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The Mucin Degrader *Akkermansia muciniphila* Is an Abundant Resident of the Human Intestinal Tract▽†

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A 16S rRNA-targeted probe, MUC-1437, was designed and validated in order to determine the presence and numbers of cells of *Akkermansia muciniphila*, a mucin degrader, in the human intestinal tract. As determined by fluorescent in situ hybridization, *A. muciniphila* accounted more than 1% of the total fecal cells and was shown to be a common bacterial component of the human intestinal tract.

The human intestinal mucosal surfaces are exposed to an enormous amount of bacteria; the concentrations approach 10^{14} organisms per individual and represent more than 1,000 species (14). This intestinal microbiota is thought to have evolved in an alliance with its host, and this symbiotic relationship is particularly relevant in the protective mucus layer that covers the epithelium and consists of water-containing mucins (1), which are high-molecular-weight glycoproteins, and other host-encoded products. Mucus offers numerous ecological advantages to intestinal bacteria as it represents a direct source of nutrients for these bacteria, especially in the colon, where carbon sources are limited (15). By incorporating mucin in a medium as a major carbon and energy source, we were able to isolate a novel bacterium, *Akkermansia muciniphila* Muc^T (6), that belongs to the *Verrucomicrobia* phylum, a recently recognized division of the *Bacteria* (9). Analysis of the results obtained for reported clone libraries generated from either feces or biopsy samples showed that eight 16S rRNA sequences showed levels of sequence similarity of ≥98% and three sequences showed <98% similarity with the 16S rRNA gene sequence of *A. muciniphila* (Fig. 1), indicating that they might represent other species in the genus *Akkermansia*. These sequences were derived from human clone libraries from healthy individuals (7, 8, 10, 12, 17; K. Saunier et al., unpublished data) and biopsies of inflammatory bowel disease patients (12; R. A. Hutson and M. D. Collins, unpublished data), as well as animals, such as herbivores (13) and mice (16). In this study, we designed, validated, and used a specific probe targeting the 16S rRNA gene sequence of *A. muciniphila* in fecal samples from healthy adults and babies to determine the prevalence and proportion of this novel mucin-degrading bacterium using fluorescent in situ hybridization (FISH) combined with flow cytometry.

Probe design and validation. Sequences of the 16S rRNA genes of *A. muciniphila* and its closest relatives (Table 1) and sequences of 96 intestinal 16S rRNA gene clones were aligned using CLUSTAL-X and checked for regions with conserved and variable sequences. Based on this alignment, a 16S rRNA oligonucleotide probe targeting part of hypervariable region V9 of the 16S rRNA gene sequence of *A. muciniphila* was designed. A region corresponding to nucleotides 1437 to 1456 of *Escherichia coli* was chosen. The newly designed probe was checked against the small-subunit rRNA database of the Ribosomal Data Project II software package using the Probe Match function (4), and a similarity search sequence of the NCBI database was performed with BLAST (www.ncbi.nlm.nih.gov/BLAST). The probe was designated S-St-Muc-1437-a-A-20 (MUC-1437) based on the nomenclature of the Oligonucleotide Probe Database (2) and had the following sequence: 5′-CCTTGCGGTTGGCTTCAGAT-3′. The oligonucleotide probe sequence has been deposited in probeBase (11). All oligonucleotide probes were purchased from Thermo Electron (Ulm, Germany) and were double labeled at both the 5′ and 3′ ends with Cy5, Cy3, or fluorescein isothiocyanate. The EUB-338 probe (5′-GCTGCCTCCCGTAGGAGT-3′) was used as the positive control (3), and the NON-EUB probe (5′-ACATCCTACGGGAGGC-3′) was used as a negative control to monitor nonspecific binding (18). EUB-338 or the nucleic acid stain TOTO-1 iodide (Invitrogen, Leiden, The Netherlands) was used for total bacterial and cell counting. TOTO-1 is a membrane-impermeant nucleic acid with very high fluorescence enhancement and quantum yield when it binds to the nucleic acids of a cell. Fifty bacterial strains belonging to the genera *Atopobium*, *Bacteroides*, *Bifidobacterium*, *Clostridium*, *Colinsella*, *Coprococcus*, *Enterococcus*, *Escherichia*, *Eubacterium*, *Faecalibacterium*, *Lachnospira*, *Lactobacillus*, *Megasphaera*, *Mitsuokella*, *Peptostreptococcus*, *Prevotella*, *Roseburia*, *Ruminococcus*, *Streptococcus*, *Veillonella*, and *Victivallis* (5) were used for optimization and validation of the FISH probe. The hybridization conditions for MUC-1437 with *A. muciniphila* were optimized by increasing the concentration of formamide in the hybridization buffer as described by Zoetendal et al. (19). To quantify hybridized cells, samples were

