

Propionibacterium acnes and *Staphylococcus epidermidis* Isolated from Refractory Endodontic Lesions Are Opportunistic Pathogens^{∇†}

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Received 30 June 2010/Returned for modification 2 August 2010/Accepted 16 August 2010

The predominant cultivable microbiota from 20 refractory endodontic lesions (9 with abscesses and 11 without abscesses) were determined, and *Propionibacterium acnes* and *Staphylococcus epidermidis* were among the most predominant organisms. The number of species identified from lesions with abscesses (14.1 ± 2.6) was significantly greater ($P < 0.001$) than the number from lesions without abscesses (7.4 ± 5.9). Comparison of perioral isolates using repetitive extragenic palindromic PCR of the same species from the same subjects demonstrated that the endodontic and skin populations were significantly different. The *P. acnes* isolates were typed on the basis of *recA* gene sequence comparison, and only three types (types I, II, and III) were identified among 125 isolates examined. However, we found that type I (type IA and IB) isolates were primarily isolated from the skin, while types II and III were significantly more likely to be isolated from the endodontic lesions ($P < 10^{-10}$). We found that the robustness of the *recA* phylotypes was not strong by comparing the partial gene sequences of six putative virulence determinants, PAmce, PAp60, PA-25957, PA-5541, PA-21293, and PA-4687. The resulting neighbor-joining trees were incongruent, and significant (ϕ test; $P = 2.2 \times 10^{-7}$) evidence of recombination was demonstrated, with significant phylogenetic heterogeneity being apparent within the clusters. *P. acnes* and *S. epidermidis* isolated from refractory endodontic infections, with or without periapical abscesses, are likely to be nosocomial infections.

Propionibacterium acnes and coagulase-negative staphylococci, including *Staphylococcus epidermidis*, have been identified among the microflora of endodontic infections (8, 12, 40, 50, 52, 55, 56, 63, 64), but their importance as endodontic pathogens has largely been ignored due to their nearly universal presence on the skin and the consequent likelihood of sample contamination. However, there is now considerable evidence that these organisms are increasingly isolated from human infections, and so their association with endodontic infections requires clarification. *P. acnes*, a non-spore-forming, Gram-positive anaerobic or aerotolerant rod, is a member of the resident microflora of the large intestine, conjunctiva, and external ear canal (10, 15) and accounts for approximately half of the total skin microbiota (59), predominating over other pilosebaceous flora (16, 39). Although traditionally considered to be relatively nonpathogenic, an increasing number of studies have implicated *P. acnes* as an opportunistic pathogen responsible for a wide range of infections and inflammatory conditions. In addition to its well-established role in the pathogenesis of acnes vulgaris (16, 34), it has also been linked to synovitis-acnes-pustulosis-hyperostosis-osteitis syndrome (44, 54), sarcoidosis (17), and prostate cancer (14). Recent studies have revealed trauma and surgery as the predisposing factors associated with numerous *P. acnes* infections, which include

brain abscesses (36), osteomyelitis after lumbar puncture (1), discitis after surgery (23), spondylodiscitis following epidural catheterization (22, 25), postoperative mediastinitis (19, 58), endophthalmitis (7), and endocarditis (21). Furthermore, it is also emerging as an important pathogen in infections related to medical foreign-body implants, such as intraocular lenses (65), ventroperitoneal shunts, orthopedic implants (9, 28, 51), silicone implants (2), prosthetic heart valves (33, 42), and prosthetic joints (38, 61).

S. epidermidis is part of the human skin microflora, where, as a commensal organism, it usually exists in a benign relationship with the host. Although it is ranked first in nosocomial and implant-based infections, perhaps due to its ubiquity on the skin, it is more likely to contaminate devices at the time of insertion. As a recognized opportunistic pathogen, it is responsible for nosocomial infections of indwelling medical devices (43), such as peripheral or central intravenous catheters (CVCs), prosthetic joints, vascular grafts, and central nervous system shunts (46), and cardiac device infections, such as prosthetic valve endocarditis (PVE) (13), as well as ventricular assist device driveline-related infections (4). Clearly, a characteristic shared by both of these organisms is an association with the infection of prosthetic devices.

The treatment of endodontic infections involves the insertion of gutta-percha into the debrided and disinfected root canal and the restoration of the tooth. This treatment may fail, with the root canal becoming infected. A range of bacteria have been isolated from such infected sites, including *P. acnes* and *S. epidermidis*, but these have generally been considered contaminants. The objective of the present study was to confirm the endodontic origin of *P. acnes* and *S. epidermidis* isolates recovered from refractory endodontic infections. We

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† Supplemental material for this article may be found at <http://jcm.asm.org/>.

[∇] Published ahead of print on 25 August 2010.

have used DNA fingerprinting of the *P. acnes* and *S. epidermidis* isolates from endodontic lesions and samples from the perioral skin of the same individual using repetitive extragenic palindromic PCRs (REP-PCRs) (3). The *recA* phylotypes of the *P. acnes* isolates were determined, as we expected to find that the predominant phylotypes from the endodontic lesions would be types II and III, which are almost exclusively associated with infections of implanted prostheses, while types IA and IB are usually isolated from skin (26, 30, 31, 62). We also investigated the phylogenetic status and robustness of the *recA* phylotypes by comparing the intrastrain relationships of partial gene sequences of six putative virulence determinants, PAmce (mammalian cell entry gene), PAp60, PA-25957, PA-5541, PA-21293, and PA-4687 (20, 35). Here we have set out to determine if *P. acnes* and *S. epidermidis* isolated from refractory endodontic lesions are contaminants arising during the sample collection process or if they represent nosocomial infections.

MATERIALS AND METHODS

Sample collection. Patients recruited into the study received treatment in the Endodontic Department of the Dental Institute at Guys' and St. Thomas's Hospital, King's College London. The selected teeth were single rooted with failed root canal treatment (refractory cases) with or without a periapical abscess. No communication between the abscess and the oral cavity or the skin surface was observed following clinical examination. All patients were medically stable and had no active, acute medical conditions, and they had not been prescribed antibiotics during the previous month. The sampled teeth had no root fracture or endoperiodontal lesion. All significant details about the history, clinical and radiographic signs, and symptoms about the involved tooth were recorded. The project was approved by the local ethics committee, and the patients gave their informed consent for their inclusion.

Twenty patients with refractory endodontic lesions ($n = 9$ with periapical abscesses and $n = 11$ without periapical abscesses) were investigated. After local anesthesia, the tooth to be treated was isolated with a rubber dam. The tooth and surrounding dam and clamp were cleaned with 30% (vol/vol) hydrogen peroxide and decontaminated with 2.5% sodium hypochlorite. After decontamination, the isolated tooth and surrounding dam were swabbed (LIP Limited, West Yorkshire, United Kingdom) to check for contamination.

The coronal restorations were removed, and the root filling was exposed. The coronal gutta-percha placed during the initial root canal treatment was removed using sterile Gates-Glidden drills, and the apical gutta-percha was removed with files. All of the material removed was transferred into 1 ml Tris-EDTA buffer (1.0 M Tris-HCl containing 0.1 M EDTA, pH 8.0, prepared in deionized water) (sample a). Radiographs were taken to ensure that all filling materials had been removed. An apex locator was used to determine the working length of the canal. Sterile saline solution was introduced into the canal, and the canal wall was filed up to the working length. The file samples were transferred into 1 ml Tris-EDTA buffer (sample b). The remaining root canal contents were absorbed onto paper points and transferred into 1 ml Tris-EDTA buffer (sample c). All samples were immediately transported on ice to the laboratory.

