Corynebacterium pyruviciproducens sp. nov., a pyruvic acid producer

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A coryneform strain, 06-17730^T (=WAL 19168^T), derived from a groin abscess sample was characterized using phenotypic and molecular taxonomic methods. Comparative analyses revealed more than 3 % divergence of the 16S rRNA gene sequence and about 10 % divergence of the partial *rpoB* gene sequence from the type strain of *Corynebacterium glucuronolyticum*. The strain could also be differentiated from *C. glucuronolyticum* by a set of phenotypic properties. A DNA–DNA relatedness study between strain WAL 19168^T and *C. glucuronolyticum* CCUG 35055^T showed a relatedness value of 13.3 % (13.7 % on repeat analysis). The genotypic and phenotypic data show that the strain merits classification within a novel species of *Corynebacterium*. We propose the name *Corynebacterium pyruviciproducens* sp. nov. for the novel species. The type strain is 06-17730^T (=WAL 19168^T =CCUG 57046^T =ATCC BAA-1742^T).

Bacteria of the genus *Corynebacterium* have been recovered extensively from mammals and the environment (Feurer *et al.*, 2004; Hommez *et al.*, 1999). Except for *Corynebacterium diphtheriae*, the aetiological agent of diphtheria, most species of the genus are regarded as opportunistic pathogens (Pavioir *et al.*, 2002). Compared with those bacteria possessing proof of clinical pathogenicity, less emphasis has been put on *Corynebacterium*. Recently, however, a report demonstrated that *Corynebacterium* was the most prevalent bacterial genus associated with chronic wound infections in diabetics (possibly skin flora) (Dowd *et al.*, 2008). In our investigation of the bacteriology of acute wound infections (Finegold *et al.*, 2008), a novel aerobic, rod-shaped, Gram-positive corynebacterium was found. It is similar to *Corynebacterium*

glucuronolyticum (Devriese *et al.*, 2000) by phenotypic and genotypic identification. The aim of this polyphasic study was to describe the isolation and characterization of this novel organism.

A groin abscess specimen was collected at Olive View–UCLA Medical Center in 2006, and studied in the Wadsworth Anaerobic Laboratory (WAL). One isolate derived from this specimen, WAL 19168^T, was analysed by phenotypic and genotypic characterization methods.

Optimal growth conditions were investigated by cultivating strain WAL 19168^T aerobically and anaerobically on trypticase soy agar with 5 % sheep blood (TSA-II; Becton Dickinson Microbiology Systems) at 20, 37 and 42 °C. Lipid requirement was determined by comparison between the culture in simple brain heart infusion (BHI) broth (MP Biomedicals) and in the same medium supplemented with 1 % Tween 80 after 72 h at 37 °C (Riegel *et al.*, 1994). Further confirmation of lipid stimulation of growth was observed by the ‘response to serum’ test (Coyle & Lipsky, 1990).

Strain WAL 19168^T and reference strains were characterized by routine tests (Devriese *et al.*, 2000; Wattiau *et al.*,

Abbreviations: CAMP, Christie–Atkins–Munch–Petersen; CFA, cellular fatty acid; ME, metabolic end product; TBSA, tuberculostearic acid.

The GenBank/EMBL/DDBJ accession numbers for the 16S rRNA gene sequence and partial *rpoB* gene sequence of strain WAL 19168^T are FJ185225 and FJ899747, respectively.

UPGMA trees based on 16S rRNA and partial *rpoB* gene sequences are available as supplementary material with the online version of this paper.

2000) including Gram staining, API Coryne and API ZYM strips (bioMérieux) and susceptibility to the vibriostatic agent O/129 (150 µg; Oxoid) (Bernard *et al.*, 2002). The Christie–Atkins–Munch–Petersen (CAMP) reaction and fermentative test from fructose were confirmed by conventional methods (Riegel *et al.*, 1994, 1995). The reference strains included in the above procedures were *C. glucuronolyticum* CCUG 35055^T, *C. urealyticum* CCUG 18158^T, *C. accolens* CCUG 28779^T and *C. jeikeium* CCUG 27192^T, received from the Culture Collection of the University of Göteborg. Additionally for strain WAL 19168^T, susceptibility to antibiotics (0.016–256 µg ampicillin ml⁻¹, 0.002–32 µg ceftriaxone ml⁻¹, 0.016–256 µg clindamycin ml⁻¹ and 0.016–256 µg erythromycin ml⁻¹) was studied by the Etest system (AB Biodisk) with MIC value confirmation according to the Clinical and Laboratory Standards Institute (CLSI) guidelines. β -Lactamase activity was assayed by the nitrocefin disc test (Biotech Diagnostics). All strains were cultured under the same conditions and tests were performed in duplicate with 24–48 h pure cultures according to the manufacturers' instructions, unless otherwise noted.

