Progressive macular hypomelanosis (PMH) treatable but often misdiagnosed
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PROGRESSIVE MACULAR HYPMELANOSIS IS ASSOCIATED WITH A PUTATIVE NEW PROPIONIBACTERIUM SPECIES
ABSTRACT

Introduction: *Propionibacterium acnes* (*P. acnes*) plays an important role in the pathogenesis of acne and progressive macular hypomelanosis (PMH). However, acne lesions are absent in most PMH patients.

Patients and Methods: We characterized bacterial isolates obtained from PMH and acne patients to detect a possible difference.

Amplified Fragment Length Polymorphism of pure cultures from skin swabs of 14 PMH and 10 acne patients resulted in 3 different DNA groups.

Results: Compared to the *P. acnes* reference strain, isolates from group 1 (8 acne and 6 PMH patients) showed a similarity between 55 and 100% suggesting the same species, isolates from group 2 (2 acne patients) showed a similarity between 30 and 55% suggesting a variant of *P. acnes* and group 3 isolates (8 PMH patients) formed a clear distinct DNA group with a similarity of less than 30%. This low level of homology suggested that these isolates belong to a different species. 16S rRNA gene sequencing and biochemical tests showed minimal differences between the three DNA groups, suggesting a subspecies of *P. acnes*.

Conclusions: The results show a correlation between the presence of group 3 strains and PMH, since these strains were exclusively found in PMH patients. Further research is needed to confirm this relationship.
INTRODUCTION

Progressive macular hypomelanosis (PMH) is a skin disorder characterized by ill-defined, nummular, symmetrically localized hypopigmented macules on sebum-rich areas of the skin of young adults, rarely extending to the head and proximal extremities (Figure 1) (Relyveld et al. unpublished data 2007). PMH has often been (mis)diagnosed as pityriasis alba (PA) or pityriasis versicolor (PV) even though there are distinct histological and clinical differences (Relyveld et al. 2008).

In 2004 Westerhof et al. hypothesized that PMH might be caused by Propionibacterium acnes (P. acnes) bacteria. This was based on the observation of red follicular fluorescence in lesional skin of PMH patients, when illuminating the skin with a Wood’s lamp in a dark room. This fluorescence was absent in the adjacent normal skin as well as in patients with proven PA (by biopsy) or PV (by KOH tests). Red follicular fluorescence is characteristic for P. acnes bacteria residing in pilosebaceous ducts of normal skin and especially in acne prone skin. P. acnes is considered to be commensal flora, and accounts for approximately half of the total skin microbiota (Tancrede et al. 1992). In PMH patients the red follicular fluorescence is only present in the hypopigmented macules and not in the adjacent normal sebum-rich skin (Westerhof et al. 2004).

Conventional cultures from follicles of lesional skin in PMH patients showed P. acnes bacteria that could not be cultured from adjacent normal skin. Furthermore the bacteria showed high-level sensitivity to penicillin, amoxicillin, amoxicillin-clavulanate, piperacillin-tazobactam, erythromycin and clindamycin. Resistance was observed for metronidazole (Westerhof et al. 2004). These phenotypic characteristics are typical for P. acnes.

Since P. acnes is particularly implicated as a cause of acne our group conducted a study in 2006 (Relyveld et al. 2006) in which we compared anti-acne therapy with anti-inflammatory therapy in the treatment of PMH. Anti-acne therapy had
significantly better treatment results than anti-inflammatory therapy. Our previous findings gave a scientific basis to our hypothesis that *P. acnes* might be the cause of PMH. However, it is notable that acne does not seem to predispose for PMH and patients with PMH do not have acne more often than the general population.\(^1\) In 1972 two phenotypes of *P. acnes* have been described (Johnson *et al*. 1972), which have been proven to be distinct phylogenetical groups (McDowell *et al*. 2005) called type I and type II *P. acnes*, but the clinical importance of these two types is still unknown. Therefore we hypothesized that the causative bacteria in PMH might be a subspecies of *P. acnes* that can not be differentiated by conventional culturing methods. We decided to further identify the bacteria we cultured from PMH patients and acne patients by molecular strain comparison. Characterization by the DNA fingerprinting method Amplified Fragment Length Polymorphism (AFLP) has proven to be superior for identification and typing of bacteria at the strain level, compared to identification by conventional culture (Savelkoul *et al*. 1999). AFLP is a genetic mapping technique that uses specific amplification of a subset of restriction enzyme digested DNA fragments to generate a unique fingerprint of a particular genome. AFLP has been widely applied in the identification and genotyping of various organisms, including *Propionibacteria*, because of its high discriminatory power and reproducibility (Savelkoul *et al*. 1999, Vos *et al*. 1995, Mohammadi *et al*. 2005).

