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# Development of 16S rRNA-Based Probes for the *Coriobacterium* Group and the *Atopobium* Cluster and Their Application for Enumeration of *Coriobacteriaceae* in Human Feces from Volunteers of Different Age Groups

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**Two 16S rRNA-targeted probes were developed: one for the *Coriobacterium* group and the other for the *Atopobium* cluster (which comprises most of the *Coriobacteriaceae* species, including the *Coriobacterium* group). The new probes were based on sequences of three new *Coriobacteriaceae* strains isolated from human feces and clinical material and sequences from databases. Application of the probes to fecal samples showed that formula-fed infants had higher numbers of *Coriobacterium* group cells in their feces than breast-fed infants. In addition, based on the presented results, it is hypothesized that with the increasing age of a person, the diversity of *Atopobium* cluster species present in the feces increases.**

In the intestinal tract of all animals, including humans, a complex community of microorganisms exists that is generally believed to play an important role in health and disease (4). Therefore, medical microbiologists and microbial ecologists have investigated the composition of this community, i.e., gut microflora, for many decades. Anaerobic culturing techniques enable cultivation of many numerically important fecal microorganisms, which can be identified by biochemical techniques (5). However, it is only since the introduction of molecular microbiological techniques in intestinal ecology that the full diversity of the human gut microflora can be assessed (2, 19, 25, 28, 29). 16S rRNA-targeted oligonucleotide probes directed at different phylogenetic levels (domain, family, genus, species) of the bacterial kingdom and with which it is possible to identify quantitatively a large number of different bacterial groups of the gut ecosystem have been developed (6, 9, 15, 16, 22, 23), even those that were hitherto nonculturable (28). One group of anaerobic bacteria, the *Coriobacteriaceae*, can readily be cultured from human feces (11). *Eggerthella lenta* and *Collinsella aerofaciens* are the best known representatives of this group, and *C. aerofaciens* is an especially well-known member of the resident microflora (5). However, these bacteria are underrepresented in clone libraries of the human gut microbial community (25, 28), and so far, no specific probe for members of the *Coriobacteriaceae* has been designed. In this report, the isolation, characterization, and phylogenetic analysis of three *Coriobacteriaceae* strains from feces and clinical material are described. The 16S rRNA sequences of these isolates formed, together with literature data, the basis for the development of new specific probes to investigate the abundance of this group of bacteria in human feces.

Two strains were isolated from human feces of two volunteers (age of each, 30 years). The isolation was done by plating dilution series on anaerobic tomato juice agar (per liter, 45 g of Eugonagar [Becton Dickinson, Cockeysville, Md.], 10 g of maltose, 5 mg of hemin, 400 ml of tomato juice [autoclaved separately]). This medium is used to enumerate and isolate bifidobacteria from human feces, and it was noted that on these plates, gram-positive rods that were not bifidobacteria also grew. The plates were incubated at 37°C in anaerobic jars with a gas mixture of 90% $N_2$–5% $H_2$–5% $CO_2$. After 3 days, small translucent colonies were picked from the $10^8$ dilution, grown on peptone-yeast-glucose (PYG) medium (10), and Gram stained. Two gram-positive isolates with an irregular rod shape (G118 and H818) were selected for further analysis. Coincidentally, a gram-positive bacterium with a similar irregular rod shape was isolated in the diagnostic laboratory from a patient's blood culture. This clinical isolate (EKSO3) was isolated from a blood culture vial by plating on Brucella blood agar anaerobic plates as previously described (26). The blood sample originated from a patient who suffered from colitis probably caused by Crohn's disease and who had, as complications, an ileus and a perforation in the colon ascendens. The new strains were deposited at the Deutsche Sammlung von Mikroorganismen und Zellkulturen (DSMZ) under numbers 13713 (G118), 13712 (H818), and 13714 (EKSO3).