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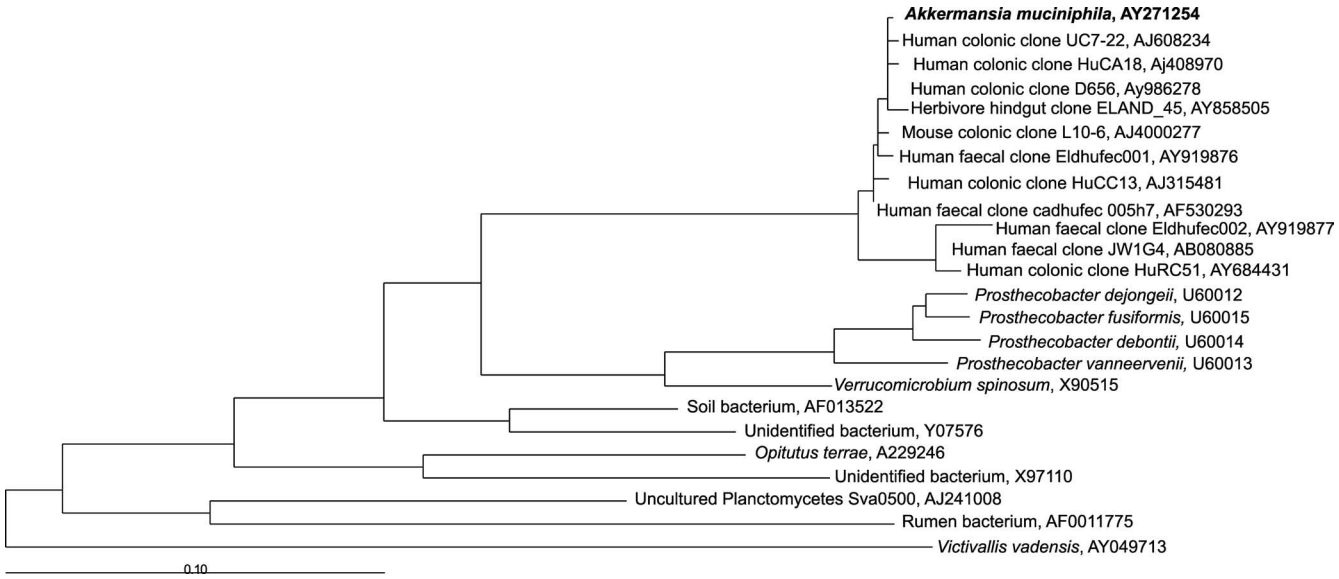

FIG. 1. Phylogenetic tree showing the position of *A. muciniphila* and the clone sequences in the *Verrucomicrobia* phylum. The numbers after the commas are accession numbers. Bar = 10% sequence divergence.

