**Propionibacterium acnes** is developing gradual increase in resistance to oral tetracyclines

Keisuke Nakase,¹ Hidemasa Nakaminami,¹ Yuko Takenaka,² Nobukazu Hayashi,³ Makoto Kawashima² and Norihisa Noguchi¹,*

**Abstract**

*Propionibacterium acnes* is an anaerobic bacterium that causes deep infection in organs and prosthetic joints, in addition to acne vulgaris. Many tetracycline-resistant *P. acnes* strains have been isolated because oral tetracyclines are frequently used as an acne treatment against *P. acnes*. In this study, we found a novel tetracycline resistance mechanism in *P. acnes*. Three doxycycline-resistant (MIC: 16 µg ml⁻¹) strains were isolated from 69 strains in acne patients in Japan between 2010 and 2011. Additionally, six susceptible strains (MIC: 1–2 µg ml⁻¹) that had reduced susceptibility compared to susceptible strains (MIC: ≤0.5 µg ml⁻¹) were identified. All doxycycline-resistant strains had a G1036C mutation in the 16S rRNA gene in addition to an amino acid substitution in the ribosomal S10 protein encoded by rpsJ. By contrast, susceptible strains had an amino acid substitution in the S10 protein but no mutation in the 16S rRNA. When the mutant with decreased susceptibility to doxycycline was obtained in vitro, only the mutated S10 protein was found (MIC: 4 µg ml⁻¹), not the mutated 16S rRNA gene. This result shows that the S10 protein amino acid substitution contributes to reduced doxycycline susceptibility in *P. acnes* and suggests that tetracycline resistance is acquired through a 16S rRNA mutation after the S10 protein amino acid substitution causes reduced susceptibility.

**INTRODUCTION**

*Propionibacterium acnes*, a Gram-positive, oxygen-tolerant anaerobic bacillus, exists in the sebaceous glands of human skin. *P. acnes* is a known exacerbation factor of acne vulgaris and is related to several opportunistic infections, such as bacteremia, endophthalmitis and medical device-associated infections [1]. Antimicrobial treatments are used against *P. acnes* in acne vulgaris. Generally, an antimicrobial treatment for acne vulgaris is administering topical agents, such as clindamycin and nadifloxacin. The internal antimicrobials minocycline and doxycycline are recommended to treat moderate and severe acne. Tetracyclines have been used to treat many bacterial infections, including acne vulgaris, worldwide. In the aftermath of this usage, many resistant *P. acnes* strains were isolated in the USA, Europe, Japan and many other countries [2, 3]. In particular, the tetracycline resistance rate in *P. acnes* isolated from acne patients in the UK was 20–30% in the 1990s, the same as from 1999 to 2002, because oral tetracyclines were frequently used for acne treatment [2, 4]. Additionally, tetracyclines were administered not only for acne, but also for sarcoidosis [5].

Tetracycline resistance is known to occur by the acquisition of an efflux pump coding gene, such as tetK, and a ribosome protective protein coding gene, such as tetM [6]. Other mechanisms were reported for mutation of the 16S rRNA and substitutions in the ribosomal proteins S3 and S10, encoded by the rpsC and rpsJ genes, respectively [7]. Tetracycline resistance mechanism in *P. acnes* was detected with only a G to C mutation at position 1036 (G1036C) in the 16S rRNA gene (corresponding to position 1058 in *Escherichia coli*), which is a drug binding site [4, 8]. Recently, tetracycline insusceptible strains that had reduced susceptibility to doxycycline and minocycline have been frequently isolated [9]. It is important that these strains (doxycycline MIC: 1–2 µg ml⁻¹) may no longer be susceptible to acne treatment because the theoretical plasma concentration of doxycycline is 1.5 µg ml⁻¹ after administration of 100 mg day⁻¹, which is the standard dose for acne treatment [10]. However, these strains do not have a 16S rRNA mutation and carry no resistance gene. Here, we report a novel resistance mechanism and resistance to tetracyclines in *P. acnes*.  