16S DNA sequencing is the golden standard for taxonomic species identification; the comparison of the 16S *rRNA* gene sequences allows differentiation between organisms at the genus level in addition to classification of strains at the species and subspecies level (Claridge 2004). The 16S *rRNA* gene sequence is composed of both variable and conserved regions. The gene is large enough, with sufficient interspecies-specific polymorphisms to provide distinguishing and statistically valid measurements. The purpose of this study was to identify, through Amplified Fragment Length Polymorphism (AFLP), 16S *rRNA* gene sequencing and biochemical characterization, the bacterial species that is related to PMH. We hypothesized that this species would be different from the bacterial species causing acne, and that it cannot be differentiated from the species that causes acne by conventional culture and biochemical methods. Identification of the bacterial species that causes PMH might lead to better understanding of the disease and better treatment modalities.
MATERIALS AND METHODS

Patients and inclusion criteria
Fourteen patients with PMH and 10 patients with acne were included in this prospective study. All patients were seen at the Netherlands Institute for Pigment Disorders, Amsterdam, the Netherlands, a tertiary referral center for pigment disorders. The Declaration of Helsinki protocols were followed and the study was approved by the medical ethical commission of the Academic Medical Center in Amsterdam, the Netherlands. All patients provided written informed consent before entering the study. Patients under the age of 18 needed parental consent to be included.

The diagnosis of PMH was based on a combination of clinical signs (symmetrically distributed, ill-defined nummular hypopigmented macules, especially on the trunk), the presence of red follicular fluorescence in the lesional skin when examined with a Wood’s lamp in a dark room and negative KOH tests. Patients with any form of acne on the trunk (mild, moderate, severe) and without hypopigmented lesions on the skin were included in the acne group. Patients were excluded from the study if they had a history of atopic dermatitis, seborrheic dermatitis and psoriasis and/or if they were allergic to the anaesthetic used. Furthermore any previous antibacterial treatment (both local and systemic) had to be discontinued at least 3 months prior to study entry.

Skin sampling
We first examined the skin lesions of all patients under normal lighting conditions, then in a dark room under Wood’s light. For PMH patients a red fluorescent hair follicle in lesional skin was marked and for acne patients a red fluorescent acne lesion was marked. The skin was then disinfected with 70% alcohol with chlorohexidin 0.5% to eliminate superficial skin flora. To ensure that the red fluorescent part was in the biopsies we took the biopsies (2 mm) from the marked skin spots in a dark room under Wood’s light. The biopsy specimens were cut transversally and each half was swapped on blood agar plates and immediately transported under anaerobic conditions to the Department of Medical Microbiology, University of Amsterdam (Amsterdam, the Netherlands) for further anaerobic processing and culturing.

Culture procedures
The inocula were spread on the agar plates and incubated under anaerobic conditions for 48 hours at 37 C. Colonies with a morphology compatible with Propionibacterium
were subcultured and subsequently identified with a Gram stain and the API 20A system for identification of anaerobic bacteria (Biomérieux). Per biopsy specimen one colony was subcultured again and stored at minus 70°C, and later regrown on blood agar plates to be sent to the VU University Medical Center, Amsterdam, the Netherlands for further molecular DNA processing.