For cellular fatty acid (CFA) and metabolic end product (ME) (short-chain volatile and non-volatile fatty acids) analyses, the strains were grown in BHI broth supplemented with 1% glucose and 5% rabbit serum (MP Biomedicals). CFAs of the strains were detected with a Hewlett Packard 5890 series II gas chromatograph, while MEs were tested by GLC equipped with a flame-ionization detector (Fingold *et al.*, 2004). Short-chain mycolic acids were investigated by a reversed-phase HPLC method (De Briel *et al.*, 1992). Microbial Identification System software (MIDI, Inc.) was applied to generate and analyse the profiles of MEs and CFAs, as described previously (Song *et al.*, 2006).

Genomic DNA of strain WAL 19168^T was extracted and purified using a QIAamp DNA Mini kit (Qiagen). PCRs were used for the amplification of the 16S rRNA gene (Song *et al.*, 2003) and the partial *rpoB* gene (with primers C2625F and C3130R) (Khamis *et al.*, 2004). The products were sequenced directly on a 3130 Genetic Analyzer (Applied Biosystems). The sequences obtained were compared with sequences in the GenBank database by using BLAST software (Brosius *et al.*, 1978); similarity searches were performed with CLUSTAL W and distance matrices were created with respect to 1409 bp of the 16S rRNA gene sequence and 508 bp of the partial *rpoB* gene

sequence. The neighbour-joining (Saitou & Nei, 1987) and UPGMA (Sneath & Sokal, 1973) methods were used to construct phylogenetic trees. The DNA G+C content of the novel organism was determined by HPLC (Mesbah *et al.*, 1989). A DNA–DNA reassociation test between strain WAL 19168^T and *C. glucuronolyticum* CCUG 35055^T was done by using photobiotin-labelled probes in microplate wells (Ezaki *et al.*, 1989).

Strain WAL 19168^T was recovered from the groin abscess with a count of 10⁸ c.f.u. ml⁻¹, accompanied by strains of *Actinomyces radingae* (10⁸ c.f.u. ml⁻¹), *Propionibacterium avidum* (10⁸ c.f.u. ml⁻¹) and *Propionibacterium acnes* (10⁷ c.f.u. ml⁻¹). As with *C. glucuronolyticum*, strain WAL 19168^T was able to grow on TSA-II plates at 37 and 42 °C aerobically as well as anaerobically after 2 days of incubation, but not at 20 °C. In addition, growth was visible in BHI broth supplemented with 1% Tween 80 but not in non-supplemented infusion, better under aerobic conditions than in an anaerobic jar, and more abundant at 37 than at 42 °C. The most profuse growth was exhibited in serum-supplemented liquid medium. In contrast to the opaque and whitish-pale colonies (1–1.5 mm in diameter) of *C. glucuronolyticum*, the colonies of strain WAL 19168^T were small (0.3–0.5 mm in diameter), circular, entire, convex and translucent (Fig. 1). Blood plates on which *C. glucuronolyticum* CCUG 35055^T was grown turned tan after approximately 24 h of incubation, while plates on which strain WAL 19168^T was inoculated did not change colour until 72 h later. Microscopy revealed that the cells of strain WAL 19168^T were Gram-positive, non-motile and rod-shaped, with occasional swellings.

According to tests on the API Coryne strip (code 2200725), the novel isolate was pyrazinamidase- and catalase-positive, weakly positive for pyrrolidonyl arylamidase and negative for gelatin hydrolysis, urease and reduction of nitrate. It could use D-ribose slightly and was able to ferment D-xylose, D-glucose, maltose and sucrose, but was unable to utilize D-mannitol, lactose (bovine origin) and glycogen. The API ZYM strip revealed that strain WAL 19168^T could produce esterase (C4), esterase lipase (C8), leucine arylamidase and a little naphthol-AS-BI-phosphohydrolase. The reactions for acid phosphatase, lipase (C14), valine arylamidase, cystine arylamidase, trypsin, α -chymotrypsin, α -galactosidase, α -fucosidase and α -mannosidase were negative. API Coryne and API ZYM strips both gave positive reactions for β -glucuronidase and negative tests for α -glucosidase, β -galactosidase, N-acetyl- β -glucosamini-

Fig. 1. Colonial appearance of *C. glucuronolyticum* CCUG 35055^T (a) and strain WAL 19168^T (b) on TSA-II plates after 2 days of incubation. Note the differences in the size of colonies and the colour of the plates.

dase, alkaline phosphatase and β -glucosidase. Strain WAL 19168^T showed negative results in the CAMP and fructose fermentation reactions by traditional procedures. In comparison with the reference strains, WAL 19168^T exhibited some distinct biochemical characteristics which are useful for clinical identification, as shown in Table 1. In addition, it could not produce β -lactamase and was susceptible to the lowest concentrations of antibiotics included. All the strains investigated in this study were sensitive to the O/129 agent.

