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Multiplex Touchdown PCR for Rapid Typing of the Opportunistic Pathogen *Propionibacterium acnes*

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The opportunistic human pathogen *Propionibacterium acnes* is composed of a number of distinct phylogroups, designated types IA_1, IA_2, IB, IC, II, and III, which vary in their production of putative virulence factors, their inflammatory potential, and their biochemical, aggregative, and morphological characteristics. Although multilocus sequence typing (MLST) currently represents the gold standard for unambiguous phylogroup classification and individual strain identification, it is a labor-intensive and time-consuming technique. As a consequence, we developed a multiplex touchdown PCR assay that in a single reaction can confirm the species identity and phylogeny of an isolate based on its pattern of reaction with six primer sets that target the 16S rRNA gene (all isolates), ATPase (types IA_1, IA_2, and IC), *sodA* (types IA_2 and IB), *atpD* (type II), and *recA* (type III) housekeeping genes, as well as a Fic family toxin gene (type IC). When applied to 312 *P. acnes* isolates previously characterized by MLST and representing types IA_1 ($n = 145$), IA_2 ($n = 20$), IB ($n = 65$), IC ($n = 7$), II ($n = 45$), and III ($n = 30$), the multiplex displayed 100% sensitivity and 100% specificity for detecting isolates within each targeted phylogroup. No cross-reactivity with isolates from other bacterial species was observed. This multiplex assay will provide researchers with a rapid, high-throughput, and technically undemanding typing method for epidemiological and phylogenetic investigations. It will facilitate studies investigating the association of lineages with various infections and clinical conditions, and it will serve as a prescreening tool to maximize the number of genetically diverse isolates selected for downstream higher-resolution sequence-based analyses.

Propionibacterium acnes is an anaerobic-to-aerotolerant Gram-positive bacterium, which exists in nature as a human commensal and opportunistic pathogen. It is a major component of the human skin microbiota but can also be recovered from the oral cavity and the gastrointestinal and genitourinary tracts (1). Although *P. acnes* is the main cause of opportunistic human infections within the cutaneous group of propionibacteria and is well known for its association with the inflammatory skin condition acne vulgaris (2, 3), its roles in other human infections and clinical conditions are likely to have been significantly underestimated (4–6). Despite this, we now see a growing recognition that the bacterium is an important cause of human disease, especially in relation to indwelling medical device-related infections (7–12), and it may also play a role in chronic conditions that cause significant morbidity and mortality, including low back pain associated with modic type I changes (13), sarcoidosis (14, 15), and prostate cancer (16, 17).

Within the last 10 years, phylogenetic studies based on single and multilocus gene sequencing (18–21), as well as whole-genome analyses of isolates from the Human Microbiome Project (HMP) and other studies (22–28), have provided valuable insights into the genetic population structure of *P. acnes*, particularly in the context of health and disease. The bacterium has an overall clonal structure, and its isolates can be classified into a number of statistically significant clades or phylogroups designated types IA_1, IA_2, IB, IC, II, and III; these types appear to display differences in their associations with specific types of infections (20, 21) and vary in their production of putative virulence determinants (19, 20, 29–32), inflammatory potential (33–36), antibiotic resistances (21, 37), aggregative properties (16), and morphological characteristics (19). In particular, a number of independent epidemiological studies have shown a strong association between clonal complexes from the type IA_1 phylogroup and moderate-to-severe acne, while the lineages from all other phylogroups appear more frequently to be isolated from medical device and soft tissue infections or be associated with health as true commensals (20, 21, 31, 38). Despite these associations, much uncertainty still exists regarding the exact clinical relevance of these phylogroups, particularly in the context of acne when skin sampling methods may not be optimal or appropriate (39), as well as the wider issue of whether isolates recovered from different clinical samples are truly representative of infection in all contexts or are simply skin contaminants/passive bystanders within a sample. These issues are common when dealing with an opportunistic pathogen that is also part of the normal microbiota, and untangling clinically relevant isolates from background contaminants can be a challenge. Future studies aimed at addressing such issues will undoubtedly provide a more solid platform on which we can make definite conclusions regard-

Received 26 August 2014 **Returned for modification** 24 September 2014
Accepted 18 January 2015

Accepted manuscript posted online 28 January 2015

Citation Barnard E, Nagy I, Hunyadkürti J, Patrick S, McDowell A. 2015. Multiplex touchdown PCR for rapid typing of the opportunistic pathogen *Propionibacterium acnes*. J Clin Microbiol 53:1149–1155. doi:10.1128/JCM.02460-14.

