During the past two decades, the breadth of bacterial diversity now recognized has increased to more than 40 identifiable phylogenetic branches or divisions (6). Many of these divisions are defined exclusively by environmentally retrieved 16S rRNA gene sequences (“candidate divisions” [6]) or by only a small number of cultured isolates. A typical example of the latter is the “Synergistes” division, which is still a poorly described phylotype (4). For instance, of the 124 16S rRNA gene sequences displayed in the Synergistes GenBank database, only two are derived from actual isolates: Synergistes jonesii, accession number L08066, isolated from the rumen of a goat (1), and Synergistes sp. strain P4G_{18P1}, accession number AY207056, isolated from the oral cavity (W. G. Wade and A. de Lillo, unpublished data). On the other hand, recent molecular methods-based approaches have provided evidence that these organisms are widespread in nature (4). Synergistes-like 16S rRNA gene sequences have been found in molecular inventories of several pollution-removal anaerobic digestors (8, 18, 23), as well as termite hindguts (5, 16), pig intestinal tract (9), petroleum reservoirs (17, 21), and the human subgingival ecosystem (7, 14). Using 16S rRNA gene-targeted PCR, Godon et al. (4) recently explored 93 anaerobic environments, including mesophilic and thermophilic anaerobic digestors, curd, pig slurry, compost, soil of 23 different types or locations, and the guts of 49 different animals, plus four specimens from human sources, and found Synergistes present in 95% of the ecosystems analyzed, though its proportion was generally below 1% (4, 12). The sequences from animal sources formed their own clustered groups, as did the sequences from digestors, soil, and human subgingival plaque, suggesting that phylogenetically defined subgroups of Synergistes group organisms (SGOs) occupy their own individual ecological niches (4).

Despite their recent discovery at various infected sites in the oral cavity (14, 19), human-associated SGOs have remained largely uncharacterized. In order to extend our knowledge of their ubiquity and phylogenetic diversity, we looked for possible candidates from the clinical culture collection of the R. M. Alden Research Laboratory, Santa Monica, Calif., focusing on isolates that could not be biochemically identified as members of any previously described species. The seven strains that we found constitute distinct lineages within the division of Synergistes. Here we provide a phylogenetic characterization of these isolates along with a first profile of biochemical activity and antimicrobial susceptibility. We have also developed a specifically targeted 16S rRNA gene PCR system to directly assess the incidence and phylotypes of SGOs in various human sites, such as feces and subgingival plaque.

**MATERIALS AND METHODS**

**Selection of bacterial strains.** Initially, 10 biochemically unidentifiable, slow-growing anaerobic gram-negative rods were selected from the culture collection of the R. M. Alden Research Laboratory, Santa Monica, CA. This laboratory, previously located at the Santa Monica University of California—Los Angeles Medical Center and now an independent facility, was founded in 1980 in order to characterize the etiology of human infectious diseases, focusing on anaerobic and fastidious bacteria. Through 16S rRNA gene sequencing (described below), seven strains were found to be phylogenetically affiliated with members of the Synergistes division, while three isolates were affiliated with Proteobacteria and
were therefore excluded from this study. The seven SGOs were from various sources: sacral wound soft-tissue infection (strain RMA 10849), diabetic foot soft-tissue infection (RMA 14551), and peritoneal fluid (RMA 14605, RMA 15677, RMA 16088, RMA 16290, and RMA 16406). All strains were subcultured at the R. M. Alden Research Laboratory on brucella agar (Anaerobe Systems, Morgan Hill, CA) and incubated at 37°C under anaerobic conditions.

**Biochemical testing.** Biochemical tests performed at the R. M. Alden Research Laboratory included determination of susceptibility to special potency disks of kanamycin (1,000 μg), vancomycin (5 μg), and colistin (10 μg) as well as catalase, spot iodole, nitrate reduction, growth on bile, urease, growth stimulation with formate/fumarate, and SIM (sulfide-indole-motility), as described previously (22). In addition, an enzymatic profile was determined using the RapID ANA II system (Remel, Lenexa, KS) according to the manufacturer’s instructions.