From 13 patients, perioral skin swab specimens (LIP Limited) were also taken for the isolation of *P. acnes* and *S. epidermidis*.

Microbial analysis of samples. Each sample (samples a to c) was dispersed by vortexing with glass beads, diluted in fastidious anaerobic broth (Lab M, United Kingdom), and plated onto nonselective medium (fastidious anaerobic agar [FAA] supplemented with 5% [vol/vol] horse blood; Lab M) and also onto a range of selective media, including Rogosa agar (Oxoid, United Kingdom) (49) for lactobacilli, mupirocin-containing Trypticase phytone yeast (MTPY) agar (45) for bifidobacterium, Tryptone yeast cystine agar (TYC) agar (Lab M) (53) for streptococci, Veillonella agar (47, 48) and MacConkey agar (Oxoid, United Kingdom) for enteric bacteria, and CHROMagar (CHROMagar, France) for yeasts. The Rogosa, MTPY, and TYC agar media were incubated anaerobically for 3 days, the Veillonella agar was incubated anaerobically for 4 days, and FAA plates were incubated anaerobically for 7 days. The MacConkey agar and CHROMagar plates were incubated aerobically for 2 days at 37°C. After incubation, the colonies were counted and a predetermined number of colonies from each medium ($n = 300$ maximum per patient) were randomly selected for identification.

The swabs taken from the prepared teeth prior to the removal of the restoration and the swabs from the perioral skin were plated directly onto FAA and incubated anaerobically for 7 days.

Identification of isolates. All randomly selected isolates were subcultured on FAA and grown for 24 h. Bacterial genomic DNA was extracted by boiling 100 μ l of a suspension of the cultured cells prepared in sterile distilled H₂O for 10 min. The suspension was cooled on ice for 10 min and centrifuged at 13,000 $\times g$ for 2 min, and the supernatant containing the genomic DNA was stored at -20°C prior to analysis (38). A partial fragment of the 16S rRNA gene of all isolates was amplified using the following reaction mixture (final volume, 25 μ l): either 0.3 μ l of a 10-pmol/ μ l concentration each 27F-YMa, 27F-YMb, 27F-YMc, and 27F-YMd forward primers (Integrated DNA Technology, United Kingdom) (18), 0.7 μ l of 1492R reverse primer (concentration, 10 pmol/ μ l), 22.5 μ l of Reddymix buffer (Thermo Scientific, United Kingdom), and 0.6 μ l DNA extract or 0.5 μ l of 9F forward primer (concentration, 10 pmol/ μ l; MWG, United Kingdom), 0.5 μ l of 907R reverse primer (concentration, 10 pmol/ μ l; MWG), 23 μ l of Reddymix buffer (Thermo Scientific), and 1 μ l DNA extract. The PCR products were cleaned with Microclean (Sigma, United Kingdom), in order to get ready for the sequence reaction. Amplicon sequencing was performed by using an ABI Prism BigDye Terminator sequencing kit (Applied Biosystems) with 30 cycles of denaturation at 96°C for 10 s, annealing at 50°C for 5 s, and extension at 60°C for 2 min. Sequencing reaction products were run on an ABI 3730xl sequencer Applied Biosystems). All DNA sequences were analyzed, trimmed, and aligned using BioEdit software (version 7.0.0; <http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). The partial gene sequences were identified by a BLAST search of the NCBI database (<http://0-www.ncbi.nlm.nih.gov/ilsprod.lib.nyu.edu/BLAST/>), the Human Oral Microbiome database (<http://www.homb.org/>), or the Ribosome Database Project database (<http://rdp.cme.msu.edu/>). Phylogenetic trees were constructed by the neighbor-joining (NJ) method, based on 16S rRNA gene sequence comparisons, using the MEGA (version 4.1) program (<http://www.megasoftware.net/>). Most isolates were identified using these sequences, but if isolates were identified as *Lactobacillus* spp., *Veillonella* spp., or "*Actinomyces naeslundii/A. viscosus*," these were more accurately identified by partial sequencing of the *pheS* (41), *rpoB* (5), and *metG* (24) genes, respectively.

Phylotyping of *P. acnes* isolates. Endodontic and skin *P. acnes* isolates identified by partial 16S rRNA gene sequencing were typed by partial *recA* gene sequencing. The *P. acnes recA* gene was amplified using primer PAR-1 (positions -96 to -75; 5'-AGCTCGGTGGGGTCTCTCATC-3') and primer PAR-2 (positions +1105 to +1083; 5'-GCTTCCTCATACCATTGGTCATC-3'), which generated a 1,201-bp amplicon (38). The reaction mixture comprised 0.5 μ l of PAR-1 (concentration, 10 pmol/ μ l; Sigma), 0.5 μ l of PAR-2 (concentration, 10 pmol/ μ l; Sigma), 23 μ l of Reddymix (Thermo Scientific), and 1 μ l DNA extract in a 25- μ l final volume. The thermal cycling conditions included initial denaturation at 95°C for 3 min, denaturation at 95°C for 1 min, annealing at 55°C for 30 s, and extension at 72°C for 90 s, repeated for 35 cycles, and a final extension at 72°C for 10 min. The amplified products could be stored temporarily at 4°C. Amplified products were run on a 0.5% agarose gel and visualized under UV transillumination. Sequencing was performed as described above, and the *P. acnes recA* sequences were compared with GenBank sequences AY642055 (type IA), EU687255 (type IB), AY642061 (type II), and DQ672252 (type III). NJ trees were constructed using the Jukes-Cantor method with MEGA (version 4.1) software (www.megasoftware.net/).

Comparison of putative *P. acnes* virulence determinants. A number of genes have been associated with the virulence of *P. acnes* (20, 35), including two invasion-associated proteins (PAmce and Pap60) and four predominant immunoreactive surface proteins (PA-25957, PA-5541, PA-21293 and PA-4687), the first two of which have homology with M-like protein of *Streptococcus equi* and have dermatan sulfate-binding activity, while the other proteins are similar to the product of the *Corynebacterium diphtheriae htaA* gene, which codes for an iron-regulated hemin binding protein. We used the previously reported primers to amplify and sequence internal fragments of PAmce and Pap60 (35) and designed primers to amplify and sequence fragments of each gene of these putative virulence determinants from a selection of *P. acnes* strains isolated in this study. PA5541 was amplified and sequenced using primers 5'-TCGACCTCAGCTTC AA-3' and 5'-AGGCGCTTGACGATAT-3', PA25957 was amplified and sequenced using primers 5'-CAATCGCAGCAATCAC-3' and 5'-CTCAGTCTT TGCGATC-3', PA21693 was amplified and sequenced using primers 5'-CCGA GTTCTATGGCAA-3' and 5'-CCTGTTTGGTCATGGT-3', while PA4687 was amplified and sequenced using primers 5'-GTATTGTTAGCCGTGC-3' and 5'-ATCGTTTTGACCCTGC-3'. The amplicon sizes for the six genes used in the analyses were 783, 1,024, 491, 386, 464, and 473 bp, respectively.