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METHODS

Bacterial strains

A total of 69 P. acnes strains were isolated from acne patients at Tokyo Women’s Medical University Hospital in Japan from 2010 to 2011 [3]. P. acnes ATCC 6919 was used as a reference strain for antimicrobial susceptibility [11] and ATCC 11828 was used for its wild-type 16S rRNA and genes encoding ribosomal proteins S3 (rpsC) and S10 (rpsJ) (Accession number: CP003084). P. acnes strains were incubated on modified Gifu Anaerobic Medium (GAM) Nissui Pharmaceutical) at 35 °C for 72 h under anaerobic conditions.

Susceptibility testing

Antimicrobial susceptibility was determined by calculating the MIC according to an agar dilution procedure in the Clinical and Laboratory Standards Institute (CLSI) document [12]. The MIC was determined after a 48 h incubation under anaerobic conditions. Tetracycline MICs for intermediate and resistance are defined as 8 and ≥16 µg ml⁻¹ by CLSI, respectively [13]. The breakpoints of doxycycline and minocycline were defined to be equal to that of tetracycline by us. We defined insusceptible strains as those with reduced susceptibility doxycycline MIC 1–2 µg ml⁻¹, which is higher than the value for susceptible strains (MICS: ≤0.5 µg ml⁻¹) and lower than intermediate strains. The antimicrobial agents tetracycline (Wako Pure Chemical Industries), doxycycline (Sigma-Aldrich), minocycline (Sigma-Aldrich) and tigecycline (provided by Pfizer) were used. To study the effect of drug efflux inhibition, the MICs in the presence and absence of 20 µg ml⁻¹ reserpine were determined [14]. We confirmed that reserpine inhibits the efflux of tetracyclines using Staphylococcus epidermidis carrying a tetM coding efflux pump in a pilot study, because no P. acnes strain carrying an efflux pump was found. Reserpine-positive efflux was defined as upregulating expression if the MIC with reserpine was less than a quarter of the MIC value without reserpine.

Sequencing the 16S rRNA, rpsC and rpsJ genes

The 16S rRNA gene mutation and the amino acid substitution resulting from mutated rpsC and rpsJ genes were determined by DNA sequencing. The 16S rRNA gene was analysed according to the method of Nakase et al. [9]. The amplification primers 8UA and 1485B and the P. acnes sequencing primers acnes 16S_seq-F and acnes 16S_seq-R were used. The rpsC and rpsJ genes were analysed using rpsC-F (GCCAAGTCCGAGACCAGAA), rpsC-R (GTCCACACAGCACCCTG), rpsJ-F (5’-CACAGTGTTCACGGGTT-3’) and rpsJ-R (5’-CACAGCCTTCTCGAAG-3’) as amplification and sequencing primers.

Phylogenetic analysis

P. acnes strains were identified by single-locus sequence typing [15]. Sequence data were analysed by single-locus sequence typing for P. acnes (http://medbac.dk/slst/pacnes).

Selection of tetracycline-resistant strains

For the isolation of resistant mutants, P. acnes ATCC 11828 was incubated in modified GAM broth (Nissui Pharmaceutical) for 48 h under anaerobic conditions. The culture broth was centrifuged (4000 g, 10 min), and the supernatant was removed. The pellet was suspended in PBS at a concentration of 10¹⁰ c.f.u. ml⁻¹, and 0.1 ml bacterial suspension was spread on modified GAM agar containing twofold serial dilutions of doxycycline (0.25–32 µg ml⁻¹). After incubation for 7 days, 12 mutant colonies were randomly selected and used in this study [16].