**Reference strains**

Besides the clinical isolates, *P. acnes* (DSM 1897, ATCC 6919), *P. granulosum* (DSM 20700, ATCC 25564), *P. avidum* (DSM 4901, ATCC 25577) and *P. propionicus* (DSM 43307, ATCC 14157) were included in this study as reference strains. These strains were purchased from the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ), Braunschweig, Germany and cultured under anaerobic conditions in accordance with the manufacturer’s instruction.

**DNA extraction**

Prior to extraction of DNA, pure bacterial colonies were suspended in 2 ml TE-1 buffer (10 mmol/l Tris-HCl, 1 mmol/l EDTA, pH 8.0) and adjusted to match a turbidity of 1 McFarland. The suspension was then incubated with 10 mg/ml lysozyme at 37°C for at least 1 h. Thereafter DNA was isolated following the tissue protocol of the QIAmp DNA mini kit (Qiagen GmbH, Hilden, Germany). Finally, the DNA was eluted in 100 µl AE buffer of the extraction kit and stored at -20°C until needed.

**Genotyping of bacterial strains**

Amplified-fragment length polymorphism (AFLP) DNA fingerprinting was performed for further molecular characterization of the bacterial strains from 10 acne and 11 PMH patients.

The AFLP procedure was carried out as described earlier. Briefly, bacterial DNA was restricted with two enzymes with simultaneous ligation of adaptors to the restriction site. The reaction mixture consisted of 10 ng DNA, of 1x T4 DNA ligase buffer, 0.5 mol/l NaCl, 0.5 µg bovine serum albumin, 2 pmol of the *EcoRI* adaptor (Isogen Bioscience BV, Maarssen, the Netherlands), 20 pmol of the *MseI* adaptor (Isogen Bioscience), 80 U of T4 DNA ligase, 0.2 U of *EcoRI*, 1 U of *MseI*. After incubation at 37°C for 3 h, the mixtures were diluted 1:20 in 0.1x TE buffer. All other enzymes were purchased from New England Biolabs (Beverly, MA, USA).

For amplification of the restriction fragments, 5 µl of the diluted mixture was added to 5 µl of PCR mixture, which consisted of 1x PCR buffer (Applied Biosystems, Foster City, CA, USA), 2 mmol/l dNTPs (Promega Benelux, Leiden, the Netherlands), 15 mmol/l MgCl₂
(Applies Biosystems), and 20 ng of Eco-A primer (5’-GACTGCGTACCAATTAC3’) and 60 ng of Mse-C primer (5’-GATGAGTCCTGAGTAAC3’). Eco-A was fluorescently labelled with carboxyfluorescein (Eurogentec, Maastricht, the Netherlands). Amplification was carried out in a GeneAmp PCR System 9700 (Applied Biosystems) under the following conditions: 2 min at 72°C, followed by 12 cycles of 30 s at 94°C, 30 s at 65°C and 1 min at 72°C and then 23 cycles of 30 s at 94°C, 30 s at 56°C and 1 min at 72°C, ended by a single extension at 72°C for 1 min.

Before analysis on an ABI Prism 3100 automatic sequencer, 2·5 µl of each PCR product was added to 22 µl Hi-Di formamide and 0·5 µl GeneScan-500 ROX standard (Applied Biosystems). Data were analyzed from 200-500 bp fragments with the Bionumerics software package, version 3·0 (Applied Maths, Sint-Martens-Latem, Belgium). Similarity coefficients were calculated with Pearson correlation and dendrograms were obtained by the unweighted pair group method using arithmetic averages (UPGMA) clustering.

**Strain variation within patients**

To exclude the presence of a mixture of different species in one patient, we resampled 3 acne patients and newly sampled 3 PMH patients, based on the same inclusion criteria as described above. This time a maximum of ten isolates per patient was submitted to DNA fingerprinting by AFLP and compared to the initial AFLP pattern, instead of only one isolate as in the prospective study.

