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"*Candidatus* comitans," a Bacterium Living in Coculture with *Chondromyces crocatus* (Myxobacteria)

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We describe the phylogenetic position and some taxonomically relevant characteristics of a small pleomorphic gram-negative bacterium that was cocultured with some strains of the myxobacterium *Chondromyces crocatus* that were isolated from the same geographic and ecological habitat. A 16S ribosomal DNA analysis revealed that the companion was a member of the "*Cytophaga-Flavobacterium-Bacteroides*" complex and was most closely related to members of the genus *Sphingobacterium*. The results of a fatty acid analysis, an isoprenoid composition analysis, and a DNA G+C content analysis and the presence of sphingolipids confirmed that this bacterium is affiliated with the genus *Sphingobacterium*. As the companion bacterium survived for only a few generations on solid media and could not be maintained in pure culture, we assign to this novel taxon that lives in close association with the myxobacterium *C. crocatus Candidatus* status as "*Candidatus* comitans."

The myxobacteria are a phylogenetically coherent group of gram-negative, rod-shaped, gliding bacteria (9, 22) that have morphogenetically complex life cycles (19). Together with the gram-negative sulfur-reducing bacteria and the bdellovibrios, these organisms belong to the delta subclass of the *Proteobacteria* phylogenetically (28).

The presence of bacteria associated with the myxobacterium *Chondromyces crocatus* was reported as early as 1963 (11). This myxobacterium is used to produce certain secondary metabolites (7, 8, 20). The accompanying flora can be recognized a few hours after the start of fermentation and is present for several days before it disappears without being totally eliminated. Attempts to eliminate the companion organisms from *C. crocatus* cultures by separating the two bacteria on culture plates have been successful but have led to the death of the myxobacterium (11). The presence of uncharacterized contamination during the production of a chemical compound is unwanted and raises questions about the relationship between the organisms present in the bacterial association. In order to investigate whether a single bacterium or several species, referred to as companion(s) below, are associated with *C. crocatus* and to determine the taxonomic status of these bacteria, we analyzed the companions of several *C. crocatus* strains by molecular and chemotaxonomic methods.

MATERIALS AND METHODS

Bacterial strains. *C. crocatus* Cm c2, Cm c3, and Cm c4 were isolated from a soil sample collected in 1982 on Madeira. The host cell-companion cell associations have been deposited in the culture collection of H. Reichenbach, GBF-Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany. The companion strains of *C. crocatus* Cm c2, Cm c3, and Cm c4 are strains CJ2, CJ3, and CJ4, respectively.

Culture conditions. *C. crocatus* was routinely grown in Pol 1 broth (7) at 30°C in a shaking Erlenmeyer flask. The companion cells were cultivated on nutrient broth (NB) agar (Oxoid).

Morphological tests. The sizes and shapes of cells were determined by phase-contrast microscopy. The motility of cells was determined by growing the cells on soft agar.

Extraction and PCR amplification of 16S rDNA. The cells in 1-ml portions of liquid *C. crocatus* cocultures were mechanically homogenized with glass beads (diameter 0.08 to 0.25 mm; Roth, Karlsruhe, Germany) by vortexing. The beads were collected by centrifugation, and the cells were lysed enzymatically as described previously (17). Extraction of genomic DNA, amplification of 16S ribosomal DNA (rDNA) by PCR, and purification of the PCR products were performed as described previously (17, 18).

Cloning of 16S rDNA amplification products. 16S rDNA amplification products were cloned into plasmid vector pCRII (TA cloning system; Invitrogen, San Diego, Calif.) by using the method described in the manufacturer's instructions. Ligation products were transformed into competent cells supplied in the TA cloning kit. Clones were resuspended in sterile water and boiled for 10 min. The preparation was centrifuged, and the supernatant was used to screen for 16S rDNA insertions by PCR by using a conserved 16S rDNA primer pair (17).

Sequencing of insertions and phylogenetic analysis. Insertions were amplified by PCR, cleaned (17), and sequenced with a DyeDeoxy terminator sequencing kit (Applied Biosystems, Foster City, Calif.). The extension products were purified by treatment with phenol-chloroform, and a model 373A automated sequencer (Applied Biosystems) was used to separate the sequence products. The sequences were aligned manually with homologous 16S rDNA sequences obtained from the Ribosomal Database Project (10). Pairwise evolutionary distances were computed by using the correction of Jukes and Cantor (6). Phylogenetic analyses were performed by using the algorithm of DeSoete (2) and the neighbor-joining method of Saitou and Nei (21). Initially, the sequences of the clones were compared with the sequences of representatives of the main lines of descent. Following identification of the phylum with which the clones clustered, a thorough phylogenetic analysis was performed with members of this phylum.

