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Characterization of Oral Strains of *Cardiobacterium valvarum* and Emended Description of the Organism

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The description of the new species *Cardiobacterium valvarum* prompted a search for additional strains of the organism. Here we report characterization of four oral *Cardiobacterium* strains from the Culture Collection of the University of Goteborg. The 16S rRNA gene sequences of the organisms exhibited 99.6% to 99.3% homology with *Cardiobacterium valvarum*. The cellular fatty acid profiles, electrophoretic patterns of whole-cell proteins, growth rate and nutritional requirement, colonial and cellular morphology, and biochemical reactions were also similar to those of *C. valvarum*. These results thus classify these organisms as oral strains of *C. valvarum*. All strains were susceptible to many antibiotics tested. The description of the species was emended. *C. valvarum* is a rare cause of endocarditis, and its relationship with periodontal diseases may need investigation.

Cardiobacterium valvarum is a newly proposed species and, like *Cardiobacterium hominis*, is a rare cause of endocarditis (4, 6, 10). *C. hominis* and *C. valvarum* are the only two species within the genus *Cardiobacterium*. The single-strain status of *C. valvarum* prompted a search for additional strains of *C. valvarum* for further validation of the species and possible reservoir. Here we report characterization of four oral *Cardiobacterium* strains from the Culture Collection of the University of Goteborg (CCUG).

MATERIALS AND METHODS

Bacterial strains and culture. Six strains of *Cardiobacterium* organisms, i.e., four oral strains and two type strains, were included in the study. The origins of the strains are shown in Table 1. In addition, three other *C. hominis* strains, CCUG31207, CCUG33980, and CCUG46845, were also sequenced for a portion of the 16S rRNA gene as an initial screening method in the search for *C. valvarum* strains. All the strains were initially identified by phenotyping and fatty acid analysis at the CCUG. Subcultures of the organisms were plated on sheep blood agar and chocolate agar (BBL; BD Microbiology Systems, Cockeysville, MD) and incubated aerobically at 35°C with 5% CO_2. Colony morphology was observed and size was measured under a dissecting microscope.

Sequencing of the 16S rRNA gene and phylogenetic analysis. The amplification of 16S rRNA genes by a PCR and subsequent sequencing of the amplicon were performed as described previously (5). Briefly, extracted genomic DNA was amplified by a set of highly conserved (universal) bacterial primers: 5′ TGCCA GCAGCCGCGGTAATAC 3′ and 5′ CGCTCGTTGCGGGACTTAACC 3′ (positions 515 to 1107 of GenBank accession J01859 of *Escherichia coli*). Sequencing of the 593-bp amplicon was performed by the dye terminator method in an ABI 377 sequencer (Applied Biosystems, Foster City, CA). All oral strains and the *C. hominis* strains were sequenced to this length. In addition, the oral strains were further amplified to 1,490 bp (near full length) through two primers: 5′ GCGTGCTTAACACATGCAAGTC 3′ and 5′ AGGAGGTGATCCAAC CGCA 3′ (positions 42 to 1539 of *E. coli* J01859). The 1,490-bp amplicon was sequenced using these and a few sets of internal primers. The sequences of *C. hominis* (M35014) and *C. valvarum* (AF506987) were determined previously (2, 4). The phylogenetic analysis was performed by using ClustalW multiple alignments (www.ebi.ac.uk/clustalw) (3).

Other studies. The cellular fatty acids were analyzed in a commercial laboratory using gas-liquid chromatography and Sherlock version 4.5 (0209B) software (Microbial ID, Inc., Newark, DE). The whole-cell proteins were analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) followed by computer pattern analysis and generation of a dendrogram for relatedness (13). The biochemical tests were performed in conventional tube medium (BBL; BD Microbiology Systems, Cockeysville, MD). The antibiotic susceptibility tests were performed using Etest (Biodisk, Solna, Sweden) on nonstandardized blood Mueller-Hinton agar (for *C. hominis*) or sheep blood agar (for other strains). The results were obtained after 48 h of incubation in the CO_2 chamber and interpreted according to the breakpoints set for *Pseudomonas aeruginosa*, *Neisseria gonorrhoeae* (penicillin), and *Haemophilus* spp. (tetracycline and erythromycin) (8).

