A new phylogenetic group of *Propionibacterium acnes*

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Immunofluorescence microscopy-based identification of presumptive *Propionibacterium acnes* isolates, using the *P. acnes*-specific mAb QUBPa3, revealed five organisms with an atypical cellular morphology. Unlike the coryneform morphology seen with *P. acnes* types I and II, these isolates exhibited long slender filaments (which formed large tangled aggregates) not previously described in *P. acnes*. No reaction with mAbs that label *P. acnes* types IA (QUBPa1) and II (QUBPa2) was observed. Nucleotide sequencing of the 16S rRNA gene (1484 bp) revealed the isolates to have between 99.8 and 99.9 % identity to the 16S rRNA gene of the *P. acnes* type IA, IB and II strains NCTC 737, KPA171202 and NCTC 10390, respectively. Analysis of the recA housekeeping gene (1047 bp) did reveal, however, a greater number of conserved nucleotide polymorphisms between the sequences from these isolates and those from NCTC 737 (98.9 % identity), KPA171202 (98.9 % identity) and NCTC 10390 (99.1 % identity). Phylogenetic investigations demonstrated that the isolates belong to a novel recA cluster or lineage distinct from *P. acnes* types I and II. We now propose this new grouping as *P. acnes* type III. The prevalence and clinical importance of this novel recA lineage amongst isolates of *P. acnes* remains to be determined.

INTRODUCTION

*Propionibacterium acnes* belongs to the ‘high GC’ group of Gram-positive bacteria and is found predominately in the sebaceous gland-rich areas of the skin in adults (McGinley et al., 1978). Although traditionally considered a relatively non-pathogenic member of the resident human microbiota, an increasing number of studies have implicated *P. acnes* as the agent responsible for various clinical conditions and infections. In addition to its well described role in inflammatory acne (Eady & Ingham, 1994), *P. acnes* is emerging as an important pathogen in relation to medical implant-related infections, such as those associated with central nervous system shunts (Brook & Frazier, 1991), silicone implants (Ahn et al., 1996), and prosthetic heart valves and hip joints (Delahaye et al., 2005; Tunney et al., 1998, 1999). Furthermore, *P. acnes* is responsible for endophthalmitis, ocular and periocular infections (Aldave et al., 1999; Clark et al., 1999; Horgan et al., 1999), as well as periodontal and dental infections (Debelian et al., 1992; LeGoff et al., 1997), and has been linked to synovitis–acne–pustulosis–hyperostosis–osteitis (SAPHO) syndrome (Kotilainen et al., 1996), sarcoidosis (Eishi et al., 2002) and prostate cancer (Cohen et al., 2005). The identification of a wide range of putative virulence determinants within the recently published genome sequence of the *P. acnes* strain KPA17202 has further brought the pathogenic potential of this organism into focus (Bruggemann et al., 2004).

Two distinct phenotypes of *P. acnes* (types I and II), which can be distinguished by serological agglutination tests and cell-wall sugar analysis, have been known for over 30 years (Johnson & Cummins, 1972). Additional studies have shown that these biovars display differences in the fermentation of sugar and sugar alcohols (Higaki et al., 2000; Kishishita et al., 1979), as well as their susceptibility to bacteriophage infection (Webster & Cummins, 1978). Sequence analysis of the *P. acnes* recA gene has revealed that types I and II correspond to phylogenetically distinct clusters or lineages (McDowell et al., 2005). These two clusters are, however, almost identical based on 16S rRNA sequencing. Analysis of the recA gene has also identified a subcluster of strains within *P. acnes* type I that have been...
designated type IB (McDowell et al., 2005; Valanne et al., 2005). These organisms do not react with the previously described mAb QUBPa1, specific for all other type I organisms (known as type IA) (McDowell et al., 2005; Valanne et al., 2005). However, variable labelling with the type II mAb QUBPa2, ranging from no reaction to a weak reaction, has been observed. In the latter case a significantly reduced fluorescence intensity and a reduction in the proportion of the bacterial population labelled is seen (McDowell et al., 2005). Type IB organisms also display differences from type IA strains in the production of putative Christie–Atkins–Munch–Peterson (CAMP) factor proteins (Valanne et al., 2005).