RESULTS

Antimicrobial susceptibility of P. acnes

Antimicrobial susceptibility of P. acnes clinical isolates were determined (Fig. 1). Doxycycline-susceptible strains (MIC: ≤0.5 µg ml⁻¹) were detected in 60 isolates (87.0 %). Insusceptible strains (MIC: 1–2 µg ml⁻¹) were detected in 6 (8.7 %) and 3 (4.3 %) isolates, respectively. Upon determining the phylogenetic types, susceptible strains were identified in 11 types (A1, A2, A5, A14, A15, C4, F4, H1, H4, K1 and K7), and the strains of cluster A included 32 isolates (53.3 %). In contrast, insusceptible strains were identified in three types (three of A2, one of A10 and K1), but one strain was not determined. One C1 and two C2 of resistant strains were detected.

Patient histories

The mean ages of patients, from whom susceptible (n=60), insusceptible (n=6) and resistant strains (n=3) were isolated, were 25±6.2, 25.5±4.4 and 20.3±3.2, respectively. The rates of patients who have taken oral tetracyclines for acne treatment and carried susceptible, insusceptible and resistant strains were 11.7, 33.3 and 100 %, respectively.

Analysis of tetracycline resistance factors

The resistance mechanisms of doxycycline-resistant and insusceptible strains were analysed (Table 1). The major resistance mechanism of tetracyclines in Gram-positive bacteria

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Fig. 1. MIC distribution of tetracyclines in P. acnes isolates.
including staphylococci is acquiring the efflux pump gene. Although the MICs in the presence or absence of reserpine as an efflux inhibitor were tested, no difference in their MICs was found. The resistance mechanism of tetracyclines due to decreased drug affinity caused by a mutated drug-binding site was analysed by sequencing the 16S rRNA gene and rpsJ gene, which encodes the ribosomal S10 protein. All three resistant strains (MIC: 16 µg ml⁻¹) had a G to C mutation at position 1036 in the 16S rRNA (G1036C). Additionally, an A to C mutation at position 1180 in the 16S rRNA (A1180C) was found, although its effect on tetracycline resistance is unclear. The insusceptible strains contained a C to T mutation at position 831 (C831T) and a T to C mutation at position 985 (T985C) in the 16S rRNA but not a G1036C mutation. It is suggested that these mutations contribute very little to tetracycline resistance because they are frequently found in susceptible strains. When the rpsJ gene was sequenced, five types of mutations were detected in nt 169–172 of rpsJ. These mutations resulted in four amino acid substitutions in the ribosomal S10 protein: Lys57Glu (Al69G), Lys57Met (A170T or G171C), Lys57Asn (G171T) and Tyr58Asp (T172G). By contrast, four susceptible strains, which were randomly selected, did not have mutations in the 16S rRNA and rpsJ genes. No difference was found in the rpsC gene sequence between the resistant and susceptible strains. Although some strains had specific mutations, all were silent mutations. These results demonstrate that the only S10 protein contributes to tetracycline insusceptibility, and both mutations in the 16S rRNA gene and S10 protein contribute to tetracycline resistance.

Selection of tetracycline-resistant strains
To confirm tetracycline resistance from the mutations in the 16S rRNA gene and the substitution in the S10 protein, we isolated doxycycline-resistant mutants in vitro. The resistant mutants were obtained on agar plates containing 1 and 2 µg ml⁻¹ of doxycycline. The colonies were randomly isolated, and tetracycline susceptibility and chromosomal mutations were studied (Table 1). No difference was found between the MIC values of the tetracyclines in the presence and absence of reserpine (data not shown). The MICs of tetracycline, doxycycline and minocycline for all mutants were 4, 4 and 2 µg ml⁻¹, respectively. Additionally, it has been reported that if the strains acquire a mutation in the S10 protein, the tigecycline MIC increases in Streptococcus pneumoniae [7]. In P. acnes, tigecycline MICs of mutants acquiring the mutation in the S10 protein increased more than that of the parental strain, which showed a MIC of 0.25–0.5 µg ml⁻¹. Mutations in the rpsJ gene were found in all mutants, while no 16S rRNA mutations were detected. The different types of mutants with S10 protein substitution were detected in eight (Lys57Glu), two (Lys57Met) and two (Tyr58Asp). No mutation of rpsC gene was found in any mutant.