For phylogenetic analysis, DNA of the three new strains was isolated as previously described (3) and the 16S rRNA gene was amplified and sequenced using universal 16S rRNA-specific primers (1). The partial 16S rRNA sequences of G118, H818, and EKSO3 were 1,432, 1,417, and 1,442 nucleotides long, respectively. They were aligned to reference sequences present in the Ribosomal Database Project (RDP) (18) and EMBL database by using the ARB software (17). A phylogenetic tree was constructed using an alignment from *Escherichia coli* positions 73 to 1480 by the neighbor joining method with Jukes Cantor correction based on distance matrix and parsimony, implemented in the ARB software. Figure 1 shows a tree of the phylogenetic relationship between the isolates and other bacterial species. This tree indicates that the fecal isolates G118 and H818 belong to the species *C. aerofaciens*, fecal bacteria forming lactic acid and formic acid, hydrogen, and ethanol from glucose (13). *C. aerofaciens* was recently renamed

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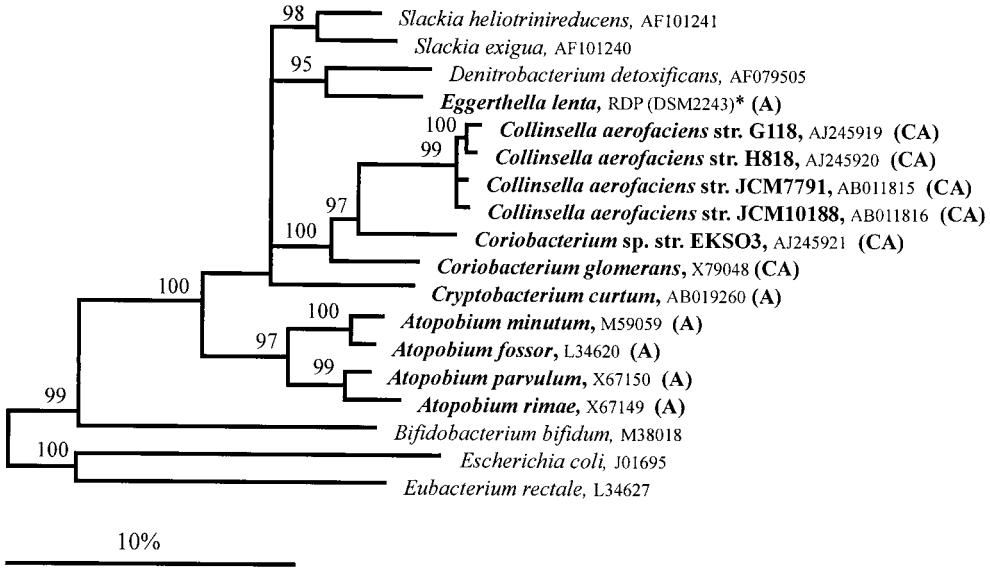


FIG. 1. Tree based on 16S rRNA sequences showing the phylogenetic relationship of the newly isolated strains G118, H818, and EKSO3 with the genus *Collinsella* and *Coriobacterium glomerans*, the family of *Coriobacteriaceae*, and three distant bacteria as an outgroup. Accession numbers of the used sequences are after the species names. *, sequence is available from the Ribosomal Database Project. (A), specificity of the ATO291 probe; (CA), the strain hybridizes with both ATO291 and COR653 probes. Numbers next to the branch nodes indicate bootstrap values (%); only values more than 90% are shown. Bar, 10% sequence divergence.

from *Eubacterium aerofaciens* after the finding that this bacterium is not related to *Eubacterium* sp. sensu stricto or clostridia but to the *Coriobacteriaceae* (13). The two new strains and the two *C. aerofaciens* strains described before have 99% sequence similarity among each other and have 93% sequence similarity with strain EKSO3 (Fig. 1). The closest relative of these five strains is *Coriobacterium glomerans*, a bacterium isolated from the intestinal tract of the red soldier bug (7), with 92% sequence similarity. These species belong to the family of *Coriobacteriaceae* of the *Actinobacteria* subdivision of gram-positive bacteria (21), to which *E. lenta* (formerly known as *Eubacterium lentum*) and the genera *Slackia* and *Atopobium* belong (14, 24, 27). The group of bacteria of the family *Coriobacteriaceae* without the genera *Slackia* and *Denitrobacterium* will be indicated in this paper as the *Atopobium* cluster (18).