Generation of companion cell mass. Cell mass was obtained by using a model TI 100 membrane fermentor (INFORS AG Einsbach, Germany). In principle, two cultures that were separated by a membrane that had defined a pore size (40 kDa) were fermented. Substances, such as proteins, could pass through the membrane by free diffusion and thus could be used by the organisms (5).

Analysis of peptidoglycan. The cell wall was examined for the presence of *meso*-diaminopimelic acid by using whole-cell hydrolysates as described previously (18).

Extraction and analysis of lipoquinones, polar lipids, and sphingolipids. Lipoquinones, polar lipids, and sphingolipids were extracted from 100 mg of freeze-dried material by the two-stage method described previously (26, 27). The respiratory lipoquinones were separated into their different classes (menaquinones and ubiquinones) by thin-layer chromatography on silica gel thin layers (catalog no. 805023; Macherey-Nagel) by using hexane–*tert*-butylmethyl ether as the solvent. UV-absorbing bands corresponding to menaquinones or ubiquinones were removed from the plate and analyzed by high-performance liquid chromatography (HPLC). The HPLC analysis was performed with a model LDC analytical HPLC (Thermoseparations) equipped with a reverse-phase 3 μm

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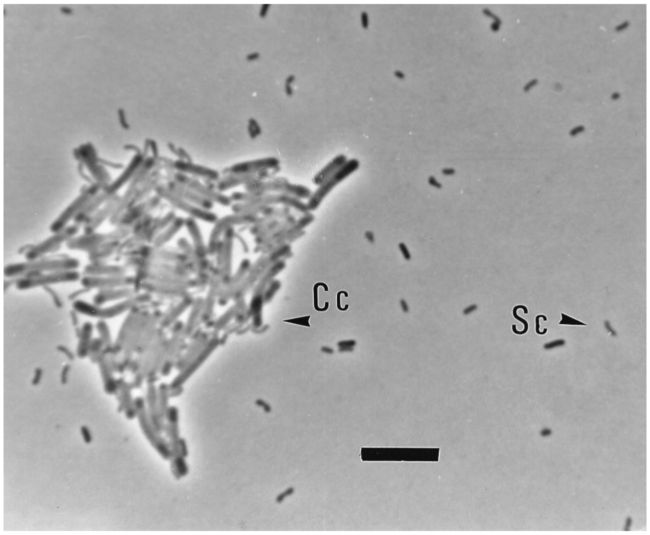

FIG. 1. Phase-contrast micrograph of cells of *C. crocatus* Cm c3 (Cc) and companion strain CJ3 (Sc). Bar = 5 μm.

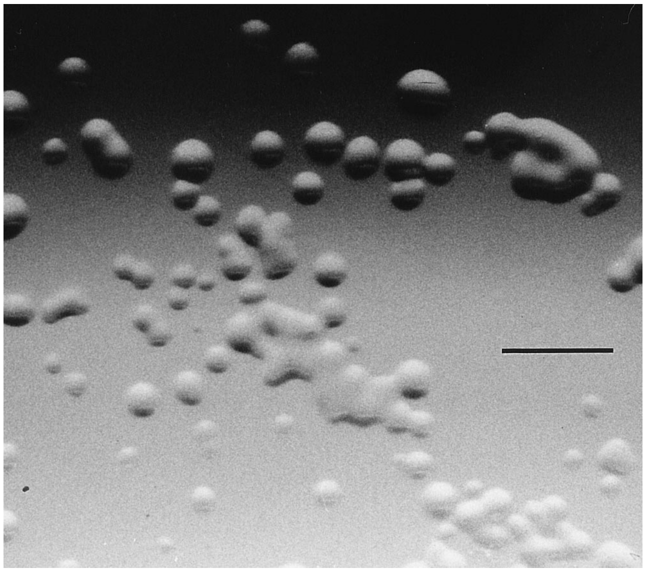

FIG. 2. Size and morphology of companion strain CJ3 colonies. Bar = 1 mm.

RP18 column (2 by 125 mm; Macherey-Nagel); methanol was used as the eluant, and the respiratory lipoquinones were detected at 269 nm.