The sequences of the same genes of the sequenced *P. acnes* strains (strains J165 and SK137 [type IA], KPA171202 and SK187 [type IB], and J139 [type II])

were downloaded from GenBank, and these were aligned with the sequences derived here using BioEdit and NJ trees for each gene and for the concatenated sequences of all the putative virulence genes that were constructed, using the Jukes-Cantor model, in MEGA (version 4.1). The concatenated sequences were tested for the presence of recombination using the phi test (11).

REP-PCR analysis of *P. acnes* and *S. epidermidis*. Skin and endodontic *P. acnes* and *S. epidermidis* isolates from the same individual ($n = 8$ for *S. epidermidis* and $n = 13$ for *P. acnes*) were compared using REP-PCR (3). Primers REPIR-Dt (5'-555 NCG NCG NCA TCN GCC-3') and REP2-Dt (5'-NCG NCT TAT CNG GCC TAC-3') (Integrated DNA Technologies, Belgium) used in this study were described previously (3). The reaction mixture contained each of the four deoxynucleoside triphosphates (dNTPs; Thermo Scientific) at a concentration of 125 μ M, 150 ng of each primer, 3.75 mM MgCl₂ (Thermo Scientific), 2.5 U of *Taq* polymerase (Thermo Scientific), 20 mM (NH₄)₂SO₄, 75 mM Tris-HCl (pH 9.0), 0.01% (wt/vol) Tween 80, and 3 μ l of DNA solution made up to a final volume of 25 μ l with sterile water. PCR amplification was performed in an automated thermocycler (Techne) with an initial denaturation (7 min at 95°C), followed by 32 cycles of denaturation (30 s at 94°C), annealing (1 min at 40°C), and extension (8 min at 65°C) and with a final extension (16 min at 65°C). The amplification products of REP-PCR (15 μ l) were analyzed with a 2% Metasieve agarose gel (Flowgen, Staffordshire, England) containing Gel Red nucleic acid stain (10,000 \times in dimethyl sulfoxide; Cambridge Bioscience) and were separated electrophoretically on gels at 80 V for 5 h in 1 \times TBE (Tris-borate-EDTA) buffer. A molecular size marker (pGEM DNA markers; Promega) was included on all gels to facilitate comparison of tracks between gels. The gels were imaged (AlphaImager), and the resulting patterns were compared visually.

Biochemical characterization of *P. acnes* isolates. *P. acnes* isolates from skin ($n = 29$) and endodontic infections ($n = 34$) and reference strains *P. acnes* type IA (NCTC 737) and *P. acnes* type II (NCTC 10390) were characterized for their ability to ferment lactose, *N*-acetylglucosamine, erythritol, ribose, and sorbitol, according to a previously described method (6). The fermentation reactions were also performed in a Rapid ID 32A system, according to the manufacturer's instructions (bioMérieux United Kingdom, Limited). β -D-Fucosidase, β -*N*-acetylglucosaminidase, sialidase, α -L-fucosidase, β -*N*-acetylglucosaminidase, α -glucosidase, β -glucosidase, β -galactosidase, α -arabinosidase, and β -galactosidase activities were determined using fluorogenic substrates, as described previously (6). The strains were also tested for the production of lipase (32), DNase, nonspecific proteinase (37), elastase (29), lecithinase (60), and hyaluronidase (57).

Statistical analysis. Data distributions were compared using χ^2 tests, means were compared using the Mann-Whitney U test in SPSSPC (version 16.0), and the analysis of the biochemical test results using canonical variates analysis was performed in the PAST-Palaeontological Statistics program (version 1.90; <http://folk.uio.no/ohammer/past/>).

RESULTS

No organisms were recovered from the samples taken from the sterilized tooth surfaces prior to removal of the restoration. The range of organisms cultured from the 20 endodontic lesions is shown in Tables 1 and 2. The lesions without abscesses (Table 1) were characterized by the presence primarily of Gram-positive facultative anaerobic organisms, and these organisms were also present in the lesions with abscesses. However, the cultivable flora of the lesions with abscesses was more complex, as these lesions also harbored obligate anaerobic Gram-positive and Gram-negative organisms. The mean number of taxa from the lesions with periapical abscesses (Table 2) was 14.1 ± 2.6 , and that from lesions without periapical abscesses was 7.4 ± 5.9 ($P < 0.001$). *P. acnes* was recovered from 10 of the 11 endodontic lesions without periapical abscesses and from 8 of the 9 lesions with periapical abscesses ($P > 0.1$). Other propionibacteria isolated from the lesions included *P. avidium*, *P. granulosum*, *P. propionicum*, and *Propionibacterium* sp. strain FMA5. The median proportion of propionibacteria recovered from the file samples in the teeth without abscesses was 26.7% (range, not detected [ND] to 92%) compared to not detected (range, ND to 76.7%) in teeth with abscesses ($P <$

0.01). Staphylococci, including *Staphylococcus caprae*, *S. condimentii*, *S. epidermidis*, *S. hominis*, *S. pasteurii* and *S. warneri*, were isolated from the endodontic lesions, with *S. epidermidis* being isolated from 5/9 endodontic lesions with abscesses and from 9/11 of the lesions without abscesses ($P > 0.1$). The median proportion of staphylococci recovered from the file samples in the teeth without abscesses was 6.7% (range, ND to 73%) compared to not detected (range, ND to 13.3%) in teeth with abscesses ($P < 0.01$).

Phylotyping of *P. acnes* isolates. Partial *recA* sequences were obtained for 65 skin and 60 endodontic *P. acnes* isolates; and on the basis of sequence alignment, 3 distinct phylogenetic lineages, type I, type II, and type III, were observed. The type I isolates segregated into two distinct groups, into which the sequences from known type IA and type IB sequences clustered. When the distribution of the different *P. acnes* types was considered (Table 3), the distribution of types recovered from the skin was significantly different from that of all *P. acnes* isolates recovered from all endodontic lesions ($P < 10^{-10}$) and from that of the *P. acnes* isolates recovered only from patients from whom samples from skin were also taken ($P = 2 \times 10^{-8}$).

Comparison of putative *P. acnes* virulence determinants. Individual NJ trees were constructed for each of the partial putative virulence gene sequences determined for the strains isolated in this study, along with the corresponding sequences derived from each of the 5 sequenced *P. acnes* genomes. In PA-4687, we found that each of the type III isolates had evidence of a 5-nucleotide (nt) deletion, such that the gene could no longer be transcribed; these nucleotides were removed from the sequences of the other phylotypes to enable the construction of the NJ trees. The NJ trees for PA-25957 and PA-4687 are shown in Fig. 1 (the remaining trees are shown in Fig. 1S in the supplemental material). From these it is apparent that the type II and III isolates formed distinct clusters with each gene. All the type I sequences for PA-5541, PA-4687, PAmce, and PA-21693 formed either single clusters or clustered together but clustered as subclusters supported by bootstrap values of ≤ 65 . The type IA and IB strains formed distinct discrete clusters with the partial sequences of PA-25957 and Pap60, except that SK187 (type IB) was in the type IA cluster, while the type IB and II strains were on the same lineage with Pap60. The trees for the different genes were clearly incongruent (Fig. 1).