Standard tests according to Holdeman et al. (10) were performed to see what phenotypic characteristics the new strains had and to what extent they differed from those of *C. aerofaciens*. Gas chromatography of the short-chain fatty acids produced after growth in PYG medium showed that all strains produced as main product $H_2$, acetic acid, lactic acid, and ethanol. The addition of 0.5% Tween 80 increased the maximum growth rate of *C. aerofaciens*, G118, and H818 on PYG medium 1.1, 1.4, and 1.3 times, respectively. EKSO3 showed almost no growth on PYG medium, but the addition of 0.5% Tween 80 resulted in a growth rate ($\mu$) of 0.468 $h^{-1}$, which was similar to the growth rate of the other three strains. Therefore, 0.5% Tween 80 was included in the PY medium for testing of carbohydrate utilization of all strains. The carbohydrates from which acid was produced are listed in Table 1. Based on the fermentation pattern of five sugars, the strains could be classified into groups as suggested by Kageyama et al. (13). The clinical isolate EKSO3 belonged to group I subgroup C, a subgroup for which so far only one isolate is described. The *C. aerofaciens* strains all belonged to group III since they utilized sucrose but not cellobiose. However, all three had different fermentation patterns so they should be classified into different subgroups, although only one subgroup has been defined so far (13).

Two specific 16S rRNA-targeted oligonucleotide probes were designed using the ARB software (17) to detect bacteria of the *Atopobium* group in fecal samples. The first probe, S-*-Cor-0653-a-A-18 (COR653) (5′-CCCTCCC(A/C)TACCG GACCC), is specific for the genera *Coriobacterium* and *Collinsella*, here referred to as the *Coriobacterium* group. The second probe, S-*-Ato-0291-a-A-17 (ATO291) (5′-GGTCGG TCTCTCAACCC), is specific for the *Atopobium* cluster, which includes the *Coriobacterium* group. The specificity of the

TABLE 1. Phenotypic characteristics of the three new isolates compared to those of *C. aerofaciens*

| Substrate | Acid production[a] | | | |
|---|---|---|---|---|
| | G118 | H818 | EKSO3 | *C. aerofaciens* type strain ATCC 25986 |
| Sucrose | + | + | + | + |
| Cellobiose | − | − | + | − |
| Aesculin | + | − | − | + |
| Salicin | + | − | + | + |
| Amygdalin | − | − | + | +[w,d] |
| Arabinose | − | − | − | +[w,d] |
| Erythritol | − | | − | +[w,d] |
| Maltose | + | + | − | + |
| Ribose | + | − | +[w] | +[w,d] |
| Starch | + | + | + | +[d] |
| Starch hydrolysis | − | − | − | − |
| Trehalose | − | − | − | +[w] |
| Xylose | − | + | + | +[d] |

[a] All four strains produced acid from glucose, fructose, galactose, lactose, and mannose. None produced acid from mannitol, glycogen, inositol, sorbitol, indole, melizitose, raffinose, or rhamnose. +, positive; −, negative; w, weak positive (pH difference between 0.25 and 0.35); d, different from literature data (10).

TABLE 2. Number of *Collinsella* and *Atopobium* cluster bacteria and bifidobacteria in human feces determined by FISH using the probes COR653, ATO291, and BIF164

| Group (*n*)[a] | *Collinsella* group | | | *Atopobium* cluster | | | Bifidobacteria | | |
|---|---|---|---|---|---|---|---|---|---|
| | No. of cells[b] | % Fluorescent[c] | <0.5% (*n*)[d] | No. of cells | % Fluorescent | <0.5% (*n*) | No. of cells | % Fluorescent | <0.5% (*n*) |
| Baby, 12 days | | | | | | | | | |
| Breast fed (6) | ND[e] | 0.5 (0–3) | 5 | ND | 0.5 (0–3) | 5 | ND | 68 (41–91) | 0 |
| Formula fed (6) | ND | 17 (0–39) | 2 | ND | 20 (0–39) | 2 | ND | 33 (0.2–46) | 1 |
| 1–10 years (12) | $7.7 \times 10^9$ | 2.5 (0.1–8) | 3 | $1.0 \times 10^{10}$ | 3.3 (0.2–12) | 1 | $1.8 \times 10^{10}$ | 5.6 (0.4–12) | 1 |
| 25–55 years (10) | $4.4 \times 10^9$ | 1.1 (0.2–3) | 1 | $8.6 \times 10^9$ | 2.6 (0.1–7) | 1 | $8.9 \times 10^9$ | 2.5 (1.2–6) | 0 |
| 75–95 years (11) | $4.1 \times 10^9$ | 4.3 (0.3–9) | 3 | $1.1 \times 10^{10}$ | 11.5 (0.3–26) | 0 | $1.1 \times 10^{10}$ | 8.5 (0.1–20) | 2 |