Editor: C.-A. D. Burnham

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Supplemental material for this article may be found at http://dx.doi.org/10.1128/JCM.02460-14.

doi:10.1128/JCM.02460-14

TABLE 1 Multiplex PCR primer characteristics

Primer[a]	Specificity	Gene(s) targeted	Sequence (5′ to 3′)	Positions	Concn (μM)	Annealing temp (°C)	Amplicon size (bp)
PArA-1	All *P. acnes*	16S rRNA	AAGCGTGAGTGACGGTAATGGGTA	442–465	0.2	66	677
PArA-2			CCACCATAACGTGCTGGCAACAGT	1118–1095			
PAMp-1	Type IA_1/IA_2/IC	ATPase	GCGTTGACCAAGTCCGCCGA	451–470	0.25	66	494
PAMp-2			GCAAATTCGCACCGCGGAGC	944–925			
PAMp-3	Type IA_2/IB	*sodA*	CGGAACCATCAACAAACTCGAA	168–189	0.6	62	145
PAMp-4			GAAGAACTCGTCAATCGCAGCA	312–291			
PAMp-5	Type IC	Toxin, Fic family	AGGGCGAGGTCCTCTTCTACCAGCG	17–41	0.1	66	305
PAMp-6			ACCCTCCAACTGCAACTCTCCGCCT	321–297			
PAMp-7	Type II	*atpD*	TCCATCTGGCCGAATACCAGG	339–360	0.15	66	351
PAMp-8			TCTTAACGCCGATCCCTCCAT	689–669			
PAMp-9	Type III	*recA*	GCGCCCTCAAGTTCTACTCA	641–660	0.25	66	225
PAMp-10			CGGATTTGGTGATAATGCCA	865–846			

[a] For protein-coding housekeeping genes, the primers relate to positions within the open reading frame. For the 16S rRNA gene, the primers relate to positions within the sequence for *P. acnes* NCTC 737 (GenBank accession no. AB042288).

ing the association of specific *P. acnes* phylogroups with human disease.

To date, a number of phenotypic and/or molecular approaches have been investigated as methods for phylogroup identification, ranging from very simple biochemical tests based on hemolysis (19) or fermentation profiles (40) to matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF MS) (41), monoclonal antibody (MAb) typing (18), and DNA-based analysis; DNA-based analysis includes ribotyping (23), DiversiLab analysis (42), direct PCR assays (43), and protein-coding gene sequencing (18, 20, 21, 44). Unfortunately, many of these approaches suffer from specificity or sensitivity limitations that constrain their diagnostic value. For example, previously described direct PCR assays do not differentiate IA_1 from type IA_2 or IC or target type III strains (43); they may also give ambiguous results (44). Single-locus nucleotide sequencing, which to date has been primarily based on the *recA* housekeeping and *tly* methyltransferase/hemolysin genes, is robust for identifying types I, II and III but displays reduced specificity for differentiating type IB from type IA_2 and some strains within the type IA_1 clade, as they contain identical alleles due to horizontal gene transfer (HGT) (20, 21, 23, 38). More recently, MAb typing with antibodies targeting types IA, IC, and II combined with *recA* sequence analysis has obviated this problem, facilitating the accurate differentiation of type IB from all type IA strains (45). MALDI-TOF MS has also been described as a valuable and powerful approach for rapid and high-throughput phylogroup identification but currently will not differentiate type IA_1 from IA_2 (41); furthermore, the technology is not available within all laboratories. At present, multilocus sequence typing (MLST) of *P. acnes* (20, 21) still represents the clear gold standard for unambiguous phylogroup identification, as well as individual strain resolution (see Table S1 in the supplemental material), and it offers significant advantages over comparable high-resolution gel-based typing methodologies, including random amplification of polymorphic DNA (RAPD) (46) and pulsed-field gel electrophoresis (PFGE) (47, 48), which have also been applied to this bacterium. The MLST method is, however, technically demanding, time-consuming, and expensive, especially when analyzing multiple isolates. Consequently, and as a result of the growing interest surrounding the role of this microbe in disease, there is a need for a rapid, less labor-intensive, and inexpensive method for the typing and stratification of *P. acnes* isolates, thus facilitating future molecular epidemiological and phylogenetic studies. Against this background, we describe here the development and validation of a multiplex touchdown PCR assay that can be used for quick, high-throughput, and accurate molecular confirmation of *P. acnes* isolates combined with a parallel disclosure of their phylogeny. This assay should prove valuable for researchers and, along with MLST, form part of a molecular typing toolbox that can be used for the analysis of *P. acnes* isolates.