This paper describes the identification of a novel lineage of P. acnes based on recA sequence analysis, which we now formally propose as P. acnes type III. The isolates also display differences from P. acnes types I and II in their cell surface antigens and cellular morphology.

**METHODS**

**Bacterial strains.** The P. acnes reference strains NCTC 737 (type IA, ATCC6919) and NCTC 10390 (type II, ATCC12930) were obtained from the National Collection of Type Cultures (NCTC) (London, UK). A total of four isolates of P. acnes type III were recovered from spine intervertebral disc material removed during microdiscectomy procedures (for relief of severe sciatica), while one isolate was recovered from a prosthetic hip joint removed during revision arthroplasty. All surgeries were performed at the Royal Orthopaedic Hospital (ROH), Birmingham, England. A further 95 isolates of P. acnes (types I & II) were also included in our study for comparative purposes. A total of 15 of these isolates were recovered from failed prosthetic hip joints, as well as associated tissue, removed during revision arthroplasties; while a further 34 isolates were isolated from spine intervertebral disc material removed during microdiscectomy procedures at ROH. A total of 13 isolates from routine blood cultures, 18 from acne lesions and 15 from normal skin were obtained from the Queen Elizabeth Hospital, Birmingham, England. Isolates of Propionibacterium granulosum and Propionibacterium avidum recovered from patients with acne lesions were a kind gift from Professor Keith Holland. Ethical approval was obtained from the Birmingham Local Research Ethics Committee and informed patient consent was obtained in all cases.

**Bacterial culture.** Strains were grown on anaerobic blood agar (ABA) (CM0972; Oxoid). Cultures were incubated at 37 °C in an anaerobic cabinet (MAG MG 1000; Don Whitley Scientific), in an atmosphere of 80% N2, 10% CO2 and 10% H2. Isolates were examined using the API ID 32A and API 20A biochemical identification systems (bioMérieux) in accordance with the manufacturer’s instructions. Fermentation analyses with the substrates sorbitol, ribose and erythritol were carried out as previously described (McDowell et al., 2005). Putative virulence factors, namely haemolytic, proteinase, lipase, lecithinase, DNase, elastase and hyaluronidase activities, were detected as reported by Balke & Weiss (1984) and Spare et al. (2003).

**Immunofluorescence microscopy (IFM).** IFM was conducted on multiwell slides as described previously (McDowell et al., 2005). Strains were examined with the mAbs QUBPa1 and QUBPa2, which label P. acnes type IA and II, respectively, as well as mAb QUBPa3, which reacts with all P. acnes strains examined to date (Tunney et al., 1999). Slides were read using a Leitz Dialux 20 fluorescence microscope. Images were captured and bacterial cell lengths determined using LUCIA G software following the manufacturer’s instructions (Laboratory Imaging).

**Nucleotide sequence analysis.** Nucleotide sequence analysis was performed on 16S rRNA and recA genes. The 16S rRNA gene (1484 bp) was amplified using the universal primers UFPL and URPL (LiPuma et al., 1999), while recA was amplified with the primers PAR-1 and PAR-2, which are directed to downstream and upstream flanking sequences of the recA ORF, respectively, and generate a 1201 bp amplicon (McDowell et al., 2005). PCR products were verified by electrophoresis on 1% (w/v) agarose gels and duplicate samples for each gene pooled before purification with a QIAquick PCR purification kit (Qiagen) (McDowell et al., 2005). Sequencing reactions were performed using ABI PRISM ready reaction terminator cycle sequencing kits (version 1.1) (Perkin-Elmer Applied Biosystems) according to the manufacturer’s instructions and the samples analysed on an ABI PRISM 3100 genetic analyser capillary electrophoresis system (Perkin-Elmer Applied Biosystems). Raw sequences from both DNA strands were obtained by using the appropriate forward and reverse primers. Internal sequencing primers were also used to facilitate determination of the larger 16S rRNA gene sequence. Initial sequences were screened using the Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.nih.gov) to confirm gene identity.

**Phylogenetic analysis.** Multiple nucleotide sequence alignments (with no gaps) of the recA gene (1047 bp) from all five atypical isolates, as well as complete recA sequences for previously published strains representative of types I and II (McDowell et al., 2005), were constructed using CLUSTAL W (Thompson et al., 1994). Phylogenetic analyses were conducted using the genetic distance-based neighbour-joining algorithms within MEGA version 3.1 (http://www.megasoftware.net/) and phylogenetic trees constructed using the Jukes–Cantor matrix model. The sequence input order was randomized and bootstrapping resampling statistics were performed using 100 datasets for each analysis.