**Antimicrobial testing.** All SGOs were tested against 14 antimicrobial agents, including ampicillin-sulbactam (Pfizer, Roerig Division, Groton, CT); amoxicillin-clavulanate and ticarcillin-clavulanate (GlaxoSmithKline, Philadelphia, PA); pipercillin-tazobactam (Wyeth Laboratories, Pearl River, NY); erythromycin, imipenem, and cefotixin (Merck & Co., Rahway, NJ); ceftriaxone (Roche Laboratories Inc., Nutley, NJ); moxifloxacin (Boyer Corporation, Mt. Prospect, IL); levofloxacin (Johnson & Johnson, Springhouse, PA); chloramphenicol and penicillin-clavulanate and ticarcillin-clavulanate (GlaxoSmithKline, Philadelphia, PA); moxifloxacin (Bayer Corporation, Mt. Prospect, IL); and colistin (Serious).

The DNA concentration (μg in diameter; Biospec, Bartlesville, OK) was added prior to the addition of the manufacturer’s instructions, with one modification: 0.8 g of zirconia-silica beads (0.1 mm) was added per milligram of DNA. The DNA concentration was calculated using a Gene Quant II photometer (Pharmacia Biotech, Cambridge, England).

**PCR amplification.** For generating almost complete 16S rRNA gene information of the respective PCR products, sequences for the periodontal pathogens were determined from cloned PCR products, as direct sequencing led to ambiguous sequences.
<table>
<thead>
<tr>
<th>Organism</th>
<th>No. of strains tested</th>
<th>Drug susceptibility</th>
<th>% Positive</th>
<th>RapID ANA II profile</th>
<th>Source (no.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Synergistes cluster I</td>
<td>5 S R R</td>
<td>K V C</td>
<td>0 0 0 100 0 0 0 0 0 0 0 0 0 0 0</td>
<td>None None IA (5)</td>
<td></td>
</tr>
<tr>
<td>Synergistes cluster II</td>
<td>2 S R R</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0 0</td>
<td>None None ST (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Desulfovibrio piger</td>
<td>2 S R R</td>
<td>0 0 50 0 0 0 0 0 100 100 100</td>
<td>None None IA (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. fairfieldensis</td>
<td>10 S R R</td>
<td>0 0 0 0 100 100 100 100 100 100 100 100 100</td>
<td>None None IA (5), BL (1), PR (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. desulfurosus</td>
<td>3 S R R</td>
<td>0 0 100 0 100 100 100 100 100 100 100 100 100</td>
<td>None None IA (2), BL (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>D. vulgaris</td>
<td>3 S R R</td>
<td>0 0 100 0 100 100 100 100 100 100 100 100 100</td>
<td>None None IA (2), ATCC 7757</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bilophila wadsworthi</td>
<td>11 S/R R R</td>
<td>100 0 100 100 100 36 0 0 100 100 100</td>
<td>URE ARG IA (11)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Selenomonas flueggei</td>
<td>4 S R R</td>
<td>0 0 0 0 25 0 0 0 0</td>
<td>None None OR (4)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>S. inflexa</td>
<td>2 S R S/R</td>
<td>0 0 100 0 0 0 0 100 0 0 0 0 0 0</td>
<td>None None OR (2)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Campylobacter showae</td>
<td>2 S R S</td>
<td>0 0 100 0 0 0 100 100 0 0 0 0 0</td>
<td>None None AG, GND OR (2), IA (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. giucilis</td>
<td>3 S R S</td>
<td>0 0 0 0 0 0 100 0 0 0 0 0 0 0</td>
<td>None None AG, GND OR (2), IA (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>C. curvis</td>
<td>1 S R S</td>
<td>0 0 0 0 100 0 0 0 100 0 0 0 0 0</td>
<td>None None AG, GND OR (2), IA (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Bacteroides ureolyticus</td>
<td>8 S R S</td>
<td>0 0 0 0 25 100 100 100 0 0 0 0 0 0</td>
<td>None None AG, GND OR (2), IA (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Sutterella wadsworthensis</td>
<td>8 S R S</td>
<td>0 0 100 100 100 100 100 100 100 100 100 100 100 100</td>
<td>None None AG, GND OR (2), IA (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Dialister pneumosintes</td>
<td>5 S R R</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0 0 0 0</td>
<td>None None AG, GND OR (2), IA (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Veillonella sp.</td>
<td>8 S R R</td>
<td>17 0 0 0 100 0 0 0 0 0 0 0 0 0 0</td>
<td>None None AG, GND OR (2), IA (1)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Mobiluncus curtisiis</td>
<td>3 S R R</td>
<td>0 0 100 0 0 0 0 0 0 0 0 0 0 0 0</td>
<td>αGLU, αGAL, PRO, ARG, BLTS, ONPG, αFUC, LGY, GLY, PAL VA (3)</td>
<td></td>
<td></td>
</tr>
<tr>
<td>M. mulieris</td>
<td>2 S S R</td>
<td>0 0 0 0 0 0 0 0 0 0 0 0 0 0 0</td>
<td>αGLU, PRO, ARG, BLTS, ONPG, αFUC, LGY, GLY, PAL VA (2)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The other taxa are slow-growing, strictly anaerobic, gram-negative bacilli that produce small colonies and are kanamycin susceptible and (except for M. curtisiis) vancomycin resistant. The biochemical data are included here for comparison with the enzymatic profiles. (Adapted from reference 22.)