The polar lipids were separated by two-dimensional silica gel thin-layer chromatography (catalog no. 818135; Macherey-Nagel); the gel was developed with chloroform-methanol-water (65:25:4, vol/vol/vol) in the first dimension and with chloroform-methanol-acetic acid-water (80:12:15:4, vol/vol/vol/vol) in the second dimension. The total lipids and specific functional groups were detected by using dodecmolybdophosphoric acid (total lipids), Zinzadze reagent (phosphate), ninhydrin (free amino groups), periodate-Schiff reagent (α-glycols), Dragendorff reagent (quaternary nitrogen), and anisaldehyde-sulfuric acid and alpha-naphthol–sulfuric acid (glycolipids).

The sphingolipids (as their free bases) were obtained by acid hydrolysis with 4 M HCl–methanol (1:1, vol/vol) at 100°C for 3 h. After the solution was made alkaline with NaOH, the free bases were extracted with *tert*-butylmethyl ether. The *N*,*O*-bis(trimethylsilyl)acetamide derivatives were analyzed by gas chromatography by using the conditions described above.

Extraction and analysis of fatty acids. Fatty acid methyl esters were obtained from freeze-dried biomass by sonification, methylation, and extraction by the method of Miller (13). The fatty acid methyl ester mixtures were separated by using a model 5898A microbial identification system (Microbial ID, Inc., Newark, Del.) (12). The following conditions were used: injection and detector port temperature, 300°C; inlet pressure, 80 kPa; split ratio, 50:1; injection volume, 1 μl; and a temperature program in which the temperature increased from 130 to 310°C at a rate of 4°C/min.

Determination of the DNA G+C content. The methods used to determine the G+C content have been described previously (12, 25). Approximately 20 μg of DNA was hydrolyzed and dephosphorylated, and the nucleosides were separated by reverse-phase HPLC. The retention times of the nucleosides were determined with synthetic compounds. Nonmethylated lambda phage DNA having a G+C content of 49.858 mol% (16) was used as the calibration reference.

Nucleotide sequence accession numbers. The 16S rDNA nucleotide sequence which we determined has been deposited in the EMBL database under accession number X91814.

RESULTS

Morphology. Microscopic investigations of *C. crocatus* broth cultures revealed that some cultures of this myxobacterium contained two different cell types; in addition to the characteristic *C. crocatus* cells, small, slender, pleomorphic cells were observed (Fig. 1). Inoculation of NB agar with contaminated *C. crocatus* liquid cultures resulted in the appearance of very small, convex, opaque colonies of different sizes after 7 days. The edges of these colonies were smooth and round (Fig. 2). The cell mass appeared to be orange after fermentation cultures were concentrated. An examination of the companion cells obtained from pure cultures on NB agar and from the cocultures revealed two different cell morphologies; while the pure cultures contained highly pleomorphic, spheroplast-like cells that were 2.0 to 4.0 μm long and up to 1.0 μm in diameter (Fig. 3), the cells in cocultures with *C. crocatus* were small, slender, only slightly pleomorphic, gram-negative rods that were 1.0 to 2.0 μm long and about 0.5 μm in diameter (Fig. 1). The highly pleomorphic cells of the pure cultures turned into slightly pleomorphic cells when they were cocultivated with pure cultures of other strains of *C. crocatus* (5). In pure cultures, cells of the companion organism grew aerobically at

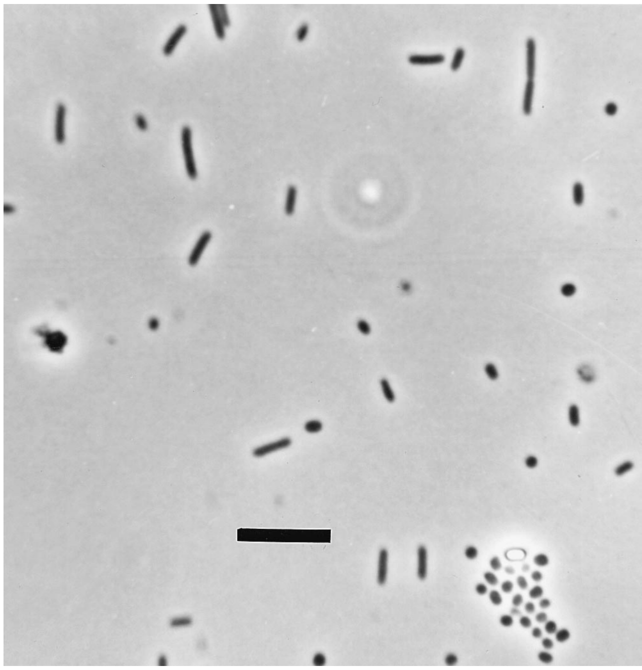

FIG. 3. Phase-contrast micrograph of a pure culture of companion strain CJ3, showing the rod-shaped, pleomorphic, and degenerate cell morphologies. Bar = 5 μm.