**Nucleotide sequence accession numbers.** Nucleotide sequences for 16S rRNA and recA gene sequences generated during this study were submitted to GenBank and the accession numbers DQ672257–DQ672261 and DQ672246–DQ672256 generated, respectively.

**RESULTS AND DISCUSSION**

**IFM.**

During routine IFM-based identification of clinical isolates presumptively identified as P. acnes by biochemical analysis, five organisms (designated Asn10–Asn14) with an atypical morphology were identified after labelling with the mAb QUBPa3, which reacts with a carbohydrate or glycolipid-containing antigen on the surface of all P. acnes cells (Tunney et al., 1999). Four of these isolates were recovered from spine intervertebral disc material (excised disc protrusion) removed during microdiscectomy procedures for severe sciatica, while one was from a prosthetic hip arthroplasty. Compared to the classical coryneform morphology normally seen with types I and II (i.e. clubs, ‘tadpole’ forms and short bifid forms), these isolates consisted of individual cells of variable length and long slender filaments that formed very large tangled aggregates.
(Figs 1 and 2). This was also observed upon Gram staining of the cells (not illustrated). No labelling with the mAbs QUBPa1 and QUBPa2, which react with a proteinaceous and carbohydrate/glycolipid-containing antigen on types IA and II (McDowell et al., 2005), respectively, was detected for any of the isolates. This provides evidence that the cell surface antigens recognized by these mAbs either are not present, or are structurally distinct to those found on types I and II giving rise to a different set of epitopes.

Analysis of cells from one of these isolates (Asn12), captured from three different fields of view of an IFM image using a × 100 objective, revealed individual cells and filaments that varied in length from 1.20 to 21.8 μm, with widths ranging from 0.71 to 0.96 μm. However, much longer filaments, which could be difficult to identify due to a greater tendency to form or to be associated with large aggregates, may also be present. For comparison, analysis of individual cells of NCTC 737 (type IA) and NCTC 10390 (type II) in a similar manner revealed lengths that ranged from 0.84 to 2.56 μm and 0.89 to 2.80 μm, respectively, with widths ranging from 0.63 to 0.88 μm and 0.66 to 0.86 μm, respectively. While filaments have not been described before in P. acnes, slender or fine branching filaments (5–20 μm or greater in length) are a characteristic feature of Propionibacterium propionicum (previously Arachnia propionica), along with short irregular rods of variable length that are commonly arranged in pairs (Holt et al., 1994). Unlike the atypical P. acnes isolates described in this study, however, P. propionicum does not show any reaction with the mAb QUBPa3 (A. McDowell & S. Patrick, unpublished data). Filaments that are associated with branching and hyphal formation are also characteristic of other members of the Actinomycetales, such as the Nocardiooses, and the less closely related Actinomyces and Rhodococcus (Holt et al., 1994). It is interesting to note that in addition to resembling Actinomycetes israelii morphologically, P. propionicum can also cause actinomycosis, a chronic, subacute suppurating granulomatous infection (Hall, 2006). Whether these P. acnes strains also share any similarities with P. propionicum and Actinomyces spp. in terms of pathogenicity remains to be determined. Their ability to form filaments may impact on the potential for colonization, particularly in relation to tissue penetration and biofilm formation. It is possible that type III isolates have remained unidentified as P. acnes due to their atypical morphology, as would be observed upon routine Gram staining.

**Fig. 1.** Micrographs of P. acnes (after growth on ABA plates) immunolabelled with the mouse IgG mAb QUBPa3 and a FITC-conjugated goat anti-mouse IgG antibody (magnification ×100): (a) Asn10 (type III), (b) Asn12 (type III), (c) NCTC 737 (type IA), (d) NCTC 10390 (type II).

**Fig. 2.** Micrograph of a large filamentous aggregate from the P. acnes type III isolate Asn12 (after growth on an ABA plate). Filaments were immunolabelled with the mouse IgG mAb QUBPa3 and a FITC-conjugated goat anti-mouse IgG antibody (magnification ×100).

