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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$\times 10^{-9}$ (4096× MIC) and 12 ± 5 cfu$\times 10^{-9}$ (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 (CAA45512). 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 vitro*[5] and, recently, *in vivo* in an animal model of foreign-body infection.[6]

Rifampicin acts by interacting with the β-subunit of the bacterial RNA polymerase encoded by the *rpoB* gene.[7] 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 pyogenes*[11] 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 mg/L, respectively.[6] 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 (10^9 cfu) of the bacteria was spread on *Brucella* agar plates containing rifampicin concentrations of 4× and 4096× 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.

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

Primer	Primer sequence (5′ → 3′)	Location[a]
PARI-1	CCATAGCGTTGTCGGCAC	+3472 to 3455
PARI-2	GGTGTCAACGAGCATCTCG	+2975 to 2958
PARI-3	CCGGTTTGCTGCAGTACG	+2912 to 2930
PARI-4	CCTTCGGAGTGACCTTGC	+2386 to 2369
PARI-5	GGCATCGTGCGTATCGGT	+2320 to +2337
PARI-6	GCTGCATATTCGCGCCCA	+1805 to +1788
PARI-7	CCATTCCTCGAGCACGAC	+1751 to 1770
PARI-8	GGCCTCAATGTCCTGCGT	+1191 to +1174
PARI-9	CCAGAACCAGTTGCGTACC	+1113 to +1132
PARI-10	CGACGGGATCACCTTACAG	+582 to 564
PARI-11	TGGTGTCCCAGTTGGTGC	+484 to +502
PARI-12	GCGTACCGCGTCGAAGAA	+12 to +29

[a]According to the *P. acnes rpoB* gene reference sequence.

For progressive rifampicin resistance selection, a protocol adapted from Entenza *et al.*[16,17] was used. Briefly, bacteria were exposed to serial 2-fold increasing concentrations of rifampicin in BHI for a total of 10 cycles, each cycle being 72 h. A series of tubes containing 2-fold increasing concentrations of rifampicin were inoculated with either 10^6 or 10^8 cfu/mL (final concentration). Following incubation, the MIC of rifampicin was read and 1 mL (for 10^8 cfu/mL) or 0.1 mL (for 10^6 cfu/mL) samples from the tubes containing the highest antibiotic concentration, and still showing turbidity, were used to inoculate a new series of tubes containing antibiotic dilutions. The experiments were performed twice.

To investigate whether a secondary agent could prevent the emergence of rifampicin resistance, experiments were performed as described above in the presence of 0.25× MIC of daptomycin, clindamycin, levofloxacin or penicillin G, for a total of 10 cycles using an inoculum of 10^6 cfu/mL. A subinhibitory concentration of the second drug was used to allow bacterial growth. The MIC of rifampicin was read after each cycle. After the last cycle, the MICs of the combination drugs were determined by macrobroth dilution, as previously described.[6] For combination studies including daptomycin, growth media were supplemented with 50 mg/L Ca^{2+}.

The stability of all rifampicin-resistant isolates was confirmed by subculture on rifampicin-free agar three times. The MIC of rifampicin was then retested by Etest (bioMérieux SA, Marcy l'Etoile, France) using an inoculum of 1 McFarland and anaerobic incubation for 48 h. All resistant isolates were identified by biochemical tests and confirmed by matrix-assisted laser desorption/ionization time-of-flight mass spectrometry with a Bruker Daltonic mass spectrometer.

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. coli*[10] and *S. aureus*,[18] 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 $MgCl_2$, 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, Illkirch, 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
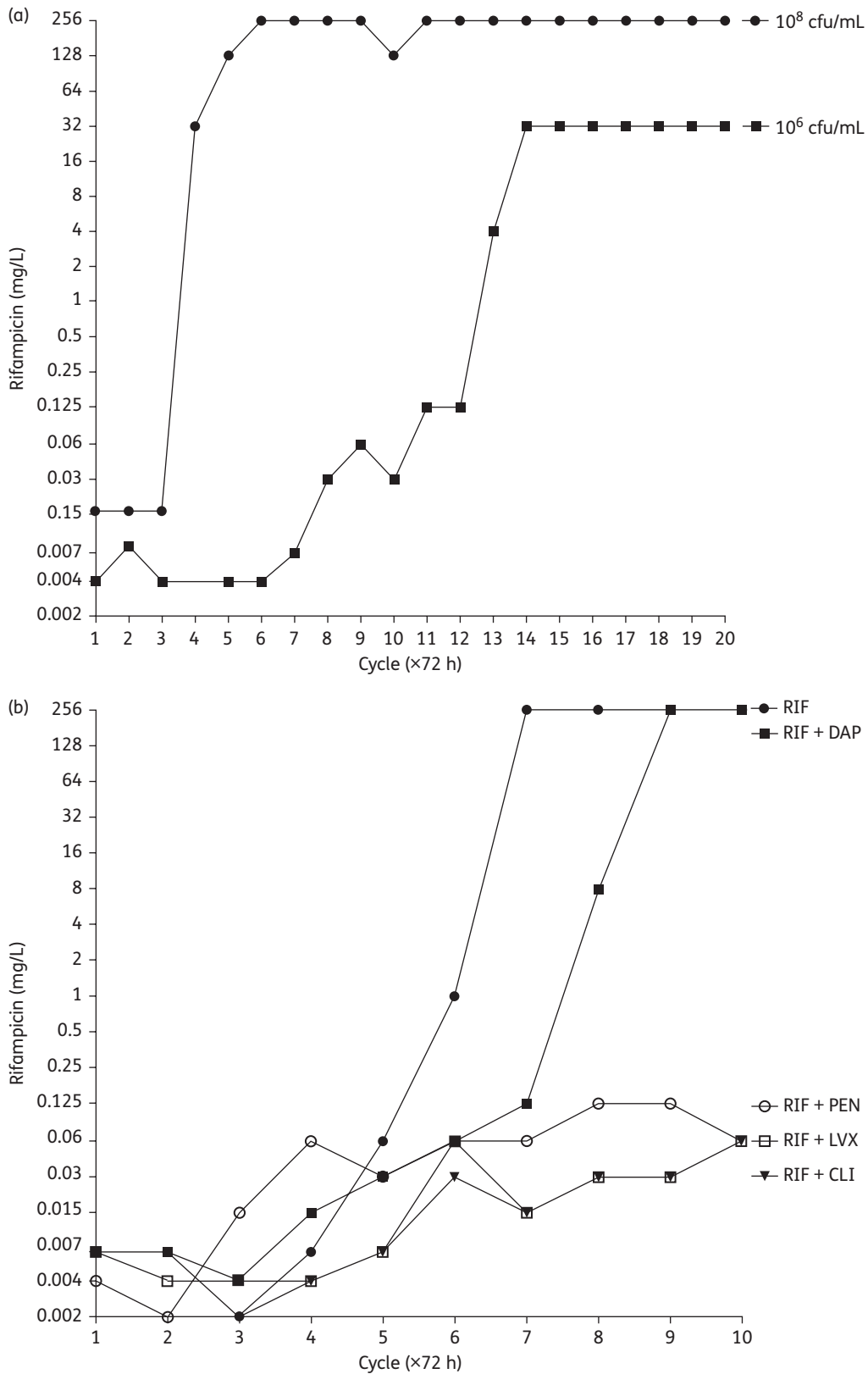
Figure 1. Progressive emergence of rifampicin (RIF) resistance in *P. acnes* using two different inocula (a) and in combination with 0.25× MIC of daptomycin (DAP), levofloxacin (LVX), clindamycin (CLI) or penicillin G (PEN) using an inoculum of 10^6 cfu/mL (b).