TABLE 1. 16S rDNA dissimilarity values for isolate CJ3 and the type strains of species belonging to the genera *Sphingobacterium*, *Flexibacter*, *Cytophaga*, *Empedobacter*, and *Chryseobacterium*[a]

Organism	% 16 S rDNA dissimilarity								
	Isolate CJ3	*Sphingobacterium spiritivorum* ATCC 33861T[b]	*Sphingobacterium mizutae* ATCC 33299T	*Sphingobacterium thalpophilum* ATCC 43320T	*Sphingobacterium heparinum* ATCC 13125T	*Flexibacter canadensis* ATCC 29591T	*Cytophaga diffluens* ATCC 23140	*Empedobacter brevis* ATCC 14234	*Chryseobacterium meningosepticum* ATCC 13253T
Sphingobacterium spiritivorum ATCC 33861T	9.4								
Sphingobacterium mizutae ATCC 33299T	9.4	6.9							
Sphingobacterium thalpophilum ATCC 43320T	10.1	7.4	6.7						
Sphingobacterium heparinum ATCC 13125T	9.5	11.2	11.1	9.4					
Flexibacter canadensis ATCC 29591T	12.8	11.3	12.7	12.5	11.0				
Cytophaga diffluens ATCC 23140	17.7	16.6	16.8	17.3	18.4	18.2			
Empedobacter brevis ATCC 14234	18.7	19.5	20.4	19.4	19.2	18.5	19.2		
Chryseobacterium meningosepticum ATCC 13253T	19.6	18.2	19.1	18.3	20.1	16.6	17.5	9.0	
Chryseobacterium indologenes ATCC 29897T	23.0	21.5	21.9	21.0	23.2	20.5	19.9	12.4	6.1

[a] Data from reference 10.
[b] T = type strain.

temperatures between 20 and 30°C but not at 37°C. No growth occurred under microaerophilic or anaerobic conditions.

Phylogenetic analyses. We determined an almost complete 16S rDNA sequence (length, 1,486 bases) for companion strain CJ2 of *C. crocatus* Cm c2 and about 800 nucleotides of the sequences of companion strains CJ3 and CJ4 of *C. crocatus* Cm c3 and Cm c4, respectively. The levels of similarity were virtually identical (>99.8%) for the common region analyzed in the three companion strains. The sequence of CJ2 was also compared with the homologous sequences of a broad range of prokaryotic taxa. The closest phylogenetic relatives were members of the “sphingobacter” subgroup of the “*Cytophaga-Falvobacterium-Bacteroides*” phylum (3). Strain CJ2 exhibited the highest levels of 16S rDNA similarity (more than 90% similarity) with strains belonging to the genus *Sphingobacterium* (Table 1 shows the dissimilarity values used to generate the distance matrix tree) and related taxa. The CJ2 sequence had all of the signature nucleotides identified for the “sphingobacter” subgroup (3). The relationships of strain CJ2 with the phylogenetically most closely related species for which sequence data are available are shown in Fig. 4.

Chemotaxonomic properties of strain CJ3. Our cell wall analyses confirmed that strain CJ3 contained *meso*-diaminopimelic acid, which is the diagnostic amino acid of gram-negative cell walls.

Menaquinone MK-7 was the major isoprenoid quinone (99% of the total isoprenoid quinones). With the exception of phosphatidylethanolamine, all of the strain CJ3 polar lipids, which differed from the polar lipids of *C. crocatus* Cm c2, could not be identified as known lipids (data not shown). Fatty acids iso-$C_{15:0}$ (33% of the total fatty acids) and iso-2-OH-$C_{15:0}$ (31%) dominated the fatty acid pattern of strain CJ3, while straight-chain fatty acids ($C_{15:0}$ and $C_{16:0}$ [7 and 3%, respectively] and iso-3-OH-$C_{17:0}$ (8%) occurred in smaller amounts. Unknown sphingolipids were also present. More detailed information could not be obtained because of the small amount of cell mass available. It should be noted that the cells used to determine chemotaxonomic properties were not grown under strictly standardized conditions, as cell mass was harvested from a bioreactor after different lengths of time (3 to 5 days). The G+C content of the strain CJ3 DNA was 35.9 mol%.