[a] Groups were divided by age.
[b] Number of cells per gram of feces (dry weight). The coefficient of variation (CV) due to the assay error of the FISH method was 0.12 for automated counting (11) and 0.18 for visual counting (6).
[c] Percentage (range) of fluorescent cells relative to the number of total cells. Total-cell numbers were determined by phase-contrast microscopy (babies) or by DAPI staining (other samples).
[d] Number of volunteers that produced samples with no cells or less than 0.5% fluorescent cells relative to the number of total cells that hybridized with the indicated probe.
[e] ND, not determined. Absolute numbers could not be determined, since an unknown amount of feces was suspended in the transport medium (8).

probes as indicated (Fig. 1) was confirmed by testing against a panel of reference strains. This panel consisted of the four strains described in Table 1, *C. glomerans*, *Atopobium minutum*, *Atopobium parvulum*, *E. lenta*, *Slackia exigua*, and a panel of 45 obligate anaerobes that were used before as reference strains (6). The probes were used for detection of *Coriobacteriaceae* organisms in fecal samples of volunteers from different age groups. Fecal samples were prepared and 4′,6′-diamidino-2-phenylindole (DAPI) stained as previously described (6) except for the fecal samples from babies which were prepared as mentioned previously (8). Fluorescent in situ hybridizations (FISH) using the COR653, ATO291, and BIF164 probes were performed on the fecal samples at 50°C without addition of formamide, and the fluorescing cells were counted automatically (12), except for the fecal samples of babies, of which the fluorescing cells were counted as previously described (8).

Table 2 shows the number of bacteria belonging to the *Atopobium* cluster and, in particular, the *Collinsella* group relative to the number of total bacteria in fecal samples of people belonging to different age groups. For comparison, the number of bifidobacteria is also shown. In a previous study with newborn infants, it was already shown that 12 days after birth, feces of breast-fed babies contained mainly bifidobacteria, while feces of formula-fed babies contained a more diverse microflora (8). These data also suggested that a bacterial group remained undetected in the fecal samples of formula-fed babies. Therefore, the samples from this previous study were evaluated again in the present study, and a striking difference between the formula-fed and breast-fed babies was found. In breast-fed babies, only one out of six babies contained a small amount of *Coriobacterium* group cells, while in formula-fed babies, four out of six babies had large numbers of *Coriobacterium* group cells in their feces, reaching up to 39% of the total bacterial population. In these babies, all *Atopobium* probe-positive bacteria also hybridized with the probe for the *Coriobacterium* group, identifying them as such. This was checked by combining the two probes with different labels in one assay. Figure 2 shows a phase-contrast image and a fluorescent image of feces from a formula-fed baby after hybridization with the two probes. The sample contained about 35% *Coriobacterium* group cells, no bifidobacteria, about 40% *Bacteroides*, and 5% *E. coli*. All positive cells show a yellowish fluorescence of both the green COR653 and the red ATO291 probes. In contrast to these baby feces samples, in fecal samples of older children and adults, not all *Atopobium* group bacteria hybridized to the *Coriobacterium* group probe. Figure 3 shows an example of a