MATERIALS AND METHODS

Bacterial strains and growth. A total of 312 *P. acnes* isolates were used to validate the multiplex PCR (145 type IA_1, 20 type IA_2, 65 type IB, 7 type IC, 45 type II, and 30 type III isolates). These isolates were previously recovered from a wide range of clinical sources and healthy skin, and their phylogroup status was determined using an MLST scheme based on eight genes (38). Representative samples of isolates from each group were also analyzed by MAb typing, as previously described (18), and their reactivities were consistent with their phylogroup designations based on MLST. Genomic DNA from a panel of 49 isolates representing 34 medically relevant bacterial species, which also included other human *Propionibacterium* species, was also used in the assessment of multiplex specificity (see Table S2 in the supplemental material). All bacterial strains were maintained at −80°C in brain heart infusion (BHI) broth containing 12% (vol/vol) glycerol. The anaerobic isolates were cultured in BHI and on anaerobic horse blood agar (ABA) plates (Oxoid Ltd., Hampshire, United Kingdom) at 37°C in an anaerobic cabinet (Mark III; Don Whitley Scientific) under an atmosphere of 10% H_2, 10% CO_2, and 80% N_2. The aerobic bacteria were cultured on horse blood agar at 37°C.

Development of phylogroup-specific primers. Housekeeping gene sequences representing *aroE* (424 bp), *atpD* (453 bp), *gmk* (400 bp), *guaA* (493 bp), *lepA* (452 bp), and *sodA* (450 bp) were retrieved from the *P. acnes* MLST database (http://pubmlst.org/pacnes/). The sequences for each gene were then aligned using the MEGA version 5.1 software and inspected for phylogroup-specific polymorphisms. Phylogroup-specific genomic regions were also investigated using the progressiveMauve algorithm (version 2.3.1) and the Artemis Comparison Tool (ACT) (http://www.sanger.ac.uk/Software/ACT/) using whole-genome sequences (WGS) currently available as part of the HMP and other sequencing projects (http://www.ncbi.nlm.nih.gov/genome/genomes/1140) (see Table S1 in the supplemental material). Based on these analyses, phylogroup-specific primer sets were developed and are listed in Table 1.

Multiplex PCR analysis. Bacterial genomic DNA was prepared using an AquaGenomic kit (MultiTarget Pharmaceuticals). PCR amplification was carried out using a MultiGene thermocycler (Labnet International,

Inc., United Kingdom). The samples contained 1× PCR buffer, 200 μM each deoxynucleoside triphosphate (Invitrogen Life Technologies, United Kingdom), six primer sets targeting each phylogroup at the concentrations described in Table 1, 1.5 mM $MgCl_2$, 1× *Redi*Load (Invitrogen Life Technologies), 1.25 U *Taq* DNA polymerase (Invitrogen Life Technologies), and 50 ng of pure genomic DNA preparation in a total volume of 10 μl. The samples were initially heated at 94°C for 1 min, followed by 14 cycles consisting of 94°C for 30 s, 66°C (decreasing incrementally by 0.3°C per cycle) for 30 s, and 72°C for 1 min, followed by 11 cycles at 94°C for 30 s, 62°C for 30 s, and 72°C for 1 min, culminating with a final cycle at 72°C for 10 min. A negative control (PCR water) and six positive-control samples representing all phylogroups were included in all experiments. The PCR products were analyzed by electrophoresis on 1.5% (wt/vol) agarose gels containing 1× Tris-acetate-EDTA buffer. The molecular size markers were run in parallel on all gels. The resolved DNA products were stained with 1× GelRed nucleic acid gel stain (Cambridge Biosciences, United Kingdom).