Physiological properties. Physiological and biochemical characteristics could not be determined by the API system (bio-Merieux Vitek, Marcy-l'Etoile, France), by the Biolog system (Biolog, Inc., Hayward, Calif.), or in any other medium suitable for the cultivation of sphingobacteria as growth of the companion organisms was not supported.

DISCUSSION

Our phylogenetic analysis showed that companion strains CJ2, CJ3, and CJ4 were related to the genus *Sphingobacterium*. This genus can be distinguished phenotypically from its phylogenetic neighbors (e.g., saprospiras, cytophagas, and flavobacteria) by the presence of sphingolipids (4, 23, 24, 29), a

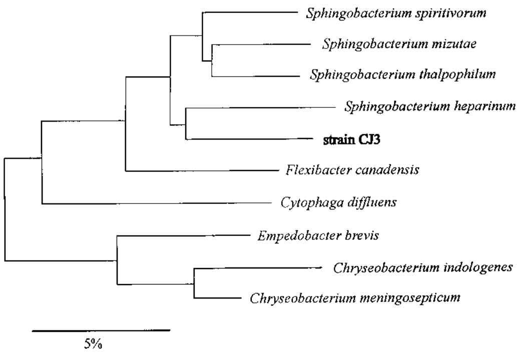

FIG. 4. Phylogenetic tree showing the positions of isolate CJ3 (designated “*Candidatus* comitans” in this study), members of the genus *Sphingobacterium*, and representatives of related genera, based on 16S rDNA sequence comparisons. Bar = 0.05 estimated nucleotide change per position.

substance also found in strain CJ3. The cell morphology, Gram staining behavior, and color of the cell mass were consistent with the genus description, as was the presence of iso-2-OH-$C_{15:0}$ as a major fatty acid (29) and the predominance of menaquinone MK-7 (1). Unfortunately, some genus-specific characteristics, such as resistance to certain antimicrobial agents, oxidation of carbohydrates, lack of indole production, and proteolytic activity, could not be studied because the companion strains did not grow on the appropriate media.

The phylogenetic position of strain CJ2 is within the radiation of the four *Sphingobacterium* species that have been studied; this strain occurs with *Sphingobacterium heparinum* on a separate line of descent. The gliding, flexible, thin, rod-shaped organism *Flexibacter canadensis* appears to be the closest non-*Sphingobacterium* relative of strain CJ2. As only one-half of the *Sphingobacterium* species that have been described have been subjected to 16S rDNA analysis so far, we could not determine whether strain CJ3 is more closely related to another *Sphingobacterium* species than to *S. heparinum*. Furthermore, because of our the inability to obtain sufficient cell material for DNA isolation, nucleic acid hybridization studies could not be performed with any of the *Sphingobacterium* type strains that have been described.

Despite the presence of *Sphingobacterium*-specific characteristics in strains CJ2, CJ3, and CJ4 and the unique ecological niche of these organisms as companions of *C. crocatus*, as well as their inability to survive without the host cells and the limited information concerning the proper taxonomic affiliation, it would not be wise to describe the companion bacterium as a new *Sphingobacterium* species. On the other hand, there is sufficient information to place the companion organism in a provisional *Candidatus* taxon, which is used to describe to record the properties of a putative taxon of prokaryotes (14, 15). The authenticity of the companion organism located in the sporangioles of *C. crocatus* has been verified by in situ hybridization with a fluorescently labelled 16S rDNA oligonucleotide (5).

"*Candidatus* comitans" [(*Bacteroides-Cytophaga-Flavobacterium* phylum, related to members of the genus *Sphingobacterium*); C, for a few generations on solid media; G−; R, nonmotile; NAS (EMBL accession number 91814), oligonucleotide sequence complementary to unique region of 16S rRNA 5′-CTCAAAGAAAGCAAGCTCTCC-3′; S (*Chondromyces crocatus*, sporangioles); aer., sphingolipids+, phosphatidylethanolamine+, iso-$C_{15:0}$ and iso-2-OH-$C_{15:0}$ fatty acids+, respiratory quinone MK-7, G+C content of the DNA 36 mol%.

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