A new *Brevibacterium* sp. isolated from infected genital hair of patients with white piedra

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Summary. A new aerobic gram-positive non-sporeforming bacillus has been isolated from infected genital hair of patients with white piedra in association with *Trichosporon beigelii*. This species has been characterised morphologically, nutritionally, by DNA base composition, cell-wall analysis and cellular fatty-acid profile on the basis of 14 isolates. The G + C content of DNA is 63.05 mol %. Cell walls possess *meso*-diaminopimelic acid (Type IV) and the sugars glucose, galactose, xylose and ribose; mycolic acids are not present. The species has a distinct colonial and microscopic morphology, is strongly proteolytic and produces methanethiol. These findings and the cellular fatty-acid profile are compatible with the genus *Brevibacterium*. A new species is proposed based on the following characters: colonial and microscopic growth and morphology; conditions for rod-to-coccus cycle; ribose utilisation; and tellurite reduction. The type strain has been named *Brevibacterium mcbrellneri* E2cr (ATCC 49030). The strong proteolytic properties may be the mechanism of pathogenesis.

Introduction

In a previous study of genital white piedra,¹ gram-positive rods were observed histologically in hair lesions and infected hair. A morphologically and biochemically distinct bacterium was isolated frequently with the yeast *Trichosporon beigelii*, and synergic growth was demonstrated between the two organisms *in vitro*.² The possibility that this bacterium may play a role in the pathogenesis of white piedra indicated the importance of its characterisation and of establishing its taxonomic position.

Aerobic non-sporeforming gram-positive rods are ubiquitous in nature and are particularly prevalent as part of the normal flora on human skin, where they are commonly designated as coryneform bacteria. Advances in chemotaxonomic methods, which include identification of cell-wall peptidoglycan, sugars and mycolic acids, whole cell fatty-acid composition, and polar lipid and menaquinone patterns, have provided a basis for differentiation of these organisms.³ From these methods and by morphology, species previously designated as coryneforms can now be assigned to other genera:⁴ *Arthrobacter*, *Cellulomonas*, *Clavibacter*, *Brevibacterium*, *Corynebacterium*, *Curtobacterium*, *Gordana*, *Microbacterium*, *Rhodococcus*. However, when dealing with environmental strains, unless an organism has become clinically relevant,

most species have remained unnamed.⁵ Even most clinical isolates, many of which are asymptomatic colonisers or part of the normal flora, defy classification.⁵ Pitcher⁶ recognised that many skin isolates of coryneform morphology belong to the genus *Brevibacterium*. The distinction between *Corynebacterium* and *Brevibacterium* can be made morphologically, nutritionally, by chemotaxonomic markers of the cell walls and by the production of methanethiol.⁷

The purpose of this study was to characterise these isolates from infected hair of patients with white piedra morphologically, nutritionally, by DNA base composition, cell-wall analysis, and fatty-acid profiles. These gram-positive non-sporeforming rods were compared with culture collection strains of *Corynebacterium* and *Brevibacterium* spp. to establish their taxonomic position and learn more of their pathogenic potential.

Materials and methods

Micro-organisms

Fourteen strains of coryneform bacteria were isolated from hair nodules from nine patients with white piedra in a previous study,¹ as previously described. Briefly, genital hairs from patients with nodules were surface sterilised by rinsing in alcohol 70 % for 1 s and then soaked overnight in phosphate-buffered saline, pH 7.2, containing Triton X100 0.1 %. The nodules

Table I. Reference strains

Species	Designation	Source
<i>Corynebacterium diphtheriae</i>	160	VAMC
<i>C. pseudotuberculosis</i>	1121	VAMC
<i>C. renale</i>	19412	ATCC
<i>C. jeikeium</i>	162	VAMC
<i>C. pilosum</i>	29592	ATCC
<i>Rhodococcus equi</i>	4	VAMC
<i>Brevibacterium epidermidis</i>	35514	ATCC
<i>Brevibacterium linens</i>	9172	type strain ATCC

VAMC, Veterans Administration Medical Center, Houston.
ATCC, American Type Culture Collection.

were removed from the hair by scraping with a scalpel, then macerated with dissecting needles. The resulting suspension was inoculated on to Casman's Sheep Blood Agar (Difco) and incubated at 35–37°C for 5 days. Isolates were compared with culture collection strains of *Corynebacterium* and *Brevibacterium* spp. (table I) which represented type strains of the two genera chosen or were strains that resembled the unknown clinical isolates. Isolates were subcultured on Casman's sheep blood agar, suspended in Trypticase Soy Broth (Difco) with glycerol 20%, and stored at –70°C.

