Bacterial biota in reflux esophagitis and Barrett’s esophagus

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Supported by R01CA97946, R21DK57941, R01GM63270, R01 DK58587, and R01CA77955, and by the General Clinical Research Center core grant to New York University School of Medicine (NIH/NCCR M01 RR00096) from the National Institutes of Health, by the Medical Research Service of the Department of Veterans Affairs, and by the Ellison Medical Foundation
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Received: 2005-04-01 Accepted: 2005-04-30

Abstract

AIM: To identify the bacterial flora in conditions such as Barrett’s esophagus and reflux esophagitis to determine if they are similar to normal esophageal flora.

METHODS: Using broad-range 16S rDNA PCR, esophageal biopsies were examined from 24 patients [9 with normal esophageal mucosa, 12 with gastroesophageal reflux disease (GERD), and 3 with Barrett’s esophagus]. Two separate broad-range PCR reactions were performed for each patient, and the resulting products were cloned. In one patient with Barrett’s esophagus, 99 PCR clones were analyzed.

RESULTS: Two separate clones were recovered from each patient (total = 48), representing 24 different species, with 14 species homologous to known bacteria, 5 homologous to unidentified bacteria, and 5 were not homologous (<97% identity) to any known bacterial 16S rDNA sequences. Seventeen species were found in the reflux esophagitis patients, 5 in the Barrett’s esophagus patients, and 10 in normal esophageal patients. Further analysis concentrating on a single biopsy from an individual with Barrett’s esophagus revealed the presence of 21 distinct bacterial species. Members of four phyla were represented, including Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria. Microscopic examination of each biopsy demonstrated bacteria in intimate association with the distal esophageal epithelium, suggesting that the presence of these bacteria is not transitory.

CONCLUSION: These findings provide evidence for a complex, residential bacterial population in esophageal reflux-related disorders. While much of this biota is present in the normal esophagus, more detailed comparisons may help identify potential disease associations.

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Key words: Bacterial biota; Esophagus; 16S rDNA PCR

Pei ZH, Yang LY, Peek RM, Jr Levine SM, Pride DT, Blaser MJ. Bacterial biota in reflux esophagitis and Barrett’s esophagus. World J Gastroenterol 2005; 11(46): 7277-7283
http://www.wjgnet.com/1007-9327/11/7277.asp

INTRODUCTION

Colonizing bacteria exist in each portion of the human digestive tract, from the oral cavity to the anus. Colonizing bacterial populations are essential for the development of the gastrointestinal mucosal immune system, for the maintenance of a normal physiological environment, and for the provision of essential nutrients[1,2]. Colonizing bacteria also play a role in a variety of disease conditions, as exemplified by the gastric colonizer Helicobacter pylori in relation to gastric cancer[3]. Conversely, loss of normal biota is responsible for the overgrowth of opportunistic pathogens that normally are inhibited, such as that occurs in antibiotic-associated colitis[4,8], or in candida vaginitis[9]. Microenvironment alterations may favor overgrowth of bacteria that produce carcinogenic metabolites[5,6], promoting tumorigenesis in inflammation-induced cancers, such as adenocarcinoma in experimental colitis mouse models[7].

A complex bacterial biota has been defined recently in the normal distal esophagus, estimated to be composed of approximately 140 species, of which 95 are identified[8].
Members of six phyla, Firmicutes, Bacteroidetes, Actinobacteria, Proteobacteria, Fusobacteria, and TM7 are represented. Firmicutes represent the most commonly identified phylum in the distal esophagus, followed by phylum Bacteroidetes. Some of the phyla, including Spirochaetes and Deferribacteres, that are commonly represented in the oral cavity, are not identified as esophageal flora, indicating that conditions in the distal esophagus are not ideal for the colonization of all oral flora. These are 14 species identified in the distal esophagus in all four persons studied, indicating that the esophageal biota are unique residents, and not identified simply as organisms transiting from the oral to the gastric cavity. Thus, although the esophagus is generally viewed as a conduit for food passage, the environment in which the bacteria reside is relatively stable.