Nucleotide sequencing. The sequencing reactions were performed using the BigDye Terminator cycle sequencing kit (version 1.1) (Life Technologies, United Kingdom), according to the manufacturer's instructions. The samples were then analyzed on an ABI Prism 3100 genetic analyzer capillary electrophoresis system (Life Technologies).

Split decomposition analysis. Split decomposition analysis was performed using SplitsTree4 version 4.13.1 (49).

RESULTS

Primer design. Polymorphisms in multiple aligned sequences of the *sodA* gene specific for types IA_2 and IB, and the *atpD* and *recA* genes specific to types II and III, respectively, were identified as candidate regions for primer development (Table 1). Primers were also developed against the ATP-binding component (ATPase; GenBank accession no. ABB20821.1) of a previously described ABC-type peptide uptake operon (GenBank accession no. DQ208967) present in the closely related type IA_1, IA_2, and IC groups but absent in type IB, II, and III strains (Table 1) (43). This operon also includes genes encoding permeases (GenBank accession no. ABB20819.1 and ABB20820.1) and a solute binding protein (GenBank accession no. ABB20823.1) alongside genes for a glycoside hydrolase (GenBank accession no. ABB20818.1) and chitinase (GenBank accession no. ABB20824). For the type IC strains, we developed a primer set targeting a Fic family toxin gene located on an approximately 7.3-kb genomic fragment present in the draft genome sequences of the *P. acnes* type IC strains PRP-38 (TICEST70_07737) and HL097PA1 (HMPREF9344_02057) but not other phylogroups (Table 1). This genomic fragment also contained restriction enzyme-associated genes and a gene encoding a DEAD/DEAH box helicase (HMPREF9344_02061). Our previously described *P. acnes*-specific 16S rRNA gene-based primers were also included in the assay to confirm species identity (Table 1) (11, 50). The primer sets incorporated phylogroup-specific mismatches at the 3′ end, and elsewhere in the sequence when available, and were designed to have identical annealing temperatures where possible and to generate amplicons with characteristic size differences that would facilitate easy visual identification on a gel after multiplexing.

Multiplex PCR development and validation. Each individual phylogroup-specific primer set was initially examined against a small panel of strains ($n = 40$) representing types IA_1, IA_2, IB, IC, II, and III. Amplicons of the predicted size were correctly generated from the targeted phylogroup, and no products were unexpectedly observed in the divisions outside those targeted by the primers (data not shown). The identity of each PCR product was confirmed by direct nucleotide sequence analysis (data not shown). Each primer set was then combined into a single multiplex touchdown PCR, which was optimized for final primer and $MgCl_2$ concentrations as well as amplification cycles, as outlined in Materials and Methods. As the *sodA* primers PAMp-3/PAMp-4 had a lower annealing temperature (62°C) than that of all the other primer sets (66°C), a touchdown PCR approach was adopted to ensure satisfactory highly specific amplification of all gene targets within the assay. Using this approach, it proved possible to reliably determine the phylogeny of an isolate based on the combination of different phylogroup-specific amplification products, as illustrated in Fig. 1.