Morphological studies

Isolates were grown on Casman's sheep blood agar, Trypticase Soy Agar (Difco; TSA), Czapek's Mineral Agar (Difco) with yeast extract 0.2% (CYA) and Yeast Malt Agar (Difco; YMA). Microscopic and colonial morphology were observed during incubation for 2 weeks at 25, 30 and 35°C. Modified Kinyoun stain⁸ was used to test for acid fastness. Anaerobic growth was tested in an anaerobic jar with the Gaspak System (BBL).

Biochemical tests

All *Brevibacterium* spp. except *B. linens* strains were tested for effect on the following substrates by standard methods⁹ and in the API 20E system (API bioMérieux Inc., La Balones les Grottes, France). API Coryne and API Rapid ANA were used to test three piedra strains, *B. epidermidis* and *B. linens*. The API Coryne strip, when used conventionally according to the manufacturers' instructions, does not differentiate among the *Brevibacterium* spp. The tests for nitrate reductase, pyrazinamidase, pyrrolidonylarylamidase, alkaline phosphatase, β -glucuronidase and acetyl- β -glucosaminidase were read after incubation for 24 h, according to the instructions, but the tests for aesculin, urea and gelatin hydrolysis and fermentation of ribose, xylose, mannitol, maltose, lactose, sucrose and glycogen were held for 7 days at 35°C in air. The Rapid ANA II system test for pre-formed enzymes was used according to the manufacturers' instructions. It

includes the following biochemical tests: hydrolysis of urea, aryl substituted arabinoside, galactoside, glucoside, sucrose, glucosaminide and phosphate; hydrolysis of leucyl-glycine, glycine, proline, phenylalanine, arginine, serine and pyrrolidonyl; and formation of indole. The effects of the test strains on starch, casein, tyrosine and xanthine were tested on commercially prepared plates (Scott, Fiskeville, RI 02823, USA). Litmus milk activity was tested on litmus-milk-agar plates.¹⁰ Hydrolysis of Tween 80 was tested on TSA containing Tween 80 0.1%. Methanethiol production was detected by the method of Pitcher and Malnick,⁶ and Laakso, as described by Jones and Keddle.⁷ Tolerance to NaCl 6.5%, 10%, 15% and 20% was tested in TSA. Reduction of tellurite was tested on Bacto Tellurite Medium (Difco).

Antibiotic susceptibility tests

These were done by the Kirby Bauer disk diffusion method¹¹ on Mueller Hinton agar with the following antibiotic disks: penicillin 2 μ g, ampicillin 10 μ g, erythromycin 15 μ g, tetracycline 30 μ g, methicillin 5 μ g, oxacillin 10 μ g, vancomycin 15 μ g, cephalothin 30 μ g, ciprofloxacin 5 μ g, gentamicin 10 μ g and trimethoprim sulphate 24 μ g. Sensitivity to ergosan was tested by inoculation on TSA containing ergosan 0.1%.

Cellular fatty-acid analysis

Three representative strains of the new organism were tested by Microbial ID Inc. (Newark, Delaware, USA) with the Hewlett-Packard System as previously described.¹² Biomass was obtained by growth on TSA at 25°C.

Cell-wall analysis

Six strains of the new species that demonstrated characteristic colonial and microscopic morphology were chosen for cell-wall analysis. These and reference strains were grown on Casman's sheep blood agar at 37°C. Culture collection strains of *Corynebacterium* spp. and *B. epidermidis* were incubated overnight and the six new isolates for 3 days. A heavy suspension of the growth was made in saline 0.85% and 0.2 ml was inoculated on to Brain Heart Infusion Agar (Difco). When maximum growth was achieved, plates were flooded with formalin 10% and left overnight. The suspension of organisms was centrifuged and the bacteria were washed twice with distilled water and once with alcohol 95% then allowed to dry in a vacuum desiccator and stored at –70°C.