The distal esophagus may be distinguished from other portions of the esophagus by the changes induced by the reflux of gastric and duodenal contents. Persistent untreated gastroesophageal reflux disease (GERD) can lead to a metaplastic and premalignant condition known as Barrett's esophagus, which carries an increased risk of esophageal adenocarcinoma. Repeated exposure of the distal esophagus to gastric acid and duodenal bile salts likely alters the biota present in the distal esophagus; as has been previously demonstrated in the stomach, changes in the microenvironment lead to alterations in colonizing bacterial populations. One possibility is that perturbation of the normal esophageal biota could contribute to the progression from GERD to Barrett’s esophagus towards the development of adenocarcinoma. Because little is known about the nature of bacterial biota in reflux esophagitis-related diseases, we sought to identify whether there exists a population of bacteria in patients with GERD and Barrett’s esophagus. Our specific goal in this pilot study was to use broad-range 16S rDNA PCR to identify the presence of colonizing bacteria in patients with reflux esophagitis or Barrett’s esophagus.

**MATERIALS AND METHODS**

**Subjects**

Patients presenting to the Department of Veterans Affairs Medical Center, Nashville, TN, USA with gastrointestinal symptoms requiring upper gastrointestinal endoscopy were eligible for this study. Those who were willing to participate in the studies of upper gastrointestinal microbiology and who signed an informed consent form were recruited for this study. Exclusion criteria included recent use of antibiotics, previous gastric/esophageal surgery, and active infection of the oral cavity. Esophagogastroduodenoscopy was performed and endoscopic findings were recorded for 24 consecutive patients who met the above criteria. Esophageal biopsies were obtained 2 cm above the squamocolumnar junction or in the case of Barrett’s esophagus, 2 cm above the gastroesophageal junction. Each biopsy was examined microscopically for morphological features of GERD and intestinal metaplasia (Barrett’s esophagus). As described, features consistent with GERD included mucosal erosions/superficial ulcerations, epithelial hyperplasia, and inflammatory infiltrate of polymorphonuclear cells or eosinophils in the mucosal layer. Features of Barrett’s esophagus included the presence of intestinal-type epithelium in the esophagus. Tissue sections of esophageal biopsies from representative patients with normal esophagus, esophagitis, or Barrett’s esophagus were examined by microscopy using Gram-Twort stain.

**Specimen processing for molecular biological studies**

Biopsies of 2 mm x 2 mm x 2 mm obtained for this study were placed in a 1.5-mL screw-top test tube and stored at -70°C. The specimens were coded so that the laboratorian performing the studies was blinded to the clinical information. DNA was extracted from the biopsy using a tissue DNA extraction kit (Qiagen) in a PCR-free clean-room and the DNA-enriched fractions were eluted in 200 microliters of buffer, as described by the manufacturer.

**PCR**

For each PCR amplification, 5 microliters of the DNA extracted from each biopsy was added to 45 μL of PCR reaction mixture containing 5 μL of 10× PCR buffer (Qiagen), 1.5 mmol/L MgCl₂, 200 μmol/L each dNTP, 50 pmol of each primer, and 5 units of Taq DNA polymerase. Reactions were run at 94 °C for 2 min, followed by 30 cycles of amplification at 94 °C for 30 s, 55 °C for 30 s, and 72 °C for 30 s and a 10-min extension at 72 °C. Primers used were fPB71 (forward): 5’-GGIACGTGAGACCACICCHACTCCT-3’ and rPB101 (reverse): 5’-CGTATTACCGCGTGTGCAC-3’, where I represents inosine, which was used at positions of nucleotide ambiguity, since it forms stable base pairs with A, G, T, and C. Use of inosine-containing primers significantly reduces the complexity accompanying the use of conventional degenerate primers. As such, both inosine-containing primers perfectly match the consensus sequence-derived 16S rDNA pools composed of 21 evolutionarily-well diversified eubacterial groups including Agrobacterium, Aquifex, Arthrobacter, Bacillus, Chlamydia, Chlorobium, Chloroflexus, Chloroplast, Clostridium, Desulfovibrio, Escherichia, Flavobacterium, Flexibacter, Gloeobacter, Helitobacterium, Leptotena, Planctomycetes, Rhodococcus, Synechococcus, Thermotoga, and Thermus, but do not have significant 3’ homology with human 18S rDNA, and human mitochondrial small subunit rDNA sequences. The expected PCR products are approximately 210 bp, depending on the species. In a study to determine the sensitivity, the above primer pair was able to amplify as little as one copy of an Escherichia coli genome (data not shown). An amplification control was designed to assess whether DNA extracted from the esophageal biopsy is of sufficient quality and quantity to be amplified by PCR. A primer pair specific for human 18S rDNA...
was designed to serve this purpose: PBH (forward), 5’-TTGCCAAGAATGTTTTC-3’ and rPBH (reverse), 5’-CGCGTAACTAGTTAGCA-3’.

