the rpoB gene of P. acnes

<table>
<thead>
<tr>
<th>Primer</th>
<th>Primer sequence (5’→3’)</th>
<th>Locationa</th>
</tr>
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<tbody>
<tr>
<td>PARI-1</td>
<td>CCATAGCCGTGTGCAC</td>
<td>+3472 to 3455</td>
</tr>
<tr>
<td>PARI-2</td>
<td>GGTGCTCAACGGAGCACTCTCG</td>
<td>+2975 to 2958</td>
</tr>
<tr>
<td>PARI-3</td>
<td>CCGCTTGGCTGAGCAGCAG</td>
<td>+2912 to 2930</td>
</tr>
<tr>
<td>PARI-4</td>
<td>CTTCTGGAGTGACCTTGCG</td>
<td>+2386 to 2369</td>
</tr>
<tr>
<td>PARI-5</td>
<td>GGCATCTGGTGACCTGCGT</td>
<td>+2320 to +2337</td>
</tr>
<tr>
<td>PARI-6</td>
<td>GCTGATATTCGGGAGCA</td>
<td>+1805 to +1788</td>
</tr>
<tr>
<td>PARI-7</td>
<td>CCATTCCTTCCAGCAGCACG</td>
<td>+1751 to +1770</td>
</tr>
<tr>
<td>PARI-8</td>
<td>GCCCTCAATGTCTGCCTGG</td>
<td>+1191 to +1174</td>
</tr>
<tr>
<td>PARI-9</td>
<td>CCAGAACCAGTAGGTGCTACC</td>
<td>+1113 to +1132</td>
</tr>
<tr>
<td>PARI-10</td>
<td>CGACGCGAGTACCCAGTGTCAAG</td>
<td>+582 to +564</td>
</tr>
<tr>
<td>PARI-11</td>
<td>TGGTGTCGCCAGTGCTG</td>
<td>+484 to +502</td>
</tr>
<tr>
<td>PARI-12</td>
<td>GCCATCCGTCGCTAGAACAA</td>
<td>+12 to +29</td>
</tr>
</tbody>
</table>

aAccording to the P. acnes rpoB gene reference sequence.
Figure 1. Progressive emergence of rifampicin (RIF) resistance in *P. acnes* using two different inocula (a) and in combination with 0.25× MIC of daptomycin (DAP), levofloxacin (LVX), clindamycin (CLI) or penicillin G (PEN) using an inoculum of 10^6 cfu/mL (b).
Results

Resistance studies

To determine the rate of spontaneous emergence of resistance, bacteria were exposed to rifampicin concentrations equal to 4× and 4096× MIC (0.03 and 32 mg/L). The mutation rate was 12±5×10⁻⁹ and 2±1×10⁻⁹ cfu for the low and high rifampicin concentrations, respectively. After three subcultures on rifampicin-free agar plates, the MIC remained 0.03 and >32 mg/L, as determined by Etest for the P. acnes derived from plates containing 4× and 4096× MIC of rifampicin, respectively.

Figure 1(a) demonstrates the results of rifampicin resistance selection during continuous exposure of bacteria to 2-fold increasing concentrations of rifampicin using two different inocula, 10⁸ and 10⁶ cfu/mL. An increase in the MIC was rapidly observed when using a high bacterial concentration, with an MIC of 32 mg/L being observed after four cycles. With a lower inoculum, the MIC increased gradually, with full resistance (MIC 32 mg/L) being observed after 13 cycles.

The addition of a secondary agent did not prevent resistance development when using a high inoculum (data not shown). Figure 1(b) shows the selection of rifampicin resistance, alone and in combination with 0.25× MIC of clindamycin, penicillin G, levofloxacin and daptomycin, using an inoculum of 10⁶ cfu/mL. The addition of clindamycin, levofloxacin and penicillin G to the cultures prevented the selection of highly resistant strains during 10 cycles and the MIC remained <0.06 mg/L. Daptomycin delayed the increase in the MIC of rifampicin, but did not prevent the emergence of resistance. No increase in the MIC of the secondary antimicrobials used in the combination studies was observed.

Molecular characterization of the rpoB gene in rifampicin-resistant isolates

The rpoB gene of rifampicin-resistant P. acnes isolates, deriving from plates containing 0.03 mg/L (named PARif1–2, exhibiting reduced susceptibility) and 32 mg/L (named PARif3–5, expressing resistance), and from cycle 10 in the cycling experiment using a high inoculum (named PARf6, expressing resistance), was sequenced. The five different amino substitutions detected in the isolates are summarized in Table 2. Figure 2 shows the alignment of the rpoB gene sequences, including clusters I and II, of E. coli (GenBank accession number EG10894), M. tuberculosis (GenBank accession number L27989), S. aureus (GenBank accession number X64172) and P. acnes (GenBank accession number NC006085) reference strains. The amino acid substitutions found in P. acnes are indicated by arrows, and previously described mutations in other species are underlined and in bold.