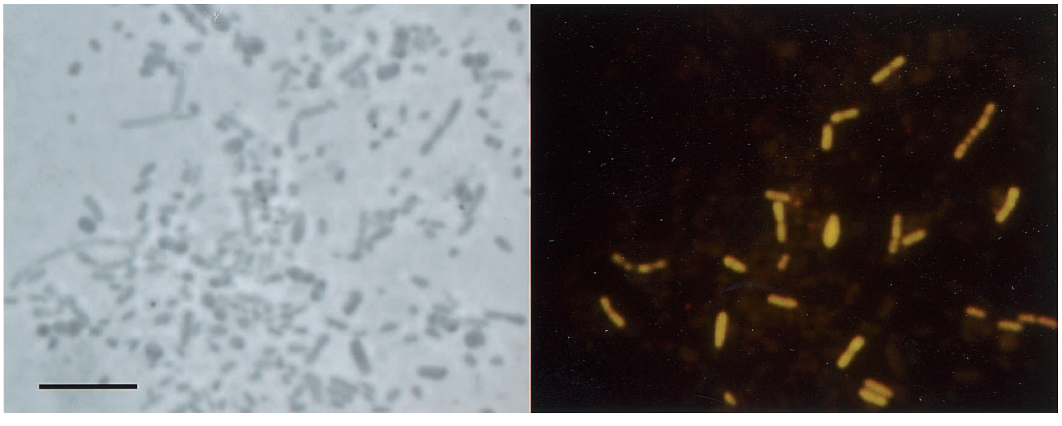

FIG. 2. FISH of a fecal sample from a formula-fed newborn infant at day 20. Two images were taken from the same microscopic field. Left, a phase-contrast image of all bacteria; right, the epifluorescence image of the bacteria that hybridized with 16S rRNA-based oligonucleotide probes COR653 (specific for *Coriobacterium* group) and ATO291 (specific for the *Atopobium* cluster). The yellowish fluorescence is a combination of the green light of the fluorescein-labeled COR653 probe and the red light of the rhodamine-labeled ATO291 probe, indicating that these are *Coriobacterium* group cells. Bar, 5 μm.

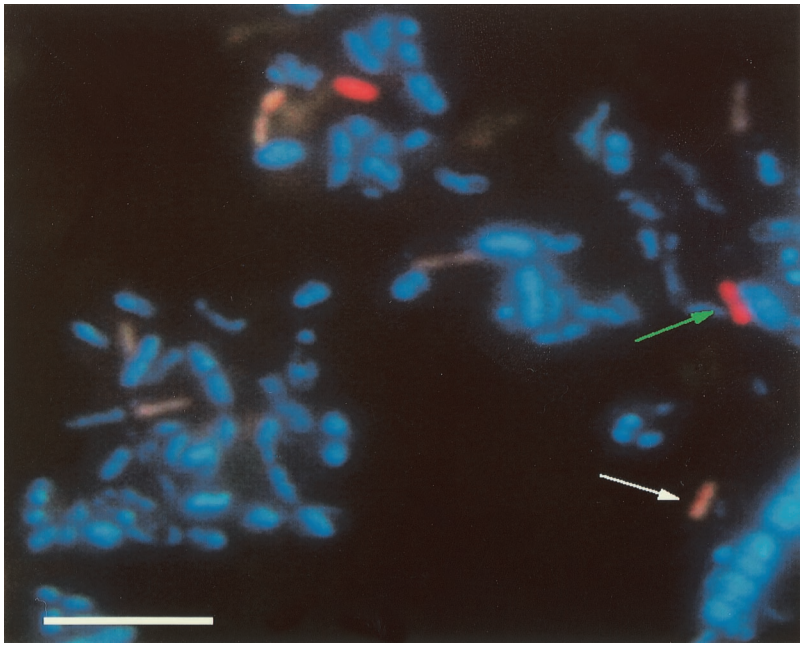

FIG. 3. FISH of a fecal sample from a young volunteer (age, 10 years). The micrograph shows merged images of the epifluorescence image after DAPI staining and hybridization with two 16S rRNA-based oligonucleotide probes. The yellowish-white cells indicated with a white arrow show fluorescence with the fluorescein-labeled probe COR653, the rhodamine-labeled probe ATO291, and DAPI fluorescence, indicating that they are *Collinsella* group cells. The red bacteria (green arrow) show only fluorescence of the probe ATO291 and DAPI fluorescence, indicating that they belong to the *Atopobium* cluster and that they are not *Coriobacterium* group cells. The blue background cells are only DAPI stained. Bar, 5 μm.