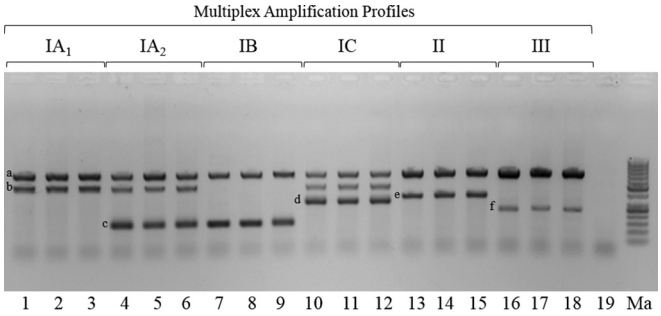

FIG 1 Multiplex PCR analysis of *P. acnes* strains (except lane 19) representing different phylogroups and STs. Lane 1, strain hdn-1 (ST1, type IA_1); lane 2, strain PRP-60 (ST20, type IA_1); lane 3, strain 76793 (ST101, type IA_1); lane 4, strain Pacn33 (ST2, type IA_2); lane 5, strain P.acn17 (ST2, type IA_2); lane 6, strain P. acn31 (ST2, type IA_2); lane 7, strain 6609 (ST5, type IB); lane 8, strain VA3/4 (ST78, type IB); lane 9, strain 74874 (ST43, type IB); lane 10, strain PRP-38 (ST70, type IC); lane 11, strain PV66 (ST85, type IC); lane 12, strain 5/1/3 (ST107, type IC); lane 13, strain ATCC 11828 (ST27, type II); lane 14, strain VA2/9N (ST28, type II); lane 15, strain 6187 (ST30, type II); lane 16, strain 12S (ST32, type III); lane 17, strain Asn12 (ST33, type III); lane 18, strain Asn10 (ST81, type III); lane 19, *Propionibacterium avidum* strain 44067. Ma, molecular size markers. ST is based on the eight-gene MLST scheme of McDowell et al. (21) and the database at http://pubmlst.org/pacnes/. Gene amplicons (left to right): a, 16S rRNA; b, ATPase; c, *sodA*; d, toxin; e, *atpD*; f, *recA*.

To assess the sensitivity and specificity of the multiplex assay, especially in relation to primers targeting genomic regions that were presumptively present/absent between phylogenetic divisions based on *in silico* analysis of the WGS data, we screened a large panel of 312 *P. acnes* isolates previously characterized by MLST and representing a total of 97 unique sequence types (ST) covering all the phylogroups. Based on this current sample cohort, the multiplex PCR displayed 100% specificity and 100% sensitivity for detecting isolates within each targeted phylogroup (Table 2); no cross-reactivity with isolates from a wide range of other medically relevant bacterial species was observed, including other cutaneous *Propionibacterium* and *Staphylococcus* species (Table 2; see also Table S2 in the supplemental material).

DISCUSSION

Since the 16S rRNA gene of *P. acnes* has very high intraspecific sequence identity (18, 19), it afforded little opportunity for the design of phylogroup-specific primers on which we could base our multiplex assay. As an alternative, we examined various protein-coding housekeeping loci and interrogated available whole-genome sequences representing all known phylogroups for unique genetic regions that could act as platforms for assay development.

TABLE 2 Multiplex PCR assay accuracy

Phylogroup[a]	No. of isolates/total no. that were[b]: Positive	Negative	Sensitivity (%)	Specificity (%)
IA_1	145/145	0/145	100	100
All others	0/216	216/216		
IA_2	20/20	0/20	100	100
All others	0/341	341/341		
IB	65/65	0/65	100	100
All others	0/296	296/296		
IC	7/7	0/7	100	100
All others	0/354	354/354		
II	45/45	0/45	100	100
All others	0/316	316/316		
III	30/30	0/30	100	100
All others	0/331	331/331		

[a] All others relates to *P. acnes* isolates outside the target phylogroup plus 49 isolates from other medically relevant species.
[b] Positive relates to the detection of the expected amplification pattern under consideration, while negative indicates that one of the alternate phylogroup profiles was detected, or no reaction was observed in the case of other species.

By adopting this approach, we were able to design primers based on the *atpD* (PAMp-7/PAMp-8) and *recA* (PAMp-9/PAMp-10) housekeeping loci that specifically identified phylogroup II and III strains, respectively. Type IC strains were identified by their reaction with the primers PAMp-5/PAMp-6 that targeted a Fic family toxin gene present on a genomic region found in type IC strains only; such toxins form part of toxin-antitoxin (TA) systems, which are believed to be important in bacterial persistence in response to specific environmental stresses, as well as in pathogenicity (51). While types IA_1, IA_2, and IC all reacted with primers targeting an ATPase gene (PAMp-1/PAMp-2) that was part of an ABC-type peptide uptake operon, the differentiation of type IA_2 and IC strains from IA_1 and each other was achieved due to their separate reactions with the primers PAMp-3/PAMp-4 and PAMp-5/PAMp-6, respectively. The restriction of an ABC-type peptide uptake operon containing chitinase to type IA and IC divisions but not other phylogroups is of particular interest and is potentially advantageous for the cleavage of chitin from the cell walls of the fungus *Malassezia* and/or *Demodex* mites, which also colonize human skin (20). Since type IA_2 isolates contain alleles of the *sodA* locus that are identical (allele 4) or very closely related (allele 5) to those present in all type IB strains (21, 38), both phylogenetic groups displayed a reaction with the *sodA* primer pair PAMp-3/PAMp-4. Their identities were, however, easily determined based on a differential reaction with the ATPase primers PAMp-1/PAMp-2; type IB isolates show no product with the PAMp-1/PAMp-2 primer set. Interestingly, as the type IA_2 clade shares *recA* and *tly* alleles with type IB isolates, it provides evidence for HGT of large genomic fragments in the natural history of the bacterium (20, 21, 38).