We next constructed an NJ tree using the concatenated sequences of the putative virulence determinants in order to determine if the *recA*-based determination of phylotype was replicated in this analysis. These data are shown in Fig. 2. In the tree derived from the *recA* sequences, the 4 lineages are well discriminated. However, in the trees derived from the concatenated sequence data (3,616 bp), the type III isolates formed a distinct lineage, as did the type II isolates, in which two subbranches supported by significant bootstrap values of > 95 were evident. The type IB isolates formed a single lineage composed of all isolates except SK187, which was found in the type IA lineage. The type IA lineage appeared to be diverse, with SK187 and SK137 forming branches distinct from the other 5 isolates in this lineage. There was statistical evidence of recombination when the concatenated sequences were tested (phi test, $P = 2.2 \times 10^{-7}$).

TABLE 1. Distribution of all 51 bacterial taxa identified among 638 isolates recovered from 11 refractory endodontic lesions without periapical abscesses

Organism	Presence in endodontic lesion (no periapical abscess) no.:											
	1	2	3	4	5	6	7	8	9	10	11	
Gram-positive organisms												
Obligate anaerobes												
<i>Clostridium sporogenes</i>	-	+	-	-	-	-	-	-	-	-	-	-
<i>Olsenella profusa</i>	+	-	-	-	-	-	-	-	-	-	+	-
Facultative anaerobes												
<i>Abiotrophia defectiva</i>	-	-	-	-	-	-	-	-	-	-	+	-
<i>Actinomyces gerencseriae</i> oral taxon 618	-	-	-	-	-	-	-	-	+	-	-	-
<i>Actinomyces massiliensis</i>	-	+	-	-	-	-	-	-	-	-	-	-
<i>Actinomyces meyeri</i>	-	-	+	-	-	-	-	-	-	-	-	-
<i>Actinomyces radidentis</i>	+	-	-	-	-	-	-	-	-	-	-	-
<i>Actinomyces</i> sp. oral taxon 169 clone AG004	-	-	-	-	-	-	-	-	-	+	-	-
<i>Actinomyces</i> sp. oral clone CT047	-	-	-	-	-	-	-	-	+	-	-	-
<i>Bacillus subtilis</i>	-	-	-	-	-	-	-	-	-	-	-	+
<i>Bifidobacterium dentium</i>	+	-	-	-	-	-	-	-	-	-	-	-
<i>Enterococcus faecalis</i>	-	-	-	-	-	+	+	-	-	-	-	-
<i>Gemella haemolysans</i>	-	-	-	-	+	-	-	-	+	+	+	+
<i>Gemella sanguinis</i>	-	-	-	-	-	-	-	-	-	+	-	-
<i>Granulicatella elegans</i>	-	-	-	-	-	-	-	-	-	+	-	-
<i>Kocuria</i> sp. oral taxon B56	-	-	-	-	-	-	-	-	-	+	-	-
<i>Lactobacillus fermentum</i>	+	-	-	-	-	-	-	-	-	-	-	-
<i>Lactobacillus paracasei</i>	+	-	-	-	-	-	-	-	-	-	-	-
<i>Lactobacillus rhamnosus</i>	+	-	-	-	-	-	-	-	-	-	-	-
<i>Lactobacillus salivarius</i>	+	-	-	-	-	-	-	-	-	-	-	-
<i>Micrococcus luteus</i> strain EHFS1	-	-	-	-	+	-	-	-	-	-	-	-
<i>Micrococcus</i> sp. oral taxon B64	-	-	-	-	-	-	-	-	-	+	-	-
<i>Propionibacterium acnes</i>	+	-	+	+	+	+	+	+	+	+	+	+
<i>Propionibacterium granulosum</i>	+	-	-	-	-	-	-	-	-	-	-	-
<i>Propionibacterium propionicum</i>	-	-	-	-	-	-	-	-	-	+	-	-
<i>Rothia dentocariosa</i>	-	-	-	-	-	-	-	-	-	+	-	-
<i>Rothia</i> sp. oral taxon 188	-	-	-	-	-	-	-	-	-	+	-	-
<i>Staphylococcus capare</i>	-	-	-	+	+	+	-	+	-	-	-	-
<i>Staphylococcus condimenti</i>	+	-	-	-	-	-	-	-	-	-	-	-
<i>Staphylococcus epidermidis</i>	+	+	+	+	+	+	-	+	-	+	+	+
<i>Staphylococcus hominis</i>	+	-	-	-	-	-	-	-	-	-	-	-
<i>Staphylococcus pasteurii</i>	+	-	-	-	-	-	-	-	-	-	-	-
<i>Staphylococcus warneri</i>	-	-	-	+	-	-	-	-	+	-	-	-
<i>Streptococcus anginosus</i>	-	+	-	-	-	-	-	-	-	-	-	-
<i>Streptococcus cristatus</i>	-	-	-	-	-	-	-	-	-	+	-	-
<i>Streptococcus gordonii</i>	-	-	-	-	-	-	-	-	-	+	-	-
<i>Streptococcus mitis</i> /S. oralis	-	+	-	-	+	-	-	-	-	+	-	-
<i>Streptococcus mitis</i> biovar 2	-	+	-	-	+	-	-	-	-	-	-	-
<i>Streptococcus mutans</i>	+	-	-	-	-	+	-	-	-	-	-	-
<i>Streptococcus sanguinis</i>	-	+	-	-	+	-	-	-	+	-	-	-
<i>Streptococcus</i> sp. oral taxon C65	-	-	-	-	-	-	-	-	-	+	-	-
Gram-negative organisms												
Obligate anaerobes												
<i>Dialister invisus</i>	-	-	-	-	+	-	-	-	-	-	-	-
<i>Prevotella buccae</i>	-	-	-	-	-	-	-	-	-	-	-	-
<i>Prevotella loescheii</i>	-	-	+	-	-	-	-	-	-	-	-	-
<i>Prevotella</i> sp. oral taxon 473 clone IK062	-	-	-	-	+	-	-	-	-	-	-	-
<i>Selenomonas noxia</i>	-	-	+	-	-	-	-	-	-	-	-	-
<i>Selenomonas sputigena</i>	-	-	-	-	-	-	-	-	-	+	-	-
<i>Tanarella forsythia</i>	-	-	-	-	+	-	-	-	-	-	-	-
<i>Veillonella parvula</i>	-	-	-	-	+	-	-	-	-	-	-	-
Facultative anaerobe												
<i>Capnocytophaga gingivalis</i>	-	+	-	-	-	-	-	-	-	-	-	-
<i>Capnocytophaga ochracea</i>	-	-	+	-	-	-	-	-	-	-	-	-
<i>Haemophilus parainfluenza</i>	-	-	-	-	+	-	-	-	-	-	-	-

TABLE 2. Distribution of all 77 bacterial taxa identified among 1,004 isolates recovered from 9 refractory endodontic lesions with periapical abscesses