**16S rRNA gene sequencing and amplification**

To confirm our AFLP findings of different (sub) species 16S rRNA gene sequencing was performed on 2 isolates (from 1 acne and 1 PMH patient) from DNA group 1, 1 (from 1 acne patient) from group 2 and 3 (from 3 PMH patients) from group 3. For sequencing of the 16S DNA, a polymerase chain reaction was performed using the universal primers described by Mohammadi *et al.* (2005), which target a conserved region of 16S ribosomal DNA. A 20 µl PCR mixture consisting of 2·5 µl 10x PCR buffer, 0·5 µl 10 mmol/l dNTPs, 1·5 µl 25 mmol/l MgCl2, 25 pmol of each of the forward and reverse primer (Eurogentec), 0·2 µl Amplitaq gold (5 U/µl) and 5 µl template DNA was amplified under the following conditions: 10 min at 95°C followed by 30 cycles of 30 s at 95°C, 30 s at 60°C and 45 s at 72°C and a final step of 10 min at 72°C. PCR products were analyzed on 2% agarose gel in 1x Tris–Borate–EDTA (TBE) buffer (Life Technologies Ltd, Paisley, UK). After amplification the PCR products were purified using the QIAquick PCR purification kit (Qiagen).
The purified products were sequenced using primers covering a 466 bp fragment (F/R universal primer) and a 900 bp fragment (gd1 (5’-TGCTTTCGATACGGGTTGAC-3’) and bak 4 (5’-AGGAGGTGATCCARCCGCA-3’) (Dasen et al. 1998) from the 16S rDNA, resulting in a consensus sequence of about 1200 bp. Primers were obtained from Eurogentec. Sequencing was performed using Big Dye terminator sequencing kit (Applied Biosystems). The programme consisted of: 10 s at 96°C, 5 s at 56°C and 4 min at 60°C for 25 cycles. The sequence products were purified and analyzed on an ABI 3100 automated DNA sequence analyser (Applied Biosystems). The 16S rRNA sequences were aligned and compared with P. acnes ATCC 6919 (GenBank accession number AB042288) with the Bionumerics software package, version 3.0.

**Biochemical analysis and antimicrobial resistance pattern**

To determine whether our findings at the DNA level correlated with biochemical characteristics 6 isolates, 2 out of each DNA group were analyzed with the rapid ID 32A multitest identification system (Biomérieux, Lyon, France). In addition we assessed the antimicrobial sensitivity pattern of the strains by conventional methods.

**RESULTS**

Patients’ characteristics are presented in Table I.

**Culture and provisional conventional identification**

Anaerobic culture on blood agar plates of biopsy specimens from both acne and PMH patients showed a remarkably homogenous growth of colonies compatible with *Propionibacteria*, with usually only very few other colony types. In all biopsy specimens the predominant colony type proved to consist of Gram positive rods that were identified as *P. acnes* by the API 20A system.

**Genotyping bacterial strains**

The AFLP patterns for *Propionibacterium* can be divided in three windows of similarity (Mohammadi et al. 2005). The first window, between 55% and 100%, defines strains of the same species; the second window, between 30% and 55%, defines strains of related species, possibly different subspecies; the third window, below 30% defines strains of different species (Savelkoul et al. 1999). Within the first window (55 to 100%) strains of the same species can be defined as identical (>90% similarity) or of different type. In our experiments strain characterization by
AFLP identified three groups (Figure 2): group 1 comprised strains isolated from 8 acne patients and 6 PMH patients. The isolates showed a similarity between 55 and 100% with the reference *P. acnes* strain. Group 2 comprised strains from 2 acne patients, showing a similarity level between 30 and 55% with the reference *P. acnes* strain. Isolates from group 3 comprised strains isolated solely from PMH patients (n = 8). For these strains a similarity level < 30% with the reference *P. acnes* strain was observed. All clinical strains showed a similarity level of < 30% when compared with *Propionibacterium avidum* (*P. avidum*), *Propionibacterium granulosum* (*P. granulosum*) and *Propionibacterium propionicus* (*P. propionicus*).