**Nucleotide sequence and phylogenetic analysis**

Systematic analysis of all five atypical P. acnes isolates was initially conducted by direct nucleotide sequencing of a 1484 bp fragment of the 16S rRNA gene amplified with the universal 16S rRNA-based primers UFPL and URPL. The GenBank accession numbers for these 16S sequences are listed in Table 1. Upon CLUSTAL W analysis, sequences obtained for all the isolates had between 99.8 to 99.9% identity to the previously published sequences for NCTC 737 (type IA, GenBank accession no. AB042288) KPA171202 (type IB, GenBank accession no. NC_006085) and NCTC 10390 (type II, GenBank accession no. AK642044), thus confirming the very close relationship between these organisms and types I and II. In contrast, when a 1473 bp sequence stretch of the 16S rRNA gene...
A 1201 bp fragment containing the recA gene was successfully amplified from all five atypical isolates, using the previously described primers PAR-1 and PAR-2 (McDowell et al., 2005), and sequenced. The GenBank accession numbers for these recA sequences are listed in Table 1. Other closely related Propionibacterium species, such as P. granulosum, do not show any reaction with this particular primer pair (A. McDowell & S. Patrick, unpublished data). Upon CLUSTAL W analysis, the recA sequences (1047 bp) for these isolates showed distinct differences from those previously published for NCTC 737 (GenBank accession no. AY642055, 98.9 % identity), KPA171202 (GenBank accession no. NC_006085, 98.9 % identity) and NCTC 10390 (GenBank accession no. AY642061, 99.1 % identity). Strains of P. acnes types I (IA & IB) and II differ in 10 highly conserved regions within the recA gene (99 % identity). For our group of atypical isolates, the recA gene sequences were found to contain a combination of four type I-specific polymorphisms (common to IA and IB), six type II-specific polymorphisms and five polymorphisms unique to the group (the sequence alignments are shown in Supplementary Fig. S1 available with the online journal). To investigate the phylogenetic relationship between these five isolates and types I and II, a recA phylogenetic tree was constructed based on the nucleotide sequences (Fig. 3). Previously published recA sequences for types IA, IB and II were included in the analysis for comparison (McDowell et al., 2005). Strains of P. acnes types I, II and this novel grouping formed highly distinct branches within the tree supported by bootstrap values of 98 % or 100 %. This demonstrates that the atypical isolates identified by IFM represent a new or novel recA phylogenetic cluster or lineage, which we propose as P. acnes type III. To facilitate further studies of the type III grouping the isolate Asn12 (recovered from intervertebral disc material) will be deposited in the NCTC bacterial cell bank as a representative of this novel phylogenetic cluster.

These results again highlight the potential limitations to the use of the 16S rRNA locus alone in understanding bacterial phylogeny. This may be especially important for studies that attempt to identify novel bacterial groupings or phylotypes within microbial communities, such as those present on the skin, using culture-independent 16S rRNA-based methods (Dekio et al., 2005). For such investigations the use of protein-encoding genes with housekeeping functions may provide more valuable information for

Table 1. Nucleotide sequence analysis of P. acnes isolates with atypical morphology

<table>
<thead>
<tr>
<th>Isolate</th>
<th>GenBank 16S rRNA accession no.</th>
<th>GenBank recA accession no.</th>
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<tbody>
<tr>
<td>Asn10</td>
<td>DQ672257</td>
<td>DQ672252</td>
</tr>
<tr>
<td>Asn11</td>
<td>DQ672258</td>
<td>DQ672253</td>
</tr>
<tr>
<td>Asn12</td>
<td>DQ672259</td>
<td>DQ672254</td>
</tr>
<tr>
<td>Asn13</td>
<td>DQ672260</td>
<td>DQ672255</td>
</tr>
<tr>
<td>Asn14</td>
<td>DQ672261</td>
<td>DQ672256</td>
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Fig. 3. Unrooted phylogenetic tree of P. acnes based on the complete recA gene sequence, illustrating the three recA lineages of the organism. Multiple sequence alignments were performed on the recA gene sequences from type III isolates and published sequences representative of type IA, IB and II isolates (McDowell et al., 2005) (with no gaps in the aligned sequence). Bootstrapping resampling statistics was performed using 100 datasets, with bootstrap values shown on the arms of the tree. The type status for the different strains analysed is also shown.
putative virulence genes (McDowell et al., 2005; Valanne et al., 2005). This demonstrates that where rates of sequence divergence are appropriate, non-housekeeping gene sequences can also provide data that may be useful for bacterial systematics. Although P. acnes isolates can be assigned to the type III phylogenetic grouping based on analysis of their cellular morphology (morphovars), we would recommend sequencing of the recA gene for unambiguous classification. On the basis of previous DNA–DNA hybridization studies (Johnson & Cummins, 1972), types I and II would appear to represent distinct sequence clusters within P. acnes rather than novel species, at least based on current definitions of a bacterial species (Vandamme et al., 1996; Cohan, 2002). Similar experiments will now have to be carried out with isolates from the type III grouping to confirm if this is also the case for these organisms.