Organism	Presence in endodontic lesion (with abscesses) no.:									
	12	13	14	15	16	17	18	19	20	
Gram-positive organisms										
Obligate anaerobes										
<i>Atopobium</i> species oral taxon 199 clone_C019	+	-	-	-	-	-	-	-	-	-
<i>Atopobium rimaiae</i>	-	-	-	-	-	+	-	-	-	-
<i>Eubacterium minutum</i>	+	+	-	-	-	+	-	-	-	-
<i>Eubacterium nodatum</i>	-	+	-	-	-	-	-	-	-	-
<i>Eubacterium infirmum</i>	-	-	-	-	-	+	-	-	+	-
<i>Eubacterium saburreum</i>	-	-	-	-	+	-	-	-	-	-
<i>Eubacterium sulci</i>	-	-	-	-	-	-	+	-	-	-
<i>Eubacterium yurii</i>	-	-	-	-	-	-	+	-	-	-
<i>Finegoldia magna</i>	-	-	-	+	-	-	-	-	-	-
<i>Lachnospiraceae</i> (G-8) sp. clone JM048	-	-	-	-	-	-	-	-	-	+
<i>Mogibacterium diversum</i>	-	-	-	-	-	-	+	-	-	+
<i>Mogibacterium neglectum</i>	-	-	-	-	-	-	+	-	-	-
<i>Mogibacterium timidum</i>	+	+	+	-	-	+	-	-	-	-
<i>Olsenella profusa</i>	-	-	-	-	-	+	-	-	-	-
<i>Olsenella uli</i>	-	+	+	-	-	+	-	-	-	-
<i>Parvimonas micros</i>	-	-	-	-	+	+	-	-	-	-
<i>Peptostreptococcus stomatis</i>	-	-	+	+	-	+	+	-	-	-
<i>Pseudoramibacter alactolyticus</i>	+	+	+	-	-	+	-	-	-	-
<i>Slackia exigua</i>	+	+	+	-	-	-	+	-	-	-
<i>Solobacterium moorei</i>	-	-	-	-	+	+	+	-	-	-
Uncultured <i>Clostridium</i> sp. clone MS027A1_C05	-	-	-	+	-	-	-	-	-	-
Uncultured bacterium clone nbw335f06c1	-	-	-	+	-	-	-	-	-	-
Facultative anaerobes										
<i>Actinomyces israelii</i>	-	-	-	-	-	-	-	+	-	-
<i>Actinomyces</i> sp. oral clone JA063	-	-	-	+	-	-	-	-	-	-
<i>Actinomyces</i> sp. oral taxon 169 clone AG004	-	-	-	-	+	-	-	+	-	-
<i>Actinomyces</i> sp. oral taxon 181 strain Hal1065	-	-	-	-	-	-	-	-	-	+
<i>Actinomyces</i> strain B27SC	+	-	-	-	-	+	-	-	-	-
<i>Actinomyces</i> strain C29KA	-	+	-	-	-	-	-	-	-	-
<i>Bifidobacterium dentium</i>	-	-	+	-	-	-	-	-	-	-
<i>Enterococcus faecalis</i>	-	-	-	-	-	-	-	+	-	-
<i>Gemella haemolysans</i>	-	-	-	-	+	-	-	-	-	-
<i>Granulicatella adiacens</i>	-	-	-	-	-	-	-	+	-	-
<i>Propionibacterium acnes</i>	+	-	+	+	+	+	+	+	+	+
<i>Propionibacterium avidium</i>	+	-	-	-	+	-	-	-	-	-
<i>Propionibacterium propionicum</i>	-	-	-	-	+	-	-	-	-	-
<i>Propionibacterium</i> sp. strain FMA5	-	-	+	-	-	-	-	-	-	-
<i>Rothia dentocariosa</i>	-	-	-	+	-	-	-	-	-	-
<i>Staphylococcus capare</i>	-	+	-	-	-	-	-	-	-	-
<i>Staphylococcus epidermidis</i>	+	+	-	-	+	-	+	-	-	+
<i>Staphylococcus warneri</i>	-	-	-	-	+	-	-	-	-	+
<i>Streptococcus anginosus</i>	-	-	-	-	-	+	+	-	-	-
<i>Streptococcus australis</i>	-	-	-	-	+	-	-	-	-	-
<i>Streptococcus constellatus</i>	-	+	+	-	-	-	-	-	-	-
<i>Streptococcus</i> genomospecies C8	-	-	-	-	+	-	-	-	-	-
<i>Streptococcus gordonii</i>	-	-	+	-	-	-	-	-	-	-
<i>Streptococcus infantis</i>	-	-	-	+	-	-	-	-	-	-
<i>Streptococcus mitis</i> /S. oralis	+	-	-	-	-	-	-	-	-	-
<i>Streptococcus mutans</i>	-	-	+	-	-	-	-	-	-	-
<i>Streptococcus sanguinis</i>	-	-	-	-	+	-	-	-	-	+
<i>Streptococcus</i> sp. oral taxon 071 strain Hans H6	-	-	-	-	+	-	-	+	-	-
<i>Streptococcus</i> sp. oral taxon 071 clone P4PA-13	-	-	+	-	-	-	-	-	-	-
Gram-negative organisms										
Obligate anaerobes										
<i>Anaeroglobus geminatus</i>	-	-	-	-	-	-	+	-	-	-
<i>Bacteroides thetaiotaomicron</i>	-	-	-	+	-	-	-	-	-	-
<i>Catonella morbi</i>	-	-	-	-	-	-	-	-	-	+
<i>Desulfobivrio fairfieldensis</i>	-	-	-	+	-	-	-	-	-	-
<i>Dialister invisus</i>	+	+	-	-	-	-	+	-	-	-
<i>Dialister pneumosintes</i>	+	-	-	-	-	-	-	-	-	-

Continued on following page

TABLE 2—Continued

Organism	Presence in endodontic lesion (with abscesses) no.:									
	12	13	14	15	16	17	18	19	20	
<i>Dialister</i> sp. oral clone BS095	—	—	—	—	—	+	—	—	—	
<i>Fusobacterium naviforme</i>	—	—	—	—	+	—	—	—	—	
<i>Fusobacterium nucleatum</i> subsp. <i>nucleatum</i>	—	—	—	—	—	—	—	+	—	
<i>Fusobacterium nucleatum</i> subsp. <i>polymorphum</i>	—	—	—	—	—	—	—	+	—	
<i>Fusobacterium nucleatum</i> subsp. <i>vincentii</i>	—	+	—	—	+	—	+	+	—	
<i>Oribacterium sinus</i>	—	—	—	—	—	—	—	—	+	
<i>Phascolarctobacterium faecium</i> ACM3679	—	—	—	+	—	—	—	—	—	
<i>Porphyromonas gingivalis</i>	+	+	—	—	—	—	—	—	—	
<i>Prevotella baroniae</i>	—	—	+	—	—	—	—	—	—	
<i>Prevotella buccae</i>	—	+	—	—	—	—	—	—	—	
<i>Prevotella melaninogenica</i>	—	—	—	—	—	—	—	—	+	
<i>Prevotella oralis</i>	+	—	—	—	—	—	—	—	—	
<i>Prevotella</i> sp. oral taxon 289	—	—	—	—	—	—	+	—	—	
<i>Prevotella tannerae</i>	+	—	—	—	—	—	—	—	—	
<i>Pyramidobacter piscolens</i>	—	—	+	—	—	—	—	—	—	
<i>Selenomonas noxia</i>	—	—	—	—	—	—	—	—	+	
<i>Tanerella forsythia</i>	+	—	+	—	—	—	+	—	—	
<i>Veillonella dispar</i>	—	+	—	—	—	—	—	+	—	
<i>Veillonella parvula</i>	—	—	—	—	—	—	—	—	+	
Facultative anaerobe										
<i>Capnocytophaga</i> sp. oral clone AA032	—	—	—	—	—	+	—	—	—	
<i>Campylobacter gracilis</i>	—	+	—	—	—	—	+	—	—	
<i>Campylobacter rectus</i>	—	+	—	—	—	—	—	—	—	
<i>Lautropia mirabilis</i>	—	—	—	+	—	—	—	—	—	