### Table I Characteristics of the study population

<table>
<thead>
<tr>
<th>Characteristics</th>
<th>acne patients</th>
<th>PMH patients</th>
</tr>
</thead>
<tbody>
<tr>
<td>No. patients</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td>Female, No.</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>Male, No.</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>Mean age[1], Y</td>
<td>30 ± 5,3</td>
<td>27 ± 6,5</td>
</tr>
<tr>
<td>Skin phototype[2]</td>
<td></td>
<td></td>
</tr>
<tr>
<td>III</td>
<td>6</td>
<td>9</td>
</tr>
<tr>
<td>IV</td>
<td>4</td>
<td>3</td>
</tr>
<tr>
<td>V</td>
<td>0</td>
<td>2</td>
</tr>
</tbody>
</table>

[1] Mean ± SD. [2] According to Fitzpatrick: determined by constitutive skin colour (the genetically determined colour or absence of colour in skin unexposed to solar irradiation and by facultative skin colour (skin colour that results from ultraviolet radiation exposure).

#### Strain variation within patients

We subjected 29 bacterial isolates cultured from the skin of 3 acne patients to the AFLP fingerprinting technique. All showed a similarity > 30% with the reference strain *P. acnes*, and fell into DNA group 1/2. Thirty colonies isolated from the skin of 3 PMH patients were also examined. All isolates (20) of 2 patients showed a similarity level < 30% with the *P. acnes* strain, comparable with bacteria in DNA group 3. The 10 bacterial isolates from the other PMH patient showed a similarity between > 55% with the reference strain *P. acnes*, comparable with bacteria in group 1 (Figure III).

#### 16S rRNA gene amplification and sequencing

Comparison of the sequence of the 16SrRNA gene of the various strains with the sequence of the 16SrRNA gene of the *P. acnes* reference strain ATCC 6919 showed one nucleotide difference at position 827 with the isolates of group 2, and
1 nucleotide difference at position 1243 with the isolates of group 3. One isolate (TY 3585) of DNA group 3 had an additional nucleotide difference at position 712 besides the one at position 1243 (Table II).

**Biochemical analysis and antibiotic resistance pattern**

Two isolates from each DNA group were analyzed with the rapid ID 32A system. Isolates from group 1 and 2 were identified as *P. acnes* with a certainty of 99.9%. The isolates from group 3 were not identified as *P. acnes* by the computer, but were labelled with an “un-acceptable profile” since both isolates showed negative results.
Table II Nucleotide differences of bacterial isolates compared to the reference strain[1]

<table>
<thead>
<tr>
<th>Nucleotide</th>
<th>Ref strain</th>
<th>TY 3574</th>
<th>TY 3589</th>
<th>TY 3569</th>
<th>TY 3582</th>
<th>TY 3585</th>
<th>TY 3591</th>
</tr>
</thead>
<tbody>
<tr>
<td>Position</td>
<td>ATCC 6919</td>
<td>group 1</td>
<td>group 1</td>
<td>group 2</td>
<td>group 3</td>
<td>group 3</td>
<td>group 3</td>
</tr>
<tr>
<td>712</td>
<td>G</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>A</td>
<td>*</td>
<td></td>
</tr>
<tr>
<td>827</td>
<td>T</td>
<td>*</td>
<td>*</td>
<td>C</td>
<td>*</td>
<td>*</td>
<td>*</td>
</tr>
<tr>
<td>1243</td>
<td>G</td>
<td>*</td>
<td>*</td>
<td>*</td>
<td>A</td>
<td>A</td>
<td>A</td>
</tr>
</tbody>
</table>

[1] 16S rRNA gene sequence analysis results of bacterial isolates from 1 acne and 1 PMH patient (group 1), 1 acne patient (group 2) and 3 PMH patients (group 3). * = reference nucleotide position; the ATCC strain belongs to group 1. A = Adenine, G = guanine, C = cytosine, T = thymine.
for Pro A, a substance that always shows positive results for \textit{P. acnes}. Sensitivity and resistance analysis of all strains were similar and typical for \textit{P. acnes}.