K, kanamycin (1 mg); V, vancomycin (5 μg); C, colistin (10 μg).

Sodium formate/sodium fumarate stimulation.

Sulfide-indole-motility medium.

S, sensitive; R, resistant.

Positive in 2 to 4 days.

URE, urea; BLTS, p-nitrophenyl-β-D-glucoside; ONPG, p-nitrophenyl-β-D-galactosidase; αGLU, p-nitrophenyl-α-D-glucosidase; αGAL, p-nitrophenyl-α-D-galactosidase; αFUC, p-nitrophenyl-α-D-fucosidase; NAG, p-nitrophenyl-N-acetyl-β-D-glucosaminidase; PO4, p-nitrophenylphosphate; LGY, leucyl-glycine-β-naphthylamide; GLY, glycine-β-naphthylamide; PAL, phenylalanine-β-naphthylamide; Arg, arginine-β-naphthylamide; SER, serine-β-naphthylamide; PYR, pyrollidonyl-β-naphthylamide; IND, tryptophane.

IA, intra abdominal; BL, blood; OR, oral; PR, perirectal; HB, human bite; ST, soft tissue; SK, skin; VA, vaginal; UK, unknown.
10849 and RMA 14551) were bile sensitive (Table 1). According to the RapID ANA II identification system, RMA 14551 was the only strain able to hydrolyze glycine-H252-naphthylamide, whereas RMA 10849 and the other strains were completely negative in all enzymatic reactions. This is in contrast to most of the other phenotypically similar anaerobic gram-negative bacilli tested (Table 1).

All seven Synergistes group isolates were susceptible to ampicillin-sulbactam, amoxicillin-clavulanate, ticarcillin-clavulanate, ertapenem, imipenem, cefoxitin, ceftriaxone, levofloxacin (MICs, ≤0.06 to 4 μg/ml; breakpoints for anaerobes have yet to be established), chloramphenicol, clindamycin, and metronidazole. However, in contrast to the soft-tissue isolates (RMA 10849 and 14551), the five levofloxacin-susceptible isolates of intestinal origin were resistant to moxifloxacin (MICs, ≥8 μg/ml). In addition, one isolate (RMA 14605) was penicillin resistant (MIC > 4 μg/ml) and nonsusceptible (intermediate) to piperacillin-tazobactam (MIC = 64 μg/ml).

**Phylogenetic analysis of novel strains.** Phylogenetic analysis was based on nearly full-length sequences (approximately 1,400 bp), except for strains RMA 16406 and RMA 15677 (approximately 500 bp). The identities of all sequences as belonging to the division Synergistes were confirmed by searching the GenBank database. Phylogenetic tree reconstruction was performed by including a representative set of publicly available reference sequences. Figure 1 depicts the evolutionary relationships of the clinical isolates at the interdivision level, while Fig. 2 shows the phylogenetic relationships among Synergistes sequence types. The five strains isolated from peritoneal fluid formed a coherent cluster (cluster I) moderately related to Synergistes jonesii, with approximately 85% similarity (Fig. 2). Within this cluster, strains RMA 16088, RMA 14605, and RMA 15677 grouped tightly with each other, while strains RMA 16406 and RMA 16290 showed a similarity of approximately 95% to the other three strains. In contrast, strain RMA 14551 (diabetic foot) formed a distinct lineage distantly related to cluster I and with approximately 82% similarity to the oral strain Synergistes sp. strain P4G_18 as its closest relative. Likewise, strain RMA 10849 (sacral wound) branched separately, showing a similarity of approximately 90% to its closest relative, the oral isolate E3_33 (deposited in the GenBank database as “Flexistipes sp. E3_33”). Although they represent different lineages, strains RMA 14551 and RMA 10849 shared a common interior branching point with a sequence similarity of approximately 78% and were thus together designated cluster II. In summary, all seven clinical isolates fell within the phylogenetic radiation of Synergistes and represented at least three distinct evolutionary lineages (Fig. 1 and 2).