REP-PCR comparison of paired isolates. When the endodontic and skin *P. acnes* isolates from the same individuals (*n* = 13) were compared using REP-PCR, it was found that all skin *P. acnes* isolates were distinct from all the endodontic isolates except in 3 patients, for whom a REP-PCR pattern was common to some of the *P. acnes* isolates from both the skin and the endodontic lesion ($\chi^2 = 56.05$; *P* < 0.001). A typical comparison of the REP-PCR patterns of skin and endodontic isolates from the same person is shown in Fig. 3A. Similarly, in the case of *S. epidermidis*, when the REP-PCR patterns of endodontic and skin isolates from the same individual (*n* = 8) were compared, all skin isolates were clearly distinct from the endodontic ones ($\chi^2 = 37.73$; *P* < 0.001). A REP-PCR pattern comparing skin and endodontic *S. epidermidis* isolates from the same patient is shown in Fig. 3B.

Phenotypic comparison of *P. acnes* phylotypes. The phenotypic properties of the *P. acnes* phylotypes are shown in Table 4. The phenotypic properties which differentiate between *P. acnes* types IA, IB, and II were investigated using canonical variates analysis (Fig. 4). Type III strains were not investigated

due to the small number of isolates. The ability to ferment sorbitol and to produce sialidase and lipase activities differentiated between types IA, IB, and II.

DISCUSSION

We have used a culture-based approach to investigate the microbiota of refractory root canal infections. With a culture-based approach, it is likely that, overall, fewer taxa than are detectable with 16S rRNA cloning or cloning combined with culture approaches may be identified. Thus, in a study of 5 primary endodontic lesions in four patients, a mean of 20.2 taxa (range, 7 to 29) was identified using a combined cloning and cultural approach (40), whereas a mean of 14.1 taxa was identified from the refractory lesions with abscesses. We investigated refractory lesions which had previously been treated, so DNA derived from dead bacteria may be present, which might lead to an overestimation of the diversity of the microbiota if a 16S rRNA cloning approach was used. However, as the purpose of this study was not just to catalogue the taxa present

TABLE 3. Comparison of distribution of *P. acnes* *recA* phylotypes from all endodontic lesions and from the 13 paired endodontic samples with the distribution of *P. acnes* phylotypes identified from the perioral samples

Isolate	No. of isolates				<i>P</i> value (χ^2 test)
	Type IA	Type IB	Type II	Type III	
<i>P. acnes</i> from paired perioral lesions	36	26	3	0	4×10^{-8} < 10^{-10}
<i>P. acnes</i> from paired endodontic lesions	7	5	25	2	
All endodontic <i>P. acnes</i> isolates ^a	9	12	35	4	

^a *n* = 20.

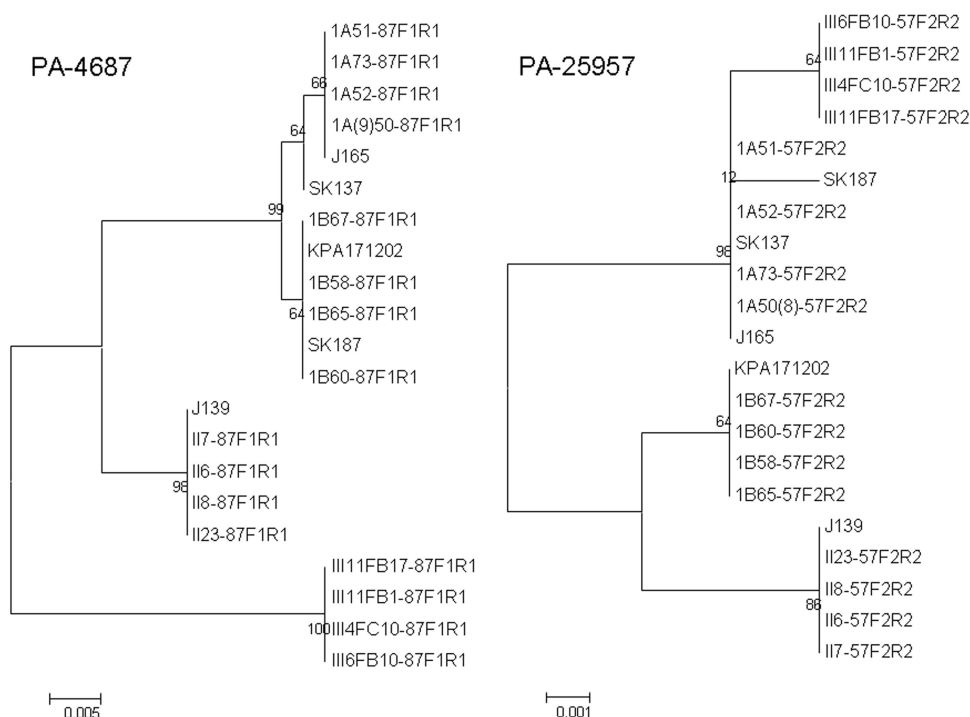


FIG. 1. Neighbor-joining trees comparing the taxonomic relationships of representatives of *P. acnes* types IA, IB, II, and III determined by analysis of partial sequences of PA-4687 and PA-25957. Bootstrap values are indicated at corresponding nodes. J165 and SK137 are type IA, SK187 and KPA171202 are type IB, and J139 is type II by *recA* sequencing. The phylotype of the isolates recovered in this study is denoted by the first letters of the strain name (1A [for type IA], 1B [for type IB], II, or III). Bars, 0.005 or 0.001 substitution per site.

at a site but to investigate the genotypic and phenotypic properties of *P. acnes* and *S. epidermidis*, it was necessary to culture the microbiota and to isolate these species.

Previous studies of the endodontic microbiota (2, 9, 28, 33, 38, 42, 51, 61) have recovered *P. acnes* and various *Staphylococcus* species, including *S. epidermidis*, but these organisms are generally regarded as contaminants since they are skin commensals. In the present study, we have used a decontamination protocol to remove contaminating organisms from the tooth surface prior to entering the diseased tooth, removed the gutta-percha points, sampled the infected root dentine bordering the root canal, and isolated *P. acnes* and *S. epidermidis*. The same species were also isolated from the perioral area and compared using REP-PCR DNA fingerprinting. The endodontic and skin isolates of both *P. acnes* and *S. epidermidis* are distinct and significantly different. These observations confirm the endodontic origin of *P. acnes* and *S. epidermidis* and the rejection of the hypothesis that these species recovered from endodontic samples are contaminants. While we did not investigate further the other *Propionibacterium* or *Staphylococcus* species, it must also be presumed that these are also infecting organisms. Foreign-body implants are one of the predisposing factors associated with numerous *P. acnes* infections (1, 14, 22, 23). *P. acnes* infections are also associated with the presence of foreign bodies in the root canals and may have been introduced into the root canals at the time of obturation of the canal with the gutta-percha or may have infected the canal subsequently, after failure. Similarly, *S. epidermidis* is associated with infections of prostheses, and it is also significant that *S. caprae*, which was isolated from 25% of patients and which is routinely

isolated from goats and may be used in cheese ripening, is also associated with infections in patients with an indwelling catheter or foreign materials, such as cardiac prostheses (27). This supports the hypothesis that the gutta-percha in the root canal may represent a foreign body susceptible to colonization by the same range of organisms that infect foreign materials or prostheses.