hybridization with feces of a young volunteer (10 years old) using the two probes and also a DAPI counterstaining which intercalates with DNA, thus staining all DNA-containing cells. The yellowish-white bacteria hybridized with both probes and were DAPI stained and are therefore *Coriobacterium* group bacteria. The remaining red bacteria are other members of the *Atopobium* group. This shows that there are also non-*Coriobacterium* group cells that are either true atopobia or related to *E. lenta*, another gram-positive rod known to be present in fecal samples (20). Our results, however, indicate that in the feces of newborn babies, only bacteria of the *Coriobacterium* group are present and no *Eggerthella* or *Atopobium* bacteria are present, while in older children or adults, *Eggerthella* or *Atopobium* can also be found. In elderly people, the variation in the number of fecal atopobia between individuals was larger than in children, and relatively more atopobia were detected that did not hybridize to the probe for the *Coriobacterium* group than in children. The numbers of atopobia in feces of adults (25 to 55 years old) were lower than in the former two groups. Currently, it is being investigated whether these age effects are related to diet, for instance, milk and carbohydrate consumption.

It is well known from culture-based studies previously described (5, 11) and confirmed with our FISH methodology that *Coriobacteriaceae* (formerly referred to as *E. lentum* and *E. aerofaciens*) form an interesting group of numerically important bacteria in the human intestinal tract. However, this group is often overlooked in other studies in which fecal samples are analyzed using molecular tools (6, 16, 28, 29) and has been mentioned only once so far (25). Surprisingly, they seem to be underrepresented in 16S rRNA clones libraries of total fecal DNA (28, 29). According to Suau et al., this could be due to the PCR conditions used or the high G + C content of the DNA of these bacteria, prohibiting the amplification of coriobacterial rDNA (25). In this study, we also report the isolation of a strain belonging to the *Coriobacterium* group from a blood culture of a patient suffering from colitis, therefore indicating its potential clinical importance. Based on the phenotypical characteristics, this strain is closely related only to a single isolate of *C. aerofaciens* grouped as I C according to Kageyama et al. (13). However, based on 16S rRNA analysis, this strain is not closely related to *C. aerofaciens* as defined previously but seems to belong to a separate genus. This is supported by phenotypic characteristics, such as the fact that strain EKSO3 grew very poorly on PYG medium without Tween 80, unlike the other strains. It would be interesting to analyze the 16S rRNA genes of strains of the different groups according to Kageyama et al. to gather more data for a further taxonomic differentiation of these groups of *Collinsella*-like strains. *C. aerofaciens* strains were also isolated from patients with colon cancer, ulcerative colitis, and Crohn's disease (13). It is tempting to suggest a relation between the *Coriobacterium* group and these diseases. However, many individuals tested had *Coriobacterium* group cells in their feces, and the beneficial or potentially pathogenic traits of these bacteria still need to be elucidated. The newly designed specific probes may help to investigate the possible involvement of *Coriobacteriaceae* in gastrointestinal diseases, e.g., bowel cancer pathogenesis, for instance, by studying their attachment to colon tissue by using FISH. The results indicate that bacteria of the *Coriobacterium* group play a role in the early development of the newborn infant's gut microflora. The molecular tools allow more focused study on the relation of the new strains to the newborn's predominant intestinal bacteria, i.e., *Bifidobacterium* spp. The data also suggest that with the increasing age of a human, the diversity of *Atopobium* cluster species present in the feces increases. This phenomenon and especially the role of *E. lenta* as a major representative of the non-*Coriobacterium* group needs to be further investigated.

The recent reclassification of *C. aerofaciens* (13) and our new specific probes give new attention to this group of fecal

bacteria. This may help to elucidate their role in food conversion and their interaction with pre- and probiotic food additives and may affect future molecular research on interactions between lactic-acid-producing bacteria in the intestinal tract.

**Nucleotide sequence accession numbers.** The sequences of the new isolates were deposited in the EMBL database under accession no. AJ245919 (G118), AJ245920 (H818), and AJ245921 (EKSO3).

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