**Cultivation-independent detection of SGOs in human samples.** As known colonizers of the animal gut, we suspected that SGOs were also present in the human intestinal tract. Total microbial community DNA from three unique fecal samples was used for PCR amplification. At first, using the primers specifically designed for the 16S rRNA genes of Synergistes, no PCR product was obtained for any of the samples. However, after preamplification using universal 16S rRNA primers and subsequently performed nested PCR with Synergistes-specific primers, a PCR amplification product of the correct fragment length was demonstrated in all three samples. These findings suggested the presence of Synergistes sequences in human fecal samples, which could not be detected using cultivation-based methods. Further studies are needed to confirm the prevalence and diversity of these organisms in the human gut and to investigate their potential role in health and disease.
size was obtained for one out of three samples tested. An ambiguity-free sequence was obtained by direct sequencing of the PCR product, which could then be assigned to *Synergistes* through GenBank database research. Phylogenetic treeing analysis grouped this sequence type, “Syn1,” tightly to cluster I, the clade exclusively represented by the isolates from peritoneal fluid, with a similarity of >99% to strain RMA16088 (Fig. 1).

Periodontal pocket samples from five different patients were also collected and analyzed. In contrast to human fecal DNA samples, all periodontal samples yielded PCR products with correct fragment sizes directly through PCR with *Synergistes*-specific primers. However, ambiguity-free sequences could not be obtained by direct sequencing of the PCR products, which indicated the presence of multiple sequence types. We therefore generated clone libraries from two samples (AP1156 and CP1177) and sequenced randomly selected clones (five clones per clone library). GenBank database research affirmed the affiliation of all sequence types to *Synergistes*, which, after phylogenetic tree reconstruction, were grouped in a separate cluster forming a unique line of descent with no close relationship to any previously cultured species (Fig. 2, cluster III). Periodontal sequences were split into two subbranches, with seven clones grouping together (including all clones from sample CP1177), sharing approximately 96% similarity to the second periodontal subgroup (groups depicted as triangles) as well as to oral clone sequence types determined in other studies (Fig. 2). The overall similarity of cluster III to cluster I and cluster II was approximately 80%.

**DISCUSSION**

**Phylogenetic diversity of SGOs of human origin.** The aim of the present study was to characterize *Synergistes* group organisms (SGOs) isolated from clinical samples and to assess whether dominant phylotypes of SGOs could be directly detected in the human intestinal tract and the oral cavity (i.e., without the need of culturing). We discovered a remarkably high diversity of 16S rRNA gene types among the seven clinical isolates that constituted at least three different evolutionary lineages. This finding considerably expands our knowledge of medically important *Synergistes* clades and demonstrates that these phylotypes are principally cultivable from clinical samples. Interestingly, exploration of DNA from fecal samples with *Synergistes*-specific primers enabled the recovery of one sequence type directly matching with the sequence types of cluster I, the clade represented by the isolates from peritoneal fluid. To our knowledge this is not only the first evidence of SGOs being present in the human intestinal tract, but their cultivation from peritoneal fluid also suggests that SGOs could possibly be involved in the etiology of peritonitis.

In contrast to the cluster I isolates, strains of cluster II were from soft-tissue infections. The fact that both strains were found in mixed cultures (strain RMA 14551 with *Aerococcus*...
species, Morganella morganii, Proteus mirabilis, Staphylococcus epidermidis, and Porphyromonas somerae; strain RMA 10849 with alpha-hemolytic streptococci, Anaerococcus tetradius, Finegoldia magna, and Porphyromonas asaccharolytica) makes it difficult to assess the origin or the principal habitat of these SGOs.