Carbohydrate analysis

Biomass was prepared as described above for cell-wall analysis. Methods of analysis for cellular carbohydrates were similar to those described previously.¹³

Cells (50 mg dry weight) were hydrolysed for 4 h in 1.5 ml of concentrated H_2SO_4 in tightly sealed reacti-vials at 100°C. After cooling, the pH was adjusted to 5.2–5.5 with saturated BaOH. The digest was centrifuged (1000 *g* for 10 min) and the supernate was evaporated to dryness under a stream of air. The residue was redissolved in 0.5 ml of distilled water and 2–4 μ l was spotted on Kodak 13255 Cellulose Chromatogram sheets. The 20 $\times$ 20 cm sheets were pre-developed and activated before use. Chromatograms were developed for 2 h with acetone:methanol:chloroform:water, 80:10:10:5 v:v:v:v. The sheets were then sprayed with 1,3-dihydroxynaphthalene ethanolic solution (20 mg/20 ml of ethanol) containing concentrated H_2SO_4 1% and placed in an oven for 10 min at 100°C. Standards used were ribose, galactose, arabinose and xylose.

Mycolic acid esters

Biomass was prepared as described above for cell-wall analysis. With slight modification, whole cell methanolysates were prepared according to the methods of Goodfellow *et al.*¹⁴; 100 mg of dry cells were methanolysed for 16 h at 50°C in 20-ml teflon-lined screw-top tubes containing methanol 5 ml, toluene 5 ml and concentrated H_2SO_4 0.2 ml. The mixture was cooled to room temperature and extracted twice with 2 ml of hexane; the upper phase was removed, dried under N_2 , and reconstituted in 1 ml of hexane for analysis. For each sample, 5 μ l was spotted on HPTLC Silica Gel 60 plates which were pre-developed and activated before use. Chromatograms were first developed in a petroleum ether (b.p. 30–60°C):diethyl ether 85:15 v:v solvent. Mycolic esters were visualised by spraying the plates with chromic acid solution and charring at 200°C. Separation of mycolic esters from fatty acids of lower mol. wt was verified by co-chromatography with fatty acid methyl ester standards, reversibly detecting the components with iodine vapour and re-developing the chromatogram in methanol:water 75:30 v:v for 6 h.

Diaminopimelic acid analysis

The method was adapted from that of Staneck and Roberts.¹⁵ Dried cells (100 mg) were hydrolysed with 1 ml of 6 N HCl for 18–22 h at 100°C in teflon-lined screw-topped tubes on a heat block. The material was filtered through Whatman No.1 filter paper, washed with 1 ml of 6 N HCl and dried in glass culture plates in a fume hood. The residue was dissolved in 0.5 ml of distilled water for chromatography on Eastman Kodak No. 6064 cellulose which was pre-developed in a thin layer chromatography tank with methanol:water:6 N HCl:pyridine 80:26:4:10 v:v:v:v. The plate was activated at 100°C and 3–5- μ l samples were spotted and developed for *c.* 4 h in methanol:water:10 N HCl:pyridine, 80:17.5:2.5:10 v:v:v:v. Diaminopimelic acid (DAP) was visualised by

spraying with ninhydrin 0.2% in acetone and heating at 100°C for 3 min.

DNA composition

G + C mol% content of DNA was determined by the American Type Culture Collection (Rockville, MD). The melting temperature was determined on a Gilford Spectrophotometer, and *Escherichia coli* strain B (Sigma D4 889) was used as a standard.

Results

Growth and morphology

Examination of toluidine blue-stained sections of infected genital hair by light microscopy with oil immersion revealed rods of coryneform morphology and yeast forms as shown in fig. 1. Direct plating of the suspension of macerated hair nodule on Casman's sheep blood agar yielded a mixed culture containing both *Trichosporon beigeli* and the gram-positive bacillus, and frequently no other organisms. All 14 strains had similar colonial morphology and growth characteristics. After incubation for 48 h at 35–37°C on Casman's sheep blood agar, colonies were 1–2 mm in diameter (fig. 2); maximum growth was achieved after incubation for 5 days when colonies were 3–5 mm in diameter, depending on the strain; β -haemolysis and an unpleasant odour were also noted at this time. Colonies were grey-cream in colour, opaque, raised with a bevelled margin and had a contoured glistening surface; the edges were crenated and the consistency was friable (fig. 3). The organisms were strict aerobes but were not fastidious, and could be cultured on nutrient agar and routine laboratory media. The characteristic colonial morphology and odour were more pronounced on blood agar. Optimum temperature for growth was 35–37°C. Microscopically, after

Fig. 1. Histological section of infected hair stained with toluidine blue showing yeast forms (Y) and bacteria (B).