DISCUSSION
Tetracyclines, broad-spectrum agents, are used to treat several types of bacterial infections by inhibiting protein synthesis by binding the aminoacyl-tRNA coding region at the 30S subunit of the bacterial 16S rRNA [17]. The tetracycline resistance mechanism in P. acnes was reported to occur only through G1036C mutation (position 1058 in E. coli) in helix 34 of the 16S rRNA coding aminoacyl-tRNA [8, 18]. The A1180C mutation was detected only in resistant strains in this study; thus, its effect on tetracycline resistance should be analysed. This is the first report of amino acid substitutions in the ribosomal S10 protein contributing to tetracycline resistance in P. acnes. The ribosomal S10 protein, which comprises two regions, is a slender protein linked with the 16S rRNA [6]. Amino acid substitutions were reported to contribute to tetracycline resistance in Neisseria gonorrhoeae and Bacillus subtilis [6, 19] (Fig. 2). The amino acid sequence of the S10 protein in P. acnes is similar to that of N. gonorrhoeae and B. subtilis with >90% similarity. The substitution of amino acid 57 in the S10 protein, which is located near the tetracycline binding site, was reported in N. gonorrhoeae and is considered to affect the tetracycline binding site by changing the structure or polarity. By contrast, it is presumed that the substitution of amino acid 46 in the S10 protein in B. subtilis causes steric obstruction in the drug-binding site because it is located close to helices 31 and 34 of the 16S rRNA. In this study, amino acid 57 and 58 mutations in the S10 protein in doxycycline-resistant and -insusceptible P. acnes strains correspond to the same sites as in N. gonorrhoeae. By contrast, no mutation of the rpsC gene was found in any tested strain; thus, it is suggested that the ribosomal protein S3 makes a small contribution to tetracycline resistance in P. acnes.

To analyse the susceptibilities of doxycycline-resistant and -insusceptible strains, the insusceptible strains with a substitution in the S10 protein showed 2–4, 1–2 and 0.25–1 µg ml⁻¹ MICs for tetracycline, doxycycline and minocycline, respectively. In vivo mutants that have only the S10 protein with an amino acid substitution showed 4, 4 and 2 µg ml⁻¹ MICs for tetracycline, doxycycline and minocycline, respectively. Laboratory-resistant strains generated in previous report showed same MICs of these agents [8], and it was presumed that they had amino acid substitution in S10 protein. Amino acid substitution of ribosomal proteins S3 and S10 was reported to contribute to the reduced susceptibility of tigecycline [7, 20]. Our results showed that tigecycline MICs were 0.25–0.5 µg ml⁻¹ in the strains carrying the mutation in the S10 protein of both acne patients isolates and in vivo mutants (Table 1). Furthermore, the MICs in the resistant strains that had double mutations in the 16S rRNA gene and S10 protein were 2 µg ml⁻¹. Tigecycline is not recommended against P. acnes, including for acne treatment. However, we first revealed the presence of P. acnes with reduced susceptibility to tigecycline.

The amino acid substitution in the S10 protein was shown to contribute to tetracycline insusceptibility. Additionally, the resistant strains that had mutations in the 16S rRNA gene and the S10 protein exhibited 32, 16 and 8 µg ml⁻¹ MICs for tetracycline, doxycycline and minocycline, respectively. According to previous reports, strains with the 16S
The top nine strains (9 to 85b) were isolated from acne patients and the bottom five strains (3b to 30 and ATCC 11828) were susceptible isolates and parental strain. The middle five strains (1-1 to 2-4) were selected by 1 or 2 µg ml⁻¹ doxycycline.