Nucleotide sequence accession number. The 16S rRNA gene sequences of CCUG31208, CCUG13150, and CCUG12990 were deposited as GenBank accessions AY596468, AY596469, and AY596470, respectively.

RESULTS

Analysis of 16S rRNA gene sequences. The 16S rRNA gene sequences of the oral strains CCUG13094, CCUG12990, CCUG13150, and CCUG31208 matched best with that of *C. valvarum* at 99.6% (1,486/1,492 bp), 99.5% (1,484/1,491), 99.3% (1,482/1,492), and 99.5% (1,484/1,492), respectively. These matches suggest that the oral strains probably belong to *C. valvarum* with minor differences, likely at subspecies level. The strains matched 96.1% to 96.4% with *C. hominis*, consistent with their being a separate species (11).

The oral strains matched 99.4% to 99.9%, with CCUG12990, CCUG13094, and CCUG13150 being closest (divergence of 1 to 3 nucleotides of the 1,490 bp). These strains were isolated from the subgingival pocket, and CCUG12990 and CCUG13150 were also associated with amelogenesis imperfecta and periodontitis, respectively (Table 1). CCUG31208 also matched completely (1,432/1,432 bp) with AF144696 (B. J. Paster and F. E. Dewhirst, unpublished), a sequence derived from the same strain (M. Kilian, personal communication). This result confirms our sequence quality. There were no significant matches (<70%) between these cardiobacteria and various *Helicobacter* species despite some similarities by fatty acid analysis (see below).

In an attempt to search for additional *C. valvarum* strains, three *Cardiobacterium* strains, CCUG31207, CCUG33980, and CCUG46845, were also sequenced for 550 bp in the middle

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TABLE 1. Origins of the six strains of *Cardiobacterium* species

Feature	CCUG12990	CCUG13094	CCUG13150	CCUG31208	CCUG48245^T	ATCC15826^T
Previous identification	*Cardiobacterium* EF group 18[a]	*Cardiobacterium* EF group 18	*Cardiobacterium* EF group 18	*Cardiobacterium* EF group 18	*Cardiobacterium valvarum*	*Cardiobacterium hominis*
Source and disease	Subgingival pocket, amelogenesis imperfecta	Subgingival pocket	Subgingival pocket, periodontitis	Tooth plaque	Blood, endocarditis	Blood, endocarditis
Yr and depositor(s)	1982, G. Dahlen, Sweden	1982, G. Dahlen, Sweden	1982, G. Dahlen, Sweden	1985, W. Frederiksen and M. Kilian, Sweden	2004, Han et al.	1964, Slotnik et al.
GenBank no., reference or note	AY596470, this study	Not deposited	AY596469, this study	AY596468, this study, also *Cardiobacterium* sp. strain B, AF144696	AF506987, Han et al., 2004	M35014, Dewhirst et al., 1990; Slotnik et al., 1964

[a] EF group 18, Enevold Falsen uncertain taxon group number 18 at the CCUG.

portion of the 16S rRNA genes, and the sequences all matched fully with *C. hominis* (data not shown). These strains also showed culture characteristics identical to those of the *C. hominis* type strain (data not shown). In addition, a previous sequence of CCUG31207 (AF144697; B. Paster, unpublished) also showed a full match with *C. hominis*.

Analysis of cellular constituents. The cellular fatty acids of the oral strains (Table 2) were nearly homogeneous (euclidean distance, 4.7), and they differed slightly, likely at subspecies level, from *C. valvarum* (euclidean distance, 13.3). The major peaks of these five organisms, i.e., C16:0 and C18:1ω7c, were nearly uniform, representing 27% to 35% (mean, 31%) and 31% to 42% (mean, 37%), respectively. These peaks, however, differed substantially from those of *C. hominis*, which were 19% and 46%, respectively. Overall, the oral strains and *C. valvarum* showed slight to moderate similarity with *C. hominis* (similarity indices [SI] of 0.491 to 0.772) and with unrelated *Helicobacter cinaedi* (SI of 0.313 to 0.675).