Fig. 2. A 48-h culture of a piedra isolate grown on Casman's sheep blood agar at 37°C; $\times 5.4$.

Fig. 3. A 4-day culture of a piedra isolate grown on Casman's sheep blood agar at 37°C; $\times 4.7$.

incubation for 4 days on Casman's sheep blood agar, the organisms were slender, more regular, gram-positive, non-sporeforming, non-acid-fast bacilli that were non-motile with a tendency to clump.

Rod-to-coccus cycle

In a comparison of *B. epidermidis* and the unknown organism, in tests with three media and temperatures of 25, 30 and 37°C, *B. epidermidis* underwent complete transformation to coccal phase after 48 h on CYA at all three temperatures (fig. 4), whereas the piedra isolate became predominantly coccal on the same medium only after incubation for 2 weeks at 37°C (fig. 5). No coccal forms were seen on YMA and blood agar at either 25 or 30°C. Although the new species would grow at 25°C, it did so only poorly on media without blood. Under these conditions, two colony types were seen, a pinpoint colony and a larger smooth colony. Microscopic morphology was identical for each colony type and subculture of each produced the same two colony types.

Biochemical characteristics

The piedra isolates were catalase positive, oxidase negative and produced methanethiol. By conventional methods, the strains were asaccharolytic. Proteolytic activity was shown by conventional methods with casein, gelatin and litmus milk. DNA was hydrolysed. Of 14 tested strains, five utilised xanthine and two hydrolysed tyrosine. In addition, *B. linens*, *B. epidermidis* and the three piedra strains tested by API Rapid ANA gave positive results for hydrolysis of leucyl-glycine, glycine, proline, phenylalanine, arginine and serine, and negative results with other test substrates. All strains tolerated NaCl 6.5% but did not

Fig. 4. Gram-stained of film the rod phase (A) from a 48-h culture of *B. epidermidis* on Casman's sheep blood agar at 37°C, and the coccal phase on CYA (B); $\times 1000$.

Fig. 5. Gram-stained film of the rod phase of a piedra isolate after incubation for 2 weeks at 37°C on Casman's sheep blood agar (A), and the coccal phase after incubation for 2 weeks at 37°C on CYA (B); $\times 1000$.

grow at higher concentrations. In contrast, *B. epidermidis* grew well on NaCl 10% but was partially inhibited at 15%. Uniformly negative results were obtained in tests for: nitrate reduction; arginine, ornithine and lysine decarboxylation; hydrolysis of Tween 80; production of H_2S , urease and indole; and growth on MacConkey agar.

Antibiotic sensitivity

All 14 strains of the new organism were uniformly sensitive to penicillin, ampicillin, vancomycin, trimethoprim sulphate and cephalothin; 10 strains were resistant to erythromycin, and six were resistant to oxacillin and tetracycline. Ergosan (Triclosan), the active agent used in many antibacterial soaps, inhibited all strains.

Cell-wall analysis

Meso-DAP and the sugars glucose, galactose, xylose and ribose were present in the cell walls. Mycolic acids and arabinose were not detected.

Table II. Fatty-acid composition* of *Brevibacterium* spp.

Organisms	Percent of structural fatty acids					
	15:0 iso	15:0 Anteiso	16:0 iso	16:0 Anteiso	17:0 iso	17:0 Anteiso
<i>B. epidermidis</i>	8.56	57.82	3.59	0.93	1.08	26.68
E2cr ^T = ATCC 49030	13.89	47.64	11.2	1.26	1.72	20.65
E7s	13.17	38.00	10.31	2.04	2.93	28.82
86-3	16.12	52.65	7.92	1.04	1.64	18.42

* Only those representing > 2% are identified.

Fatty-acid composition

The major fatty acids present in three strains of the new organism and *B. epidermidis* are shown in table II. The major component in all organisms was anteiso 15:0 with anteiso 17:0 as the second component. Smaller percentages of iso 15:0 and iso 16:0 were present, although percentages were higher in the new organism than in *B. epidermidis*.

DNA analysis

The G+C composition of strain E2cr was 63.05 mol% which was within the range of values cited for other species of *Brevibacterium*.

The major similarities and differences between the new species and skin isolates of the genera *Corynebacterium* and *Brevibacterium* are shown in table III.