### Biochemical analysis

Unequivocal identification of the type III isolates as P. acnes (99.9% identity) was observed on the basis of analysis with the API ID32A and API 20A biochemical galleries. All type III isolates were positive for indole and nitrate reduction characteristic of P. acnes, but not P. granulomae or P. avidum (Holt et al., 1994). The isolates were negative for sorbitol and erythritol fermentation, but could ferment ribose (biotype 4), as well as glucose and glycerol. The production of acid from mannose was variable between the isolates. In keeping with other P. acnes strains, the type III isolates did not produce acid from lactose, salicin, xylose, maltose, arabinose, cellobiose, melezitose, raffinose or rhamnose, and were negative for urease and β-glucosidase activity. All type III organisms were positive for catalase activity, but arginine dihydrolase activity was variable.

In addition to studies focused on understanding the metabolic profile of type III organisms, we also investigated possible differences in the production of virulence factors between all type III isolates and 95 P. acnes isolates representing types I (n=75) and II (n=20) (Table 2). These isolates, which were recovered from different sources at the ROH and Queen Elizabeth Hospital, had been routinely identified to the level of type I or II on the basis of sugar fermentation profiles (Higaki et al., 2000), random amplification of polymorphic DNA fingerprints (Perry et al., 2003) and, for some samples, IFM (with QUPPa1 and QUPPa2) (McDowell et al., 2005). All type III isolates were positive for lipase activity, but variable in their production of proteinases and hyaluronidase. They were, however, negative for lecithinase activity, as well as α- and β-haemolytic activities. Type I isolates were found to be variable for α- and β-haemolysis, while type II isolates were negative for β-haemolysis, but did display variable α-haemolytic activity. The production of lipase, lecithinase, proteinases and hyaluronidase activities was variable for type I and II isolates (Table 2). No DNase or elastase activity was detected in any of the P. acnes isolates examined. PCR analysis of type III isolates revealed the presence of the cohaemolysin or CAMP factor gene family, as well as the tly gene encoding a putative haemolysin/cytotoxin (not illustrated), which have already been described in types I and II (Valanne et al., 2005; McDowell et al., 2005). Further studies with a greater range of isolated type III strains will be required to confirm all these observations. Also, continued investigation of the virulence profile of the various P. acnes types is warranted, especially as distinct phylogenetic groups of an organism can display differences in their pathogenic potential (Ishii et al., 2007).

In conclusion, we have identified a novel phylogenetic grouping or lineage of P. acnes that we propose as type III. The identification of a third phylogenetic cluster in P. acnes further challenges our understanding of this organism, highlights potential caveats in the use of only one isolate type in laboratory studies of P. acnes virulence, and raises the possibility that other phylogenetic groups of the organism may exist.

### ACKNOWLEDGEMENTS

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**Table 2. Production of virulence factors by P. acnes types I, II and novel group III**

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<tr>
<th>Phylogenetic group</th>
<th>No. of isolates producing each putative virulence factor (percentage total of each type)*</th>
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<tbody>
<tr>
<td></td>
<td>α-Haemolysis</td>
</tr>
<tr>
<td>I (n=75)</td>
<td>2 (3)</td>
</tr>
<tr>
<td>II (n=20)</td>
<td>4 (20)</td>
</tr>
<tr>
<td>III (n=5)</td>
<td>0 (0)</td>
</tr>
<tr>
<td>Total (n=100)</td>
<td>6 (6)</td>
</tr>
</tbody>
</table>

*All isolates negative for DNase and elastase activity.
REFERENCES