**DISCUSSION**

In the present study we showed by AFLP DNA typing that bacteria cultured from 8 out of 14 PMH patients were substantially different from the \textit{P. acnes} bacteria seen in acne. We classified these bacteria as DNA group 3. By conventional culturing techniques, both in this study and in a previous study (Westerhof et al. 2004), the microbial species (\textit{P. acnes}) found in PMH could not be differentiated from that found in acne. Acne, however, is rarely seen in patients with PMH and vice versa. Interestingly, these group 3 bacteria were not detected in any of the acne patients. Furthermore, 16\textit{S} r\textit{RNA} gene sequencing and biochemical tests confirmed that there are differences between the groups of bacteria, although the differences are minor. We therefore believe that there is a relationship between PMH and the \textit{Propionibacteria} clustering in group 3.

In 2005 Mohammadi et al. also described three different types of \textit{P. acnes} strains. They conducted a study to determine the source of bacterial contamination of platelet concentrates (PCs). \textit{P. acnes} isolates derived from PCs and corresponding red blood cells concentrates (RBCs) were analyzed by AFLP procedures and by 16\textit{S} r\textit{RNA} gene sequencing. These authors describe three bacterial groups that are comparable to the bacterial groups we found in our study: group I and II were \textit{P. acnes} (sub) species (30-90% homology with the reference \textit{P. acnes} strain) and group III showed < 30% homology with the reference strain \textit{P. acnes}, suggesting a different species. Furthermore, 16\textit{S} r\textit{RNA} gene sequencing showed similar differences as those we detected: at position 827 group I bacteria showed a T nucleotide while group II bacteria showed a C nucleotide. These findings also correspond to the findings of McDowell et al. 2005 who observed the same difference in nucleotides at position 827. 16\textit{S} r\textit{RNA} gene sequencing results of the group 3 bacteria in our study corresponded with the group III bacteria described by Mohammadi et al. 2005. In their study, at position 1243 group III bacteria showed an A nucleotide, while the reference \textit{P. acnes} strain showed a G nucleotide.

Analysis by AFLP showed that the \textit{P. acnes} bacteria (group 1) were quite different from the bacteria found solely in PMH patients (group 3). The level of identity was that of a different \textit{Propionibacterium} species. This observation was confirmed by the differences in 16\textit{S} r\textit{RNA} gene sequencing and biochemical tests; the differences
were minimal however, in combination with the similar antimicrobial sensitivity pattern of the two bacterial groups, this suggests that we might be dealing with a bacterium of the genus *Propionibacterium* but of a yet undefined species. McDowell *et al.* 2008 recently also described a new phylogenetic group of *P. acnes* which they called *P. acnes* type III. This strain had a 99.8 to 99.9% identity to type I and type II *P. acnes* strains (Relyveld *et al.* 2006), when analyzed by 16S rRNA gene sequencing. However immunofluorescence microscopy, sequencing of the 16S rRNA gene and the specific recA gene (a protein-encoding gene with housekeeping functions), and biochemical analysis of the type III *P. acnes* bacteria showed obvious differences between these bacteria and the type I and II bacteria. When we compare their 16S rRNA sequences to our results, the same point mutation at nucleotide position 1243 is observed, indicating that the *P. acnes* type III corresponds to our DNA group 3.

It has to be noted that in 6 PMH patients we found group 1 *P. acnes* bacteria and not group 3 bacteria. However, this does not exclude group 3 bacteria from being present on the skin of these patients. Possibly they were present in low numbers, or sampling error may have occurred.

Group 3 bacteria were isolated solely in PMH patients, and not in acne patients. This suggests a relationship between PMH and these bacteria. The findings may help explain why not all acne patients have PMH and vice versa, and why PMH can best be treated with antimicrobial therapy. Furthermore this study shows that conventional identification methods are not sufficient to distinguish some species or subspecies of specific genera that may play an important role in the pathogenesis of diseases. Further research is necessary to substantiate the importance of the role of this putative new *Propionibacterium* species in PMH.