Internet (http://blast.ncbi.nlm.nih.gov/Blast.cgi, http://www.genome.jp/tools/clustalw/ and http://web.expasy.org/translate/).

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 $12 \pm 5 \times 10^{-9}$ and $2 \pm 1 \times 10^{-9}$ 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, 10^8 and 10^6 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 10^6 cfu/mL. The addition of clindamycin, levofloxacin and penicillin G to the cultures prevented the selection of highly resistant isolates 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 MICs 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 PARif6, 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),[20] *M. tuberculosis* (GenBank accession number L27989),[21] *S. aureus* (GenBank accession number X64172)[22,23] and *P. acnes* (GenBank accession number NC006085)[19] 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.

Table 2. Amino acid substitutions found in rifampicin-resistant *P. acnes* isolates

Isolate	Rifampicin MIC (mg/L)[a]	Amino acid substitution	GenBank accession number
PARif1	0.03	Ser-485 → Leu	JX501524
PARif2	0.03	Leu-444 → Ser	JX501525
PARif3	>32	His-437 → Tyr	JX501526
PARif4	>32	His-437 → Tyr	JX501526
PARif5	>32	Ser-442 → Leu	JX501527
PARif6	>256	Ser-442 → Leu	JX501528
		Ile-483 → Val	

[a]The MIC was determined by Etest, except for PARif6, where microbroth dilution was used.

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*.[9,18] In *M. tuberculosis*, mutations conferring rifampicin resistance are mainly located in an 81 bp hot-spot region of cluster I.[12] 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)[18] and *E. coli* (572 and 574).[10]

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.[24]

We observed that rifampicin resistance emerged rapidly, if the bacterial load was high, expressing a mutation rate of $2 \pm 1 \times 10^{-9}$. Compared with rifampicin resistance mutation rates of 10^{-7}–10^{-8} in *S. aureus* and *E. coli*,[9,25] 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.

		Cluster I		
E. coli	507	**G** **S** **S** **Q** **L** **S** **Q** F M **D** Q **N** **N** P **L** **S** E I T **H** K **R** **R** I **S** **A** **L**	533
M. tuberculosis	432	***** T ***** ***** ***** ***** ***** ***** ***** ***** * * ***** * ***** ***** G L * ***** * * * L ***** * *****	458
S. aureus	462	* * ***** * * * ***** * * ***** * A * * * **A** * L * ***** * * * ***** L * * *	488
		(↓ at three positions)	
P. acnes	418	* T * * * * * * * * * * * * * A * M * ***** * * * L ***** * *****	444
		substitutions: Y … L … S	
		Cluster II		
E. coli	560	P I E **T** **P** E G P **N** I G L **I** **N** **S** L	575
M. tuberculosis	485	* * * * * * * * * * * * G * *	500
S. aureus	515	* * * * * * * * * * * ***** * ***** *	530
		(↓ at two positions)	
P. acnes	471	* * * * * * * * * * * * ***** G ***** *	486
		substitutions: V … L	

Figure 2. Amino acid sequence comparison of clusters I and II of the *rpoB* gene of *E. coli*, *M. tuberculosis*, *S. aureus* and *P. acnes*. Identical amino acids are indicated by an asterisk. Downward-pointing arrows indicate amino acid substitutions detected in rifampicin-resistant *P. acnes* isolates. Positions involved in rifampicin resistance in *E. coli*, *M. tuberculosis* and *S. aureus* are underlined and in bold.

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.[26] 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*,[6] 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.

Transparency declaration
None to declare.

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