This hypothesis was supported when the *recA* phylotypes of the isolates were considered. We confirmed the previous observations, as nucleotide sequencing of the *recA* housekeeping gene of *P. acnes* isolates from both endodontic lesions and samples from perioral skin revealed only 3 different phylogenetic lineages (37, 38). In agreement with these observations, we found that *P. acnes* type I was the predominant phylotype on skin, whereas types II and III have rarely been recovered from samples from skin (37). In the present study, *P. acnes* type II and type III were the prevalent phylotypes in the endodontic infections, and the distribution of type II was similar to that reported for type II isolates from failed prosthetic hip implants (38), orthopedic implants (51), and radical prostatectomy specimens from subjects with prostate cancer (14). Type III strains have previously been recovered only from spine intervertebral disc material and from a prosthetic hip joint removed during revision arthroplasty. Here we failed to isolate any type III strains from the skin, but 4 isolates were identified from the infected root canals of 3 patients. The natural habitat of types II and III is not known, but these phylotypes apparently exhibit a tropism for inert foreign surfaces, or at least an ability to bind to such materials. Although *P. acnes* isolates of the type II phylotype were the most prevalent endodontic isolates in our

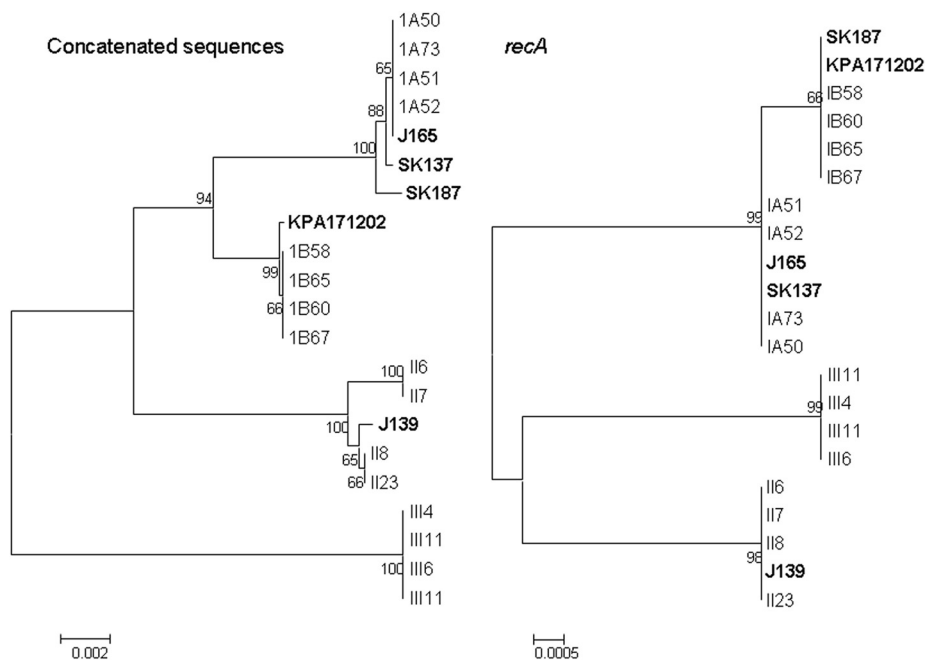


FIG. 2. Neighbor-joining trees comparing the taxonomic relationships of representatives of *P. acnes* types IA, IB, II, and III determined by analysis of a partial *recA* sequence and the concatenated partial sequences of 6 putative virulence determinants. Bootstrap values are indicated at the corresponding nodes. J165 and SK137 type are IA, SK187 and KPA171202 are type IB, and J139 is type II by *recA* sequencing. The phylotype of the isolates recovered in this study is denoted by the first letters of the strain name (1A [for type IA], 1B [for type IB], II, or III). Bars, 0.002 or 0.0005 substitution per site.

study, the virulence determinants have not been identified and none of the differential phenotypic characteristics investigated here appear to be relevant; and all isolates tested, irrespective of their phylotype, possessed genes coding for each of the 6 putative virulence determinants. However, all of the type III isolates have a 5-nt deletion in PA-4687, involved in iron transport, suggesting that this gene did not yield a functional product, which was supported by our inability to demonstrate

TABLE 4. Biochemical test results for 65 *P. acnes* isolates, including reference strains *P. acnes* type IA (NCTC 737) and *P. acnes* type II (NCTC 10390)^a

Phenotypic test	% of <i>P. acnes</i> isolates			
	Type IA (n = 20)	Type IB (n = 19)	Type II (n = 22)	Type III (n = 4)
Lactose	5	0	0	0
<i>N</i> -Acetylglucosamine	55	53	95	100
Erythritol	5	47	9	0
Ribose	95	89	100	100
Sorbitol	100	100	5	25
Urease	5	0	0	0
Arginine dihydrolase	95	100	95	100
β-Galactosidase	75	84	64	25
α-Glucosidase	15	84	77	50
β-Glucuronidase	5	16	0	0
β- <i>N</i> -Acetyl-glucosaminidase	100	100	95	100
Mannose fermentation	100	100	86	75
Raffose fermentation	0	5	0	0
Nitrate reduction	95	100	91	75
Indole production	95	79	59	50
Arginine arylamidase	40	37	59	25
Leucyl glycine arylamidase	10	11	18	0
Phenylalanine	0	0	5	0
Leucine arylamidase	0	0	5	0
Pyroglutamic acid arylamidase	5	11	9	0
Alanine arylamidase	100	95	82	50
Serine arylamidase	95	84	100	50
β- <i>N</i> -Acetyl-galatosaminidase	70	26	59	0
Sialidase	100	37	14	25
Lipase	95	26	0	25

^a Major differential characteristics distinguishing between types IA, IB, and II are highlighted in boldface. Data from type III strains were not included in the analysis due to the small number of isolates tested. All isolates were positive for glycine arylamidase activity and negative for β-fucosidase, α-fucosidase, β-glucosidase, α-galactosidase, α-arabinosidase, DNase, nonspecific proteinase, elastase, lecithinase, and hyaluronidase activities.