By combining these different primer sets along with the 16S rRNA gene primers PArA-1 and PArA-2 into a single multiplex assay, we have been able to provide researchers with a robust method for the rapid and high-throughput molecular identification of presumptive *P. acnes* isolates, combined with valuable phylogenetic typing information. This assay should prove to be a useful tool for epidemiological studies and the stratification of isolates for various downstream analyses. It offers enhanced discrimination and specificity over comparable molecular (PCR and single-gene sequencing) and MAb typing approaches that have been described in the literature, and it is also less time-consuming, especially compared to methods that require multiple, separate analyses for each isolate. The multiplex PCR will facilitate future retrospective and prospective studies aimed at investigating the association of specific phylogenetic lineages with different human infections, clinical conditions, and antibiotic resistances and will now also provide a technically undemanding way to rapidly map multiple isolates from the same clinical sample so that the presence and pattern of mixed population types can be determined, especially at different body sites/niches. The method should also provide a useful complement to the more detailed and technically complicated study of *P. acnes* populations in complex microbiotas that is based on metagenomic analysis (23). In this study, although we utilized purified genomic DNA as the template for multiplex PCR, the future optimization of the method for direct analysis of bacterial colonies (colony PCR) would further enhance the rapid nature of the assay. Furthermore, of the *P. acnes* isolates tested, the numbers representing types IA_2 and IC were lower than those from other phylogroups, especially type IC, which are infrequently recovered. As a consequence, the further analysis of additional isolates from these clusters will be important to confirm the multiplex PCR specificity and sensitivity results for these types.

To date, our understanding of the population structure of *P. acnes* within and between different body habitats of individuals is poor. These sites include not only various areas of, and regions within, the skin but also the oral cavity and genitourinary tract. Such data may prove especially valuable in our attempts to better understand the potential origin of different lineages associated with clinical samples, particularly in relation to blood culture, as well as whether the pattern of isolates recovered from primary surgical samples matches those on the overlying/surrounding skin, thus indicating potential contamination. In the surgical setting, the current methods used for preoperative skin antisepsis do not always prevent microbial contamination of surgical wounds with viable bacteria (52, 53). Although acute surgical site infections may not ensue due to effective intravenous (i.v.) prophylactic antibiotic administration, these bacteria may still cause downstream chronic biofilm-associated implant infections. It seems reasonable to assume that contamination from the skin would result in a mixture of different phylogenetic groups within a sample, while significant counts of one type may be more indicative of infection. Under such circumstances, the multiplex assay might prove to be a valuable and simple molecular screening tool to highlight such a scenario and thus aid in the diagnosis of biofilm-associated implant infections and bacteremia, etc. within a clinical setting (54). Furthermore, the detection of phylogroups with a potentially greater propensity to cause infection within a clinical sample, such as IA_1, may also be more indicative of infection than those from phylogroups, such as types II and III, believed to be associated with a more commensal existence (21).