analyzed using a FACSCalibur flow cytometer (Becton Dickinson Immunocytometry Systems, San Jose, CA) equipped with an air-cooled argon ion laser providing 15 mW at 488 nm combined with a 670-nm red-diode laser with the standard filter setup. For total cell enumeration, samples were incubated in the presence of 1 nM TOTO-1 (Molecular Probes, Leiden, The Netherlands) for 5 min at room temperature. Unlabeled beads (diameter, 6.0 μm) provided with a bacterial counting kit (Molecular Probes BV, Leiden, The Netherlands) were added to each sample stained with TOTO-1 at a final concentration of 10^6 beads/ml and were used as an internal standard to calibrate the sample volume. The relative abundance (proportion) of *A. muciniphila*-like bacteria was estimated by determining the ratio of the number of cells hybridized with Cy5-labeled MUC-1437 to the number of TOTO-1-stained cells. The analysis was performed using the WinMDI version 2.8 software (http://facs.scripps.edu/software.html) or the CellQuest Pro program (Becton Dickinson Immunocytometry Systems, San Jose, CA).

Optimal discrimination between the 50 strains (5) and *A. muciniphila* was achieved using a formamide concentration of 20%, and under these conditions none of the nontarget organisms showed cross-hybridization with the MUC-1437–Cy5 probe (data not shown). Hence, these conditions were considered specific for *A. muciniphila* in further FISH analysis of fecal samples. Remarkably, when cells were hybridized to the general bacterial probe EUB-338, the percentage of hybridized cells was intermediate between the values for the NON-EUB and MUC-1437 signals (see Fig. S1, panels A to C, in the supplemental material). This could be explained by the two mismatches between the sequences from the EUB-338 probe and the corresponding region of the 16S rRNA sequence from *A. muciniphila*. It is noteworthy that we also found in the 16S rRNA sequence of *A. muciniphila* two mismatches with the bacterial probe (EUB-338) commonly used as a positive control in FISH analysis, and *A. muciniphila* cells showed only a weak signal after EUB-338 hybridization. In retrospect, this suggests why *A. muciniphila* has not been identified previously.

TABLE 1. Aligned sequences of the oligonucleotide probe and the 16S rRNA gene sequences of the closest relatives of *A. muciniphila* in the *Verrucomicrobia* phylum

Probe or target	Sequence[a]
S-St-Muc-1437-a-A-20 ....3′	TAGACTTCGGTTGGCGTTCC 5′
Akkermansia muciniphila ....5′	ATCTGAAGCCAACCGCAAGG 3′
Clones HuCA18 and HuCC13 ....5′	●●●●●●●●●●●●●●●●●●●● 3′
Verrucomicrobium spinosum ....5′	GC●C●C-●●●G●●●●●●●● 3′
Prosthecobacter dejongeii ....5′	GCG●●●-●T●●●●●●●●●● 3′
Prosthecobacter vanneervenii ....5′	GCG●●●-●T●●●●●●●●●● 3′
Prosthecobacter debontii ....5′	GCG●●●-●T●●●●●●●●●● 3′
Prosthecobacter fusiformis ....5′	GCG●●C-●●●●●●●●●●●● 3′

[a] Filled circles represent consensus with the *A. muciniphila* sequence; dashes represent missing bases.

Quantification of *A. muciniphila* in feces. Fresh fecal samples were collected from 50 Finnish babies who were 6 and 12 months old (25 females and 25 males), 13 healthy Dutch adults (8 females and 5 males; ages, 26 to 40 years), and 13 Finnish adults (all females; ages, 26 to 34 years). These volunteers had not been subjected to any feeding trial, specific diet, or antibiotic treatment for the previous year. Samples were processed immediately after collection, and fixed samples were hybridized in 20% (vol/vol) formamide buffer as described above. For total cell enumeration, samples were incubated in the presence of TOTO-1 iodide as described above. Paired and unpaired *t* tests were used for statistical analysis of comparisons of the numbers of *A. muciniphila* cells in fecal samples. Cytometric dot plots obtained after staining with TOTO-1 and hybridization with Cy5-labeled NON-EUB and MUC-1437 are shown in Fig. S1, panels D to F, in the supplemental material. *A. muciniphila* cells (see Fig. S1, panel F, in the supplemental material) were represented in the FL4 signal (Cy5 fluorescence) as the black population separated from the nonhybridized cells