The whole-cell proteins of the six studied strains and a few other strains were also analyzed by SDS-PAGE. The dendrogram of the analysis (Fig. 1) showed that the oral strains and *C. valvarum* formed a tight cluster that was substantially distant from the cluster of *C. hominis* strains, consistent with species difference.

Culture and biochemical features. The culture and biochemical features are shown in Table 3. All strains grew optimally in the presence of 5% CO_2 and slightly in a microaerophilic atmosphere (Campy jar) but not anaerobically. Sheep blood agar was the preferred medium, and slight alpha-hemolysis was observed for CCUG12990, CCUG13094, CCUG31208, and *C. hominis* but not for CCUG13150 and *C. valvarum*. In a 48-h culture, the colonies were round, opaque, smooth, and glistening with sizes of 0.6 to 0.8 mm for CCUG13150, CCUG31208, and *C. hominis* and 0.2 to 0.3 mm for the other strains. The colonies of CCUG12990 were also elevated. On Gram stain, all were gram negative with rare to occasional teardrop morphology. CCUG12990, CCUG13094, and CCUG31208 were long rods (1 by 3 to 8 μm), and *C. valvarum* and CCUG13150 were regular rods (1 by 2 to 5 μm). *C. hominis* was a pleomorphic short rod, however, that turned to bacillary (0.7 by 2 to 4 μm) form when cultured on chocolate agar. The oral strains barely grew on chocolate agar and were pleomorphic short rods. Agar pitting was observed for *C. hominis* and *C. valvarum* but not for the oral strains. None grew on MacConkey agar.

The biochemical test results showed some variations among the strains, likely caused by their fastidious growth. Like *C. hominis* and *C. valvarum*, the oral strains were positive for cytochrome oxidase and hydrogen sulfide production but negative for catalase, urea hydrolysis, esculin hydrolysis, and nitrate reduction. Unlike *C. hominis* and *C. valvarum*, however, the oral strains were all negative for indole test. For the oxidative and fermentative utilizations of 11 sugars, all five strains consistently used fructose and mannose but not galactose, lactose, raffinose, or xylose. *C. hominis* also utilized dextrose, maltose, mannitol, sorbitol, and sucrose, but *C. valvarum* and the oral strains used these sugars variably. On a miniaturized API 20NE test, the organisms were mostly nonreactive (code 2000004 or 0000004), resulting in misidentification as *Pasteurella multocida*. Thus, API 20NE was unsuitable for these fastidious organisms.

TABLE 2. Cellular fatty acid analysis of six strains of *Cardiobacterium* species[a]

Fatty acid profile ST, or interpretation	CCUG12990	CCUG13094	CCUG13150	CCUG31208	*C. vaivarum* CCUG48245^T	*C. hominis* ATCC15826^T
12:0[b]	6.44	5.52	5.60	5.89	9.20	8.15
13:0	0.19	0.19	0.21	0.34	0.49	0.53
14:0	14.45	11.84	14.11	13.01	19.10	13.67
15:0	—	0.08	—	—	0.20	1.35
14:0 3-OH/16:1 ISO	2.77	3.00	2.28	3.41	3.62	2.90
16:1ω7c/16:1ω5c	3.32	2.95	2.52	2.72	3.24	3.57
16:1ω5c	—	0.15	—	—	—	0.16
16:0	**30.26**	**29.16**	**34.71**	**31.87**	**26.53**	19.34
15:0 3-OH	—	—	—	—	—	0.14
17:1ω8c	—	—	—	—	—	0.11
17:1ω6c	—	—	—	—	—	0.23
17:0	—	0.08	—	—	0.19	0.25
16:0 3-OH	1.55	2.33	1.53	2.43	2.27	2.19
18:2ω6,9c/18:0 ANTE	0.32	0.31	0.48	0.34	1.11	0.27
18:1ω9c	—	—	0.47	—	0.73	—
18:1ω7c	**38.28**	**41.83**	**35.50**	**37.72**	**30.98**	46.11
18:0	2.43	2.19	2.59	2.27	2.36	0.90
20:4ω6,9,12,15c	—	0.12	—	—	—	0.13
Total (%)	100.01	99.75	100.0	100.0	100.02	100.0
SI to *C. hominis*	0.772	0.746	0.580	0.678	0.491	0.984
SI to *Helicobacter cinaedi*	0.524	0.507	0.675	0.581	0.313	0.00
Interpretation	Inconclusive	Inconclusive	Inconclusive	Inconclusive	Inconclusive	*C. hominis*