The main CFA detected in strain WAL 19168^T was C_{18:1} ω 9c, comprising 28 % of the total fatty acids. No tuberculostearic acid (TBSA; 10-methyl C_{18:0}) was found; other CFAs detected at 10 % or more of the total fatty acids were C_{16:0} and summed feature 8 (C_{17:1} ω 8c and/or C_{17:2}). Short-chain mycolic acids (C₂₂–C₃₆) were present. Strain WAL 19168^T could utilize glucose and produced a small amount of propionic acid; the major MEs were acetic acid (volatile fatty acid; 494 mg ml⁻¹) and pyruvic acid (non-volatile fatty acid; 817 μ g ml⁻¹).

Table 1. Biochemical characteristics of strain WAL 19168^T and the type strains of related species

Strains: 1, *C. pyruviciproducens* sp. nov. WAL 19168^T; 2, *C. glucuronolyticum* CCUG 35055^T; 3, *C. urealyticum* CCUG 18158^T; 4, *C. accolens* CCUG 28779^T; 5, *C. jeikeium* CCUG 27192^T. +, Positive; –, negative; w, weakly positive reaction. Data were obtained in this study.

Characteristic	1	2	3	4	5
API Coryne code	2200725	7241125	2101004	7100324	2100304
CAMP reaction	–	+	–	–	–
Reduction of nitrates	–	+	–	+	–
Production of:					
β -Glucuronidase	+	+	–	–	–
Alkaline phosphatase	–	–	w	+	+
β -Glucosidase	–	+	–	–	–
Urease	–	+	+	–	–
Cystine arylamidase	–	+	–	–	–
Acid phosphatase	–	+	–	–	+
Leucine arylamidase	+	+	–	+	–
Lipase (C14)	–	–	w	–	+
Esterase (C4)	w	w	+	+	+
Fermentation of:					
Fructose	–	+	–	+	–
D-Ribose	w	–	–	+	+
Maltose	+	+	–	+	–
D-Glucose	+	+	–	+	+
Sucrose	+	+	–	–	–
D-Xylose	+	–	–	–	–

Comparisons based on the GenBank database showed that the closest match to strain WAL 19168^T was *C. glucuronolyticum* CCUG 35055^T, with 96.8 % sequence similarity for the 16S rRNA gene sequence and 90.6 % sequence similarity for the partial *rpoB* gene sequence. The DNA G+C content of strain WAL 19168^T is 62 mol%. This is within the range described for the genus *Corynebacterium* (51–70 mol%; Cerdeño-Tárraga *et al.*, 2003). A DNA–DNA reassociation study between strain WAL 19168^T and *C. glucuronolyticum* CCUG 35055^T showed a reassociation value of 13.3 % (13.7 % on repeat analysis), confirming that they are members of distinct species. Phylogenetic trees derived from alignments of the 16S rRNA and *rpoB* gene sequences are shown in Fig. 2 and Supplementary Fig. S1 (available in IJSEM Online).

The novel coryneform currently described is evidently lipophilic, based on our research on its growth conditions. We therefore chose the type strains of several medically significant lipophilic *Corynebacterium* species, i.e. *C. jeikeium*, *C. urealyticum* and *C. accolens*, as reference strains in addition to genetically related species. They are the members of coryneform CDC groups JK, D and G, respectively (Coyle & Lipsky, 1990; Pitcher *et al.*, 1992; Neubauer *et al.*, 1991). In our research, strain WAL 19168^T possessed a strongly positive catalase reaction, in common with other members of the genus *Corynebacterium*. However, it is known that the identification of members of the genus *Corynebacterium* is difficult in clinical and reference laboratories (Clarridge, 1986; Sabbe *et al.*, 1999). The API Coryne strip of strain WAL 19168^T in our study gave the result '*Corynebacterium glucuronolyticum* with 99.9 % ID'. Therefore, more supplementary tests should be performed to help classification, such as the CAMP test, fermentative activity from fructose and production of cystine arylamidase and acid phosphatase. In addition, the novel organism can be differentiated from important lipophilic strains by β -glucuronidase production and D-xylose fermentation tests. Regarding the CFAs of the novel strain, we did not find significant differences from the profiles of other *Corynebacterium* species (Van den Velde *et al.*, 2006). It should be noted that this strain was able to metabolize glucose and produce pyruvic acid at high concentrations, which can be useful in identifying the novel organism, although they are not unique characteristics within the genus *Corynebacterium*. Pyruvic acid is the major metabolic intermediate during the metabolic cycle of corynebacteria and can be produced by several species (Rollin *et al.*, 1995), but most of these are mutant strains developed for industrial purposes. So far, no *Corynebacterium* species recovered from human sources has been reported to have this ability.