To date, two MLST schemes based on eight ($MLST_8$) and nine ($MLST_9$) different protein-coding genes have been described for *P. acnes* (20, 21); the methods are essentially concordant with respect to the clustering of strains into different clonal complexes (CCs), although more subtle differences in the resolution of particular lineages within these CCs exist (21, 38). While MLST provides high-resolution typing of *P. acnes* and generates not only phylogroup information but also sequence type (ST) data that are

highly amenable to phylogenetic and evolutionary analyses, the method is laborious and time-consuming when investigating multiple isolates. The development of new approaches to help streamline the MLST workflow are very attractive, and recently, we described how cross-referencing a refined four-gene MLST allelic profile to the full eight-gene version available in the MLST database (see http://pubmlst.org/pacnes/) can be used to correctly predict and assign phylogroup, CC, and, in the vast majority of cases, ST for a *P. acnes* isolate (38). In this context, the rapid prescreening of isolates by multiplex PCR might also prove to be an extremely valuable way to maximize the number of genetically diverse isolates selected for downstream MLST analyses, thus reducing sequencing costs. Furthermore, MLST and whole-genome analyses have shown that types IA_2, IB, IC, and III represent tight phylogenetic clusters, especially when compared to types IA_1 and II (21, 22, 38) (see Table S1 in the supplemental material); this is reflected in a more restricted number of STs, some of which are highly dominant and widely disseminated. As a consequence, high-resolution MLST typing after multiplex PCR provides less-useful phylogenetic information for type IA_2, IB, IC, and III isolates than that for types IA_1 and II, which are genetically more heterogeneous and contain deeper levels of phylogenetic structure. In keeping with the desirability of a simpler approach to high-resolution typing of *P. acnes*, a single-locus typing scheme (SLST) for the bacterium based on nucleotide sequencing of an amplified target region (484 bp) immediately upstream of the *camp1* gene (identified by a genome mining approach) was described during the preparation of this paper (55). While the $MLST_8$ and $MLST_9$ schemes resolve a greater number of genotypes than does SLST (see Table S1), SLST does correctly cluster isolates into phylogenetic groupings that are congruent with a core genome reference tree (55). In addition, there is little evidence of recombination within the locus based on a network tree analysis (phi test, $P = 0.976$) (see Fig. S1 in the supplemental material). The SLST method is, therefore, a valuable complement and technically simpler approach to current MLST methods for typing *P. acnes*.

The rapid screening of isolates by multiplex PCR will also aid the discovery of novel taxa via atypical PCRs. For example, a sole reaction with the 16S rRNA gene primer set PArA-1/PArA-2 may indicate a new closely related species of *Propionibacterium* with high 16S rRNA gene identity to *P. acnes* or a novel *P. acnes* phylogroup or ST that contains base mismatches at primer binding sites within the protein-coding genes of the assay. Indeed, as a direct result of multiplex PCR screening of skin-derived isolates, we recently came across such a scenario and identified a new *Propionibacterium* species that has very high 16S rRNA gene identity to *P. acnes* (and reacts with PArA-1/PArA-2) but is quite distinct from it, and other cutaneous propionibacteria, based on whole-genome analysis (J. Hunyadkürti, A. Vörös, B. Bálint, R. Herczeg, E. Urbán, M. Göker, M. Kostrzewa, A. McDowell, and I. Nagy, unpublished data).

In conclusion, the multiplex PCR described here facilitates the rapid molecular confirmation of presumptive *P. acnes* isolates along with parallel phylogenetic typing. It should provide researchers with a flexible typing tool that can be used in isolation or as an adjunct to more detailed sequence-based analysis, depending on the epidemiological questions being asked and the resolution required. It is also a technically simple methodology for the rapid analysis of mixed *P. acnes* populations and should therefore help improve our understanding of the roles of different *P. acnes* lineages in clinical conditions.

ACKNOWLEDGMENTS

A.M. was supported by a Health and Social Care Northern Ireland Research & Development Division grant awarded to S.P. (grant HSCNI RRG 9.41) and E.B. by a Prostate Cancer UK grant awarded to A.M. and S.P. (grant 110831). Funding to I.N. was from the French-Hungarian Associated European Laboratory (LEA) SkinChroma (grant OMFB-00272/2009); I.N. was also supported by the János Bólyai Research Scholarship of the Hungarian Academy of Sciences.

The *Propionibacterium humerusii* isolates were a kind gift from Susan Butler-Wu, University of Washington, USA. We also thank Derek Fairley (Belfast Health and Social Care Trust) for kindly providing genomic DNAs from a range of other bacterial species.

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