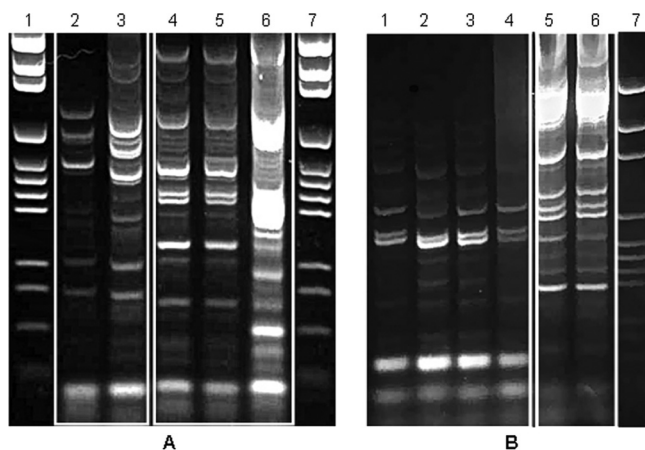


FIG. 3. REP-PCR patterns obtained from the *P. acnes* isolates of patient 6 (A) and the *S. epidermidis* isolates of patient 13 (B). (A) Lanes 1 and 7, molecular size marker (pGEM DNA markers; Promega); lanes 2 and 3, skin isolates; lanes 4 to 6, isolates from the endodontic sample. (B) Lane 7, molecular size marker; lanes 5 and 6, isolates from the endodontic sample; lanes 1 to 4, skin isolates.

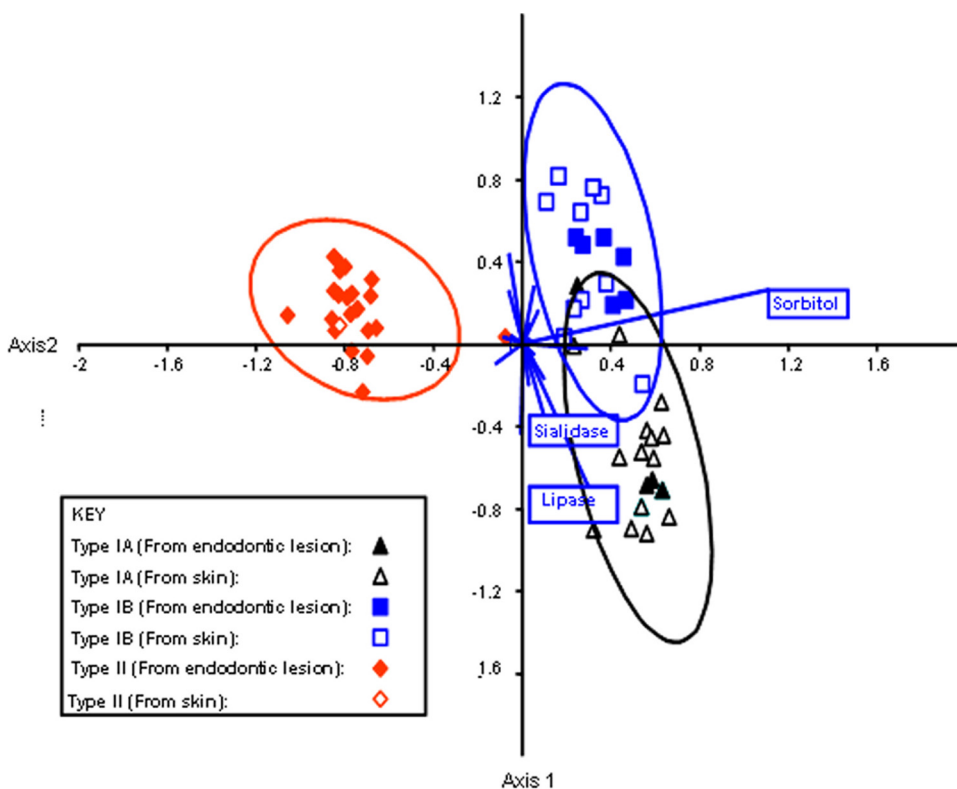


FIG. 4. Phenotypic differences between *P. acnes* type IA, type IB, and type II. The chart is a canonical variance analysis scatter plot of isolates along the first two canonical axes, showing maximal separation between the three groups. The null hypothesis of no phenotypic difference between the three groups was tested by multivariate analysis of a variance ($P < 0.01$, Bonferroni-corrected P values for comparison of pairs of groups) and nonparametric analysis of similarities ($P < 0.001$, Euclidean distance measure). The biplot (blue lines) displays the contribution of individual tests to the separation; the three most important tests are indicated.

mRNA for this gene in the reverse transcription-PCR experiments. The role of these genes in bacterial virulence remains unclear.

The *recA* typing of *P. acnes* isolates appears to be robust, as all isolates fell neatly into the three phylotypes. The significance of this typing is not clear, but it does provide a classification for *P. acnes* isolates which matches their site of isolation. Extensive DNA-DNA homology testing of *P. acnes* isolates suggests that the species is homogeneous (30), although it cannot be known if strains equivalent to types II and III were tested. To test the robustness of the *recA* typing scheme, we determined internal sequences of six putative virulence factors and used the concatenated sequences of these partial gene sequences in phylogenetic analyses. From previous investigations, it was apparent that the relationships found with *recA* sequencing were not always mirrored when the sequences of other *P. acnes* genes were compared. Thus, in a small study of 3 *P. acnes* strains, the sequences of CAMP factors 1, 2, 4, and 5 exhibited the same phylogenetic relationship between the type IA, IB, and II strains as that determined previously with *recA*; however, for CAMP factor 3, the type IA sequence had a closer relationship to the type II sequence, with the type IB sequence being more distinct (62). From the present analysis of the concatenated sequences, it is clear that types II and III remain distinct from the type I lineage. The type III strains are identical, despite being isolated from 3 different, unrelated

patients; but within the type II lineage, two distinct subclusters were evident, and this was supported by significant bootstrap values. The type I lineage shows greater rearrangement; notably, SK187 (type IB) is found within the type IA subcluster. Within both the type IA and IB subclusters, there is evidence of significant branching, with KPA17202, SK187, and SK137 being on discrete branches. These data demonstrate that while *recA* phylotyping permits facile classification of *P. acnes* strains, the use of more partial or complete gene sequences to establish phylogenetic relationships between strains gives rise to a more complicated population structure than was initially apparent. The reasons for the disparity between the *recA* typing and the relationships evident when the concatenated sequences were used to type the strains may be intraspecies recombination, for which evidence was obtained in the analyses presented here. This is also supported by the observation that not all the individual gene trees were congruent, with types IB and II being in the same lineage with Pap60 and all type I and II strains being on the same lineage with PA-5541, yet type II strains were on discrete lineages with each of the other four genes. It may be that the type II and III lineages have evolved, presumably from type I, to be better able to cause infections associated with foreign-body implants.

The gutta-percha used as root canal filling material is marketed unsterilized and may also be contaminated during root canal filling procedures, as may be the paper points used to dry

root canals prior to filling of the root canal. The results of the present study suggest that *S. epidermidis* and *P. acnes* isolates from the refractory lesions might be the result of nosocomial infections occurring at the time of the root canal treatment. Root canal treatments of infected teeth can be undertaken using a single- or a multivisit approach. The nosocomial nature of some endodontic infections would support the single-visit technique, as this would reduce the likelihood of such infections occurring.

ACKNOWLEDGMENTS

We acknowledge support from the Higher Education Commission of Pakistan; from the Dental Institute, King's College London; and from the Department of Health via the National Institute for Health Research (NIHR) Comprehensive Biomedical Research Centre Award to Guy's and St Thomas' NHS Foundation Trust in partnership with King's College London and King's College Hospital NHS Foundation Trust.

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