Previously, it has been recognized that a 16S rRNA gene sequence divergence of 3 % or more was significant, and DNA–DNA reassociation was generally regarded as the primary molecular tool to delineate species when a novel species was being studied (Stackebrandt & Goebel, 1994). However, a recent study has revealed that sequence

Fig. 2. Neighbour-joining phylogenetic trees showing the position of strain WAL 19168^T (=06-17730^T), based on comparisons of 16S rRNA gene sequences of 1409 bp (a) and partial *rpoB* gene sequences of 508 bp (b). Bootstrap percentages (based on 1000 replications) are shown at branching points. GenBank accession numbers for each sequence are given in parentheses. Bars, 0.2 (a) and 1 (b) substitutions per site.

similarity of the single-copy *rpoB* gene can be an efficient supplement to these traditional taxonomic methods, since cases of distinct species exhibiting 16S rRNA gene sequence similarity >99% are often found (Adékambi *et al.*, 2008). Furthermore, an *rpoB* gene sequence similarity of >97.7% has already been confirmed to be useful in delineating species. In our study, 16S rRNA gene sequence comparison and a similarity value of 90.6% for the partial *rpoB* gene sequence indicated that the unknown bacterium isolated from a clinical wound specimen may well represent a new genospecies of *Corynebacterium*. All of the phylogenetic analyses demonstrate that the novel strain appears to represent a new subline of the genus *Corynebacterium*, for which the name *Corynebacterium pyruviciproducens* sp. nov. is proposed.

Description of *Corynebacterium pyruviciproducens* sp. nov.

Corynebacterium pyruviciproducens (py.ru.vi'ci.pro.du'cens. N.L. n. *acidum pyruvicum* pyruvic acid; L. part. adj. *producens* producing; N.L. part. adj. *pyruviciproducens* pyruvic acid-producing).

Gram-positive, non-spore-forming rods; non-motile. Lipophilic. Can grow aerobically or facultatively anaerobically at 37 and 42 °C. Optimum growth at 37 °C under aerobic conditions. Colonies are small (0.3–0.5 mm in diameter), circular, entire, convex and translucent after 2 days of incubation on blood agar. Strongly catalase-positive, but urease-negative. Cannot reduce nitrates and CAMP test is negative. Acid is produced from D-ribose, D-xylose, D-glucose, maltose and sucrose, but not from fructose, D-mannitol, lactose (bovine origin) or glycogen. Cells produce esterase (C4), esterase lipase (C8), leucine arylamidase, pyrazinamidase, a small amount of naphthol-AS-BI-phosphohydrolase and pyrrolidonyl arylamidase, but no acid phosphatase, β -galactosidase, α -glucosidase, N-acetyl- β -glucosaminidase, lipase (C14), valine arylamidase, cystine arylamidase, α -chymotrypsin, α -galactosidase, α -fucosidase, alkaline phosphatase, α -mannosidase or β -glucosidase. Trypsin and gelatin are not hydrolysed. β -Lactamase-negative. Susceptible to the vibriostatic agent O/129 and highly sensitive to ampicillin, ceftriaxone, clindamycin and erythromycin. The major cellular fatty acids are C_{18:1} ω 9c, C_{16:0} and summed feature 8 (C_{17:1} ω 8c and/or C_{17:2}); no TBSA is produced. Short-chain mycolic acids (C₂₂–C₃₆) are present. Major MEs from glucose are acetic acid and pyruvic acid.

The type strain is 06-17730^T (=WAL 19168^T =CCUG 57046^T =ATCC BAA-1742^T), isolated from a groin abscess specimen taken at Olive View-UCLA Medical Center in 2006. The DNA G + C content of the type strain is 62 mol%.

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