[a] Boldface numbers represent characteristic peaks for the *C. valvarum* strains; —, not detected.
[b] Fatty acid profile values are percentages.

Antibiotic susceptibility. All strains were susceptible to amikacin with MICs of 0.25 to 1.5 μg/ml, ampicillin (0.016 to 0.047 μg/ml), cefepime (0.064 to 0.25 μg/ml), ciprofloxacin (0.016 to 0.047 μg/ml), erythromycin (zone of 14 to 23 mm by Kirby-Bauer method), imipenem (0.012 to 0.032 μg/ml), penicillin (0.016 to 0.064 μg/ml), tetracycline (0.25 to 1.5 μg/ml), ticarcillin-clavulanate (<0.016 to 0.094 μg/ml), and trimethoprim-sulfamethoxazole (0.032 to 0.064 μg/ml).

DISCUSSION

The preceding phylogenetic and phenotypic analyses suggest that the oral *Cardiobacterium* strains belong to *C. valvarum* with likely subspecies difference. In view of the limited number of strains, further classification is practically unnecessary at present. Thus, we emend the description of the species as follows.

Emended description of *C. valvarum*. *C. valvarum* is a fastidious gram-negative bacillus of human origin. The bacterium grows best on sheep blood agar with CO_2, and visible colonies appear after an incubation of 2 to 3 days. It also grows microaerobically but not anaerobically. The colonies are round, opaque, smooth, and glistening with various sizes depending on strains. Optimally cultured cells measure 1 by 2 to 5 μm to 1 by 3 to 8 μm, depending on strains. *C. valvarum* is positive for cytochrome oxidase and H_2S production but negative for catalase, urea hydrolysis, esculin hydrolysis, and nitrate reduction. The type strain CCUG48245^T is positive for indole production, whereas the oral strains are negative. It consistently uses fructose and mannose oxidatively and/or fermentatively but not galactose, lactose, raffinose, or xylose. The utilization of dextrose, maltose, mannitol, sorbitol, and sucrose varies with strains. Cellular fatty acid analysis can differentiate *C. valvarum* from *C. hominis* by dominant peaks, such as C18:1ω7c

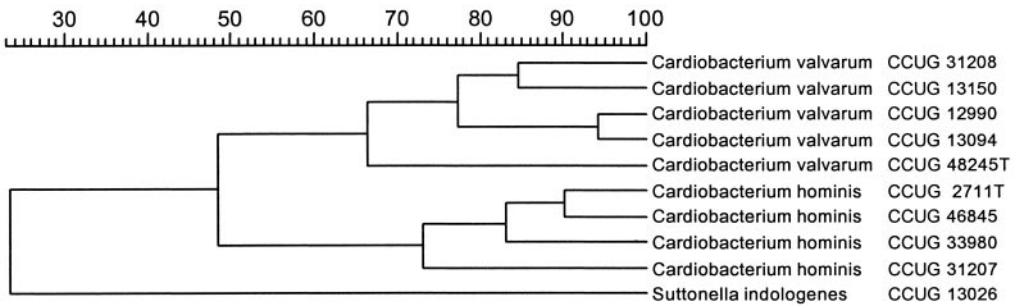

FIG. 1. Relatedness of several *Cardiobacterium* strains by SDS-PAGE pattern analysis of whole-cell proteins. *C. hominis* strain CCUG2711^T equals ATCC 15826^T.