Sequencing of isolates deriving from previous cycles (cycles 3–10) in the cycling experiment revealed that the amino acid substitutions in PARif6 Ser-442→Leu (cluster I) and Ile-483→Val (cluster II) had occurred after four and nine cycles of exposure, respectively.

Discussion

We describe, for the first time to our knowledge, the amino acid substitutions conferring rifampicin resistance in P. acnes. Three substitutions were detected in cluster I of the rpoB gene associated with either high- or low-level resistance. Interestingly, the position of the amino acid change His-437→Tyr found in PARif3 and PARif4 (MIC 32 mg/L) corresponded to the position of the His-481→Tyr substitution conferring high-level rifampicin resistance in S. aureus. In M. tuberculosis, mutations conferring rifampicin resistance are mainly located in an 81 bp hot-spot region of cluster 1. Sequence alignment of the rpoB gene of M. tuberculosis and P. acnes revealed that the substitutions Ser-442→Leu (PARif6) and Leu-444→Ser (PARif2), associated with high and low resistance, respectively, were located within this conserved region. Two amino acid changes were detected in cluster II: Ile-483→Val and Ser-485→Leu, of which the first was a secondary mutation in the already resistant P. acnes isolate, PARif6, and the second was detected in an isolate exhibiting only low-level resistance. These codons (483 and 485) correspond to codons previously described to confer rifampicin resistance in S. aureus (527 and 529) and E. coli (572 and 574).

During progressive exposure to rifampicin, a double-mutant was obtained and sequencing demonstrated that the first mutation (Ser-442→Leu) occurred in cluster I after four cycles of exposure, leading to a major increase in the rifampicin MIC, whereas the second mutation (Ile-483→Val) occurred in cluster II after nine cycles of exposure and did not increase the MIC further. The frequency of the Ile-483→Val mutation is presumably lower and could imply there is a fitness cost associated with the drug resistance.

We observed that rifampicin resistance emerged rapidly, if the bacterial load was high, expressing a mutation rate of 2±1×10⁻⁹. Compared with rifampicin resistance mutation rates of 10⁻⁷–10⁻⁸ in S. aureus and E. coli, the low mutation rate in P. acnes may reflect its slow growth rate. Mutation rates in high-density P. acnes biofilms have not yet been investigated. The emergence of rifampicin resistance in P. acnes biofilms needs further investigation, since this risk has important consequences in the treatment of implant-associated infections, such as prosthetic valve endocarditis, neurosurgical shunt and prosthetic joint infections.
To prevent the emergence of resistance, rifampicin is administered in combination with another antimicrobial agent. In an animal model of foreign-body infection, the emergence of rifampicin resistance in methicillin-resistant S. aureus was prevented when the drug was administered in combination with levofloxacin or daptomycin. In this study, we investigated the potential of levofloxacin and daptomycin, as well as two other antimicrobials commonly used in the treatment of P. acnes infections (clindamycin and penicillin G), for preventing the emergence of rifampicin resistance in vitro. High-level rifampicin resistance was prevented by the addition of clindamycin, levofloxacin and penicillin G when the bacterial inoculum was in the range of 10^6 cfu/mL, whereas none of the antimicrobials tested was able to prevent resistance if the bacterial concentration was elevated (10^8 cfu/mL). Daptomycin was not able to completely prevent rifampicin resistance; however, no increase in the MIC of daptomycin was observed despite continuous exposure to a subinhibitory concentration of the drug.

The impact of rifampicin resistance in P. acnes in clinical practice is unknown. Importantly, the rifampicin resistance in our experimental setting was stable when the antibiotic pressure was removed, both for low-level and high-level resistance. In future studies, testing of the stable isolates in a foreign-body infection animal model, which was adapted for P. acnes, will allow investigation of the influence of low- and high-level rifampicin resistance on the treatment outcome. In addition, the use of rifampicin combinations, especially with clindamycin, levofloxacin and penicillin G, needs to be evaluated in vivo to determine the potential of these antimicrobials for the prevention of resistance.

In conclusion, we demonstrated that rifampicin resistance in P. acnes can easily be selected in vitro and can be prevented by combination with levofloxacin, clindamycin and penicillin G. Rifampicin resistance was associated with point mutations concentrated in clusters I and II of the rpoB gene and occurred in codons conferring rifampicin resistance in other bacterial species.

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