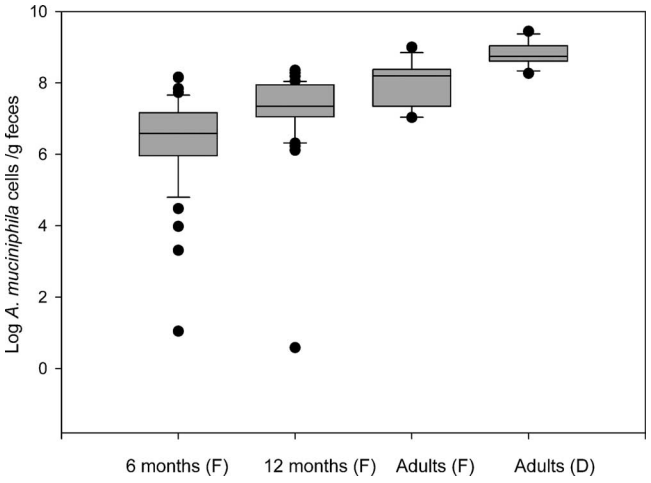

FIG. 2. Quantification of *A. muciniphila* (log cells/g [wet weight] of feces) in feces from Finnish (F) babies and adults and Dutch (D) adults, with the results represented by box and whisker plots. Each box extends from the 25th percentile to the 75th percentile, and a line indicates the median. The whiskers show the highest and lowest values after exclusion of the outliers, which are represented by dots.

(gray). The percentage of *A. muciniphila* in the 13 samples from Dutch individuals was 92%, and this organism was clearly detected in 12 of the samples. For these 12 subjects, dot plot analysis based on the bead method resulted in a mean value of 1.26% ± 0.85% of the total fecal cells, corresponding to $8.24 \times 10^8 \pm 7.35 \times 10^8$ cells g (wet weight)$^{-1}$. The level of *A. muciniphila* cells ranged between the detection limit (<0.1%) and 3.08% of the total fecal cells and did not differ significantly for male and female individuals (data not shown). Subsequently, *A. muciniphila* was also quantified using feces from 13 Finnish subjects. The results indicated that *A. muciniphila* accounted for $1.32 \times 10^9 \pm 1.50 \times 10^9$ cells g (wet weight)$^{-1}$ and represented 2.04% ± 0.90% of the total bacterial cells. The differences between Dutch and Finnish individuals (Fig. 2) were found to be not significant ($P = 0.08$). Feces from a total of 50 6- and 12-month-old babies were used to quantify *A. muciniphila* by FISH-flow cytometry using MUC-1437. The concentration of *A. muciniphila* increased significantly between 6 and 12 months ($P < 0.001$) (Fig. 2), and the levels of this organism were $1.41 \times 10^8 \pm 3.23 \times 10^8$ and $4.92 \times 10^8 \pm 4.20 \times 10^8$ cells g (wet weight) feces^{-1} for the 6- and 12-month-old babies, respectively, representing 0.86% ± 1% and 1.56% ± 0.58% of the bacterial cells. These data indicate that *A. muciniphila* colonizes the intestinal tract early in life and that within 1 year the level is close to the level observed in adults.

Perspectives. In conclusion, we describe for the first time validation and application of a novel phylogenetic probe, MUC-1437, targeting an intestinal organism. The data indicate that the mucus-utilizing organism *A. muciniphila* colonizes the intestinal tract early in life and that within 1 year the level is close to the level observed in healthy adults, representing approximately 1 to 3% of the total microbiota. The abundance of *A. muciniphila* in the human intestine provides new perspectives for further investigating its role in health and disease in relation to mucin degradation.

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