**Cloning and sequencing**

The PCR products were separated from free PCR primers using a PCR purification kit (Qiagen), then ligated with the pGEM® T Easy (Promega) vector, and used to transform into *E. coli* DH5α competent cells. The cloned inserts underwent sequence analysis using vector-based primers.

**Phylogenetic analysis**

Primer sequences were removed from all sequence files, and only inter-primer sequences were used in subsequent analyses. The sequences were analyzed using standard nucleotide BLAST (Blastn) search of GenBank for homology with known bacterial 16S rDNA sequences. In this study, 16S rDNA sequences with >97% identity with known sequences were considered as homologous with known bacterial species, as described[24]. 16S rDNA sequences were aligned using ClustalW[25] and phylograms of nucleotide alignments generated using Paup 4.0b10 (Paup 4.0b2. Phylogenetic Analysis Using Parsimony and Other Methods, Version 4, Sinauer Associates, Sunderland, MA, USA) neighbor-joining method based on HKY85 distance matrices[26]. All novel sequences were deposited in GenBank (accession numbers: AY212255-21225564).

**RESULTS**

**Microscopic examination of bacterial flora in the distal esophagus**

The 24 patients examined included 9 with normal esophagus, 12 with esophagitis, and 3 with Barrett’s esophagus (Table 1). Because chronic gastritis is generally associated with the presence of overlaying *H pylori* in the lumen[27], we examined the inflamed distal esophagus to determine whether bacterial cells might be visible. Such a study, if positive, can provide morphological evidence for an indigenous esophageal biota, and provide a rationale for its further characterization. Of the 24 biopsies, 21 had sufficient tissue materials remaining for Gram-Twort stain, including 6 from patients with normal esophagus, 12 with esophagitis, and 3 with Barrett’s esophagus. Bacteria were observed in 52% of the biopsies (from 4 of 6 with normal esophagus, 5 of 12 with esophagitis, and 2 of 3 with Barrett’s esophagus). Bacteria appeared to be closely associated with the epithelial cell surfaces (Figure 1). All bacteria observed in the two biopsies with Barrett’s esophagus were Gram-positive cocci, while in the non-Barrett’s biopsies, all were Gram-negative cocci or bacilli.

**Bacterial 16S rDNA in esophageal biopsy specimens**

To examine the nature of the bacterial populations present in the distal esophagus and to define their ancestry, we performed universal bacterial 16S PCR on biopsies

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**Table 1 Clinical and pathological features of 24 patients included in this study**

| Case# | Age | Symptoms of heartburn | Duration | Endoscopic diagnosis | Histopathology |
|-------|-----|-----------------------|----------|----------------------|----------------|
|       |     | Frequency (time/week) |          |                      |                |
|       |     | Duration              |          |                      |                |
| Normal esophagus (n = 9) |     |                       |          |                      |                |
| 247   | 63  | 3                     |          | >1 Y                 | Esophagitis    |
| 252   | 51  | 3                     |          | NA                   | Normal         |
| 254   | 57