SGOs from the periodontal pockets formed a phylogenetically separate cluster (cluster III), remote from cluster I and II but related to sequence types found in 16S rRNA gene molecular inventories from the oral microflora (7, 15). While the sequence diversity among clones from sample CP1177 is low and might reflect interoperon variability of one particular strain, sample AP1156 consisted of at least two different phylotypes within cluster III (Fig. 2). However, the diversity of oral SGOs is even greater, since two other (not further described) SGOs is even greater, since two other (not further described) strains, “Synergistes sp. P4G_18P1” (W. G. Wade and A. de Lillo, unpublished) and “Flexistipes sp. E3_33” (15), were both isolated from the oral cavity grouped within cluster II (Fig. 2). In fact, they were the closest relatives to the soft-tissue strains RMA 14551 and RMA 10849, respectively (Fig. 2). Interestingly, cluster II shares a common interior branching point with cluster I (Fig. 2; tree topology being supported by a maximum likelihood tree). This means that the oral SGOs from cluster II are actually more closely related to the isolates from soft-tissue infections, and also to the SGOs from peritoneal fluid, rather than to their oral “partners” from cluster III. This in turn indicates the high number of Synergistes phylotypes that can be found in the oral cavity, which apparently provides several ecological niches and as such might be one major reservoir of genetic diversity for human SGOs.

Sequence-based detection of SGO. The seven clinical isolates described in this study (i.e., clusters I and II) demonstrate that at least some members of these clades are cultivable in the clinical laboratory. However, so far no cultured isolates have been described for cluster III, which is exclusively represented by clone sequences, and the ability of clinical laboratories to culture these various human phenotypes may be limited. In addition, besides being slow growing, the clinical isolates reported here proved to be biochemically inert (Table 1), which constitutes a potential for misclassification when using biochemical test kits such as the RapID-ANA System (Table 1). Thus, molecular methods-based approaches may be the method of choice both for enhancing our understanding of the range and nature of human-associated SGOs and for identifying potential clinical isolates.

We developed an rRNA primer system with intended target specificity for Synergistes and were able to demonstrate the presence of different phylotypes in periodontal pockets and in human feces. This primer system appears to have advantages over the primer pair used by Godon et al. (4), since the latter did not detect SGOs in human feces even when nested PCR had been performed. Furthermore, in contrast to Godon et al. (4), our assay detects a relatively small 16S rRNA gene fragment of approximately 600 bp in size, sufficient for genotypic identification but also suitable for quantification using real-time PCR (RTQ-PCR).

In a first attempt, we quantified SGOs in the fecal and the oral samples by RTQ-PCR using our specific primer pair, along with determination of the total microbial flora using a broad-ranged primer pair as previously described (20 and data not shown). The proportion of SGOs relative to the total microbiota was 0.01% in the fecal sample and 0.04% in the oral samples. These values are consistent with the findings of Godon et al. (4), who found SGOs in a large variety of anaerobic ecosystems with an abundance below 1% according to the detection frequency in clone libraries. However, as determined by cultivation, the proportions of SGOs in peritoneal fluid and soft-tissue infections was higher, ranging from 0.5% to 20% according to the number of CFU of SGOs and total bacterial flora. These preliminary observations provide some support for claiming that SGOs may have a role as human pathogens. Work is currently under way to deeply assess the quantity of SGOs in defined anaerobic infectious processes (e.g., endodontic infections) by RTQ-PCR.

Final considerations. SGOs in humans are most likely involved in the anaerobic metabolism of protein amino acids, as most cultivated strains have been shown to degrade amino acids (2, 4, 11). For instance, Synergistes jonesii, the closest relative to the isolates of cluster I, uses arginine and histidine as major energy-yielding substrates (13) and is able to detoxify non-protein amino acids, such as dihydroxypropionate, in the rumen of cattle (1). Since many plants produce a variety of potentially toxic amino acids (4), niche specialization of distinct groups of SGOs in plant eaters (including humans) might be in part a function of the host’s diet. Such niche adaptation might be associated with the broad diversity of SGOs throughout several environmental habitats (4). The present study expands the view of Synergistes as a diverse and ubiquitous member of the human-associated bacterial ecosystem. The novel culture isolates now available will enable us to study their physiological properties, helping define the role that Synergistes isolates might play as human colonizers or pathogens.

ACKNOWLEDGMENTS

We thank Ilse Seyfarth, Morgana Eli Vianna, and Vreni Merriam for various forms of assistance. This work was supported in part by a grant from LCL Biokey GmbH, Aachen, Germany, and the START program of the Faculty of Medicine, RWTH Aachen, Germany.

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