Discussion

Aerobic, non-sporeforming, gram-positive bacilli of environmental origin frequently evade a taxonomic position. In some studies of clinical isolates, in which classical methods alone were used, over half of the coryneforms were unnamed.⁵ The more analytical methods added information, but left many groups taxonomically unassigned. With the advent of whole-cell fatty-acid analysis and the API Coryne system, many of the clinically relevant species, such as *C. diphtheriae*, *C. jeikeium* and *C. cystitidis* (D2) can be identified reliably.¹⁶⁻¹⁸

There are several morphologically similar genera from which medically significant coryneform isolates

Table III. Differences between species of *Brevibacterium*

Character	<i>B. epidermidis</i>	<i>B. linens</i>	Piedra isolates
Morphology			
—colony	smooth	smooth	contoured friable
—emulsifiable	yes	yes	no
—time for rod-to-coccus	48 h	6 days	2 weeks
—haemolytic	+	—	+
Growth at 25°C	good	good	slow
Growth at 37°C	good	poor	good
Tellurite reduction	—	—	+
Nitrate reduction*	+	+	—
Pyrazinamidase*	—	+	—
Mannose*	—	+	—
Ribose*	—	—	+
Xanthine utilisation*	+	+	5/14
Tyrosine hydrolysis	+	+	2/14

* Results from API Coryne.

must be distinguished. We have shown that these white piedra isolates are in the genus *Brevibacterium* by: rod-to-coccus cycle conversion; fatty-acid profile; the presence of DAP and absence of mycolic acids and arabinose in the cell wall; strict aerobic growth; NaCl tolerance; G+C mol% content; production of methanethiol; strong proteolysis; and lack of saccharolytic activity in conventional tests.

The fatty-acid profiles of *Corynebacterium* spp. are predominantly straight chain saturated and mono-unsaturated, whereas the major component of the new organism is iso 15:0 with anteiso 17:0 as the second component.¹⁹ The DNA G+C mol% composition of strain E2cr was 63.05% which is similar to that of other species of *Brevibacterium*: *B. epidermidis*, 63.3%; *B. linens*, 63.4%; and *B. iodinum*, 62.5%.²⁰

Many environmental, poorly defined species were originally included in the genus *Brevibacterium*. With more exacting studies, many of these have now been re-assigned, such as *B. divaricatum*, which has mycolic acid and arabinose in the cell wall and was found to resemble *C. glutamicum*. Similarly, the fatty-acid components of *B. acetylicum* are incompatible with *Brevibacterium* spp. On the other hand, the unassigned CDC Gp.B resembles the genus *Brevibacterium* in its whole cell fatty-acid composition.¹⁷ Currently, in contrast with previous classifications, there are only four recognised species: *B. linens*, *B. iodinum*, *B. casei* and *B. epidermidis*.²¹ The latter is the only species isolated from skin; the others have been associated with dairy products, have lower optimum temperature for growth, and are pigmented.

The white piedra isolate described here resembles *B. epidermidis* in habitat but differs from it in several significant ways (table III). The colonial and microscopic morphology are distinctive and the cultural conditions necessary to initiate the rod-to-coccus cycle are different. Biochemically, the new species can reduce tellurite and utilise ribose. We propose a new species, *B. mcbrellneri*, and the type strain, E2cr, has been deposited with the ATCC (49030).

The potential pathogenicity of the strains in this report is of particular interest, as, so far, no pathogenic species of *Brevibacterium* has been described. Salt tolerance, thought to be a factor in skin colonisation by *Brevibacterium* spp., is lower for the new strains than for other skin isolates,²² but the proteolytic properties may be significant in the attachment and invasion of keratin structures such as hair. The relationship of the white piedra strains to coryneform bacteria previously cultured from infected hair of cases of trichomycosis axillaris needs to be clarified.²³⁻²⁵ Ellner² postulated two separate diseases—white piedra, caused by the synergic activity between *Trichosporon beigeli* and *B. mcbrellneri* (proposed name), and trichomycosis axillaris, caused by a variety of undefined coryneform bacteria. However, since for these latter isolates, neither cell-wall analysis nor DNA G+C mol% content has been studied to establish a distinct taxonomic identity, it is possible that some could be *Brevibacterium* spp. Further studies are necessary to elucidate the taxonomic position of isolates from trichomycosis axillaris to evaluate the pathogenic potential of *Brevibacterium* spp. for skin and hair.

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