TABLE 3. Biochemical reactions of six strains of *Cardiobacterium* species[a]

Characteristic	CCUG12990	CCUG13094	CCUG13150	CCUG31208	*C. valvarum* CCUG48245^T	*C. hominis* ATCC15826^T
Colony size (mm) at 48 h on SBA	0.2–0.3	0.2–0.3	0.6–0.8	0.6–0.8	0.2–0.3	0.6–0.8
Hemolysis	Slight α	Slight α	−	Slight α	−	Slight α
Gram stain morphology	GNR, 1 × 3–8 μm	GNR, 1 × 3–8 μm	GNR, 1 × 2–5 μm	GNR, 1 × 3–8 μm	GNR, 1 × 2–5 μm	GNR, 0.7 × 2–4 μm (on CAP)
Motility	−	−	−	−	−	−
Production of indole	−	−	−	−	+ strongly	+ weakly
Cytochrome oxidase	+	+	+	+	+	+
H_2S production	+	+	+	+	+	+
Catalase	−	−	−	−	−	−
Nitrate reduction	−	−	−	−	−	−
Urea hydrolysis	−	−	−	−	−	−
Esculin hydrolysis	−	−	−	−	−	−
Gas from glucose	−	−	−	−	−	−
API 20NE	Reactive only for oxidase, code 0000004	Reactive only for oxidase, code 0000004	Reactive only for oxidase, code 0000004	Reactive only for oxidase, code 0000004	Reactive for only tryptophanase and oxidase, code 2000004	Reactive only for tryptophanase and oxidase, code 2000004
Oxidation/fermentation of:						
Dextrose	±/±	+/+	−/±	−/−	+/±	+/+
Fructose	+/+	−/+	±/±	+/+	+/−	+/+
Galactose	−/−	−/−	−/−	−/−	−/−	−/−
Lactose	−/−	−/−	−/−	−/−	−/−	−/−
Maltose	±/+	+/+	+/+	+/+	−/−	+/+
Mannitol	−/−	−/−	−/−	±/±	−/−	+/+
Mannose	±/+	+/+	−/±	+/+	+/+	+/+
Raffinose	−/−	−/−	−/−	−/−	−/−	−/−
Sorbitol	−/−	±/+	−/−	+/+	+/+	+/+
Sucrose	+/−	+/+	+/±	+/+	−/−	+/+
Xylose	−/−	−/−	−/−	−/−	−/−	−/−

[a] +, positive; −, negative; ±, weakly positive; CAP, chocolate agar plate; GNR, gram-negative rod; SBA, sheep blood agar.

(31% to 42%), C16:0 (27% to 35%), and C14:0 (12% to 19%). The 16S rRNA gene sequences, i.e., AF506987 for the type strain and AY596468, AY596469, and AY596470 for oral strains, define this organism. *C. valvarum* is a rare cause of endocarditis. It is susceptible to a number of antibiotics tested.

The rarity and fastidiousness of *C. valvarum* and the lack of uniform biochemical reactions likely explain why this organism has been underrecognized. Thus, establishing this species may facilitate future studies of the organism by distinguishing it from *C. hominis*. In addition to causing endocarditis, *C. valvarum* may be also significant for periodontal diseases, although the possibility of incidental isolation of oral strains could not be excluded. The previous patient with *C. valvarum* endocarditis also had a dental procedure 2 weeks before the onset of disease (4), and *C. valvarum* DNA has been detected in advanced noma lesions (9). These findings, together with the current oral *C. valvarum* strains and the known oral origin of *C. hominis*, form the preliminary evidence of the oral origin of *C. valvarum*.

Our data and a previous study (7) have also shown that *C. valvarum* and *C. hominis* are exquisitely susceptible to many antibiotics tested, which may be useful for medical and dental practices. The previous *C. valvarum* endocarditis was treated successfully with piperacillin-tazobactam (4), a recommended drug for *C. hominis* endocarditis (12). Thus far, approximately 70 cases of *C. hominis* endocarditis have been reported and reviewed (1, 12). The phenotypic similarities between *C. valvarum* and *C. hominis*, the sole species from 1964 to 2004, make us speculate that some of those cases might have been caused by *C. valvarum*.

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