Table 1. Tetracycline resistance profiles of clinically isolated and laboratory-selected strains of P. acnes

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>MIC (µg ml⁻¹)*</th>
<th>Tetracycline</th>
<th>Doxycycline</th>
<th>Minocycline</th>
<th>Tigecycline</th>
<th>16S rRNA</th>
<th>rpsJ</th>
<th>S10 protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>9</td>
<td>0.5</td>
<td>0.06</td>
<td>0.06</td>
<td>≤0.06</td>
<td>≤0.06</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>11</td>
<td>0.5</td>
<td>0.06</td>
<td>0.06</td>
<td>≤0.06</td>
<td>≤0.06</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>15b</td>
<td>0.5</td>
<td>0.06</td>
<td>0.06</td>
<td>≤0.06</td>
<td>≤0.06</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>34b</td>
<td>0.5</td>
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<td>0.06</td>
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<td>≤0.06</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>45</td>
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<td>0.06</td>
<td>≤0.06</td>
<td>≤0.06</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>75b</td>
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<td>0.06</td>
<td>0.06</td>
<td>≤0.06</td>
<td>≤0.06</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>77b</td>
<td>0.5</td>
<td>0.06</td>
<td>0.06</td>
<td>≤0.06</td>
<td>≤0.06</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>83b</td>
<td>0.5</td>
<td>0.06</td>
<td>0.06</td>
<td>≤0.06</td>
<td>≤0.06</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>1-1</td>
<td>0.5</td>
<td>0.06</td>
<td>0.06</td>
<td>≤0.06</td>
<td>≤0.06</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>2-1</td>
<td>0.5</td>
<td>0.06</td>
<td>0.06</td>
<td>≤0.06</td>
<td>≤0.06</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>2-2</td>
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<td>0.06</td>
<td>0.06</td>
<td>≤0.06</td>
<td>≤0.06</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>2-4</td>
<td>0.5</td>
<td>0.06</td>
<td>0.06</td>
<td>≤0.06</td>
<td>≤0.06</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
<tr>
<td>30</td>
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<td>0.06</td>
<td>0.06</td>
<td>≤0.06</td>
<td>≤0.06</td>
<td>WT</td>
<td>WT</td>
<td>WT</td>
</tr>
</tbody>
</table>

*≤ or or less.

rRNA mutation had tetracycline, doxycycline and minocycline MICs of 2–64, 1–16 and 0.5–4 µg ml⁻¹, respectively [2, 8]. However, the mutation in the S10 protein of the resistant strains was unknown. We could not analyse susceptibilities for strains with only 16S rRNA mutations because no mutants with the 16S rRNA mutation were isolated in vivo as previously reported [8]. We hypothesized two processes for the mechanisms of tetracycline resistance in P. acnes. First, it is presumed that tetracycline resistance in P. acnes occurs when the susceptible strains first become insusceptible by acquiring the S10 protein substitution in rpsJ. Thereafter, insusceptible mutants become resistant by acquiring the mutation of 16S rRNA because all resistant strains have mutations in both the 16S rRNA gene and the S10 protein. The other hypothesis states that strains having the G1036C mutation in 16S rRNA become resistant after acquiring the mutation in the S10 protein because a G1036C mutation was found in only a specific gene clade [21]. Interestingly,
three resistant strains acquiring a G1036C mutation belonged to the specific clades C1 and C2 in this study. We attempted to isolate 16S rRNA mutants from the S10 protein mutants in vivo; however, we could not obtain strains with mutations in both the 16S rRNA gene and S10 protein. Therefore, the data suggest that the resistant strains, which had mutations in both the 16S rRNA gene and S10 protein, were induced by the strains originally having the G1036C mutation in 16S rRNA.

In either case, our data strongly suggest that tetracycline resistance contributes to 16S rRNA mutations and the S10 protein amino acid substitution. However, we were able to isolate only a few resistant and insusceptible strains. We need to collect more P. acnes isolates and analyse them for further study.

This is the first report to demonstrate resistance mechanisms of tetracyclines by substitution in the S10 protein caused by rpsJ mutations in P. acnes. Additionally, our results strongly suggest that the doxycycline and minocycline MICs for P. acnes significantly increase and exceed the resistance threshold through a double mutation in the 16S rRNA gene and S10 protein.

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Conflicts of interest
The authors declare that there are no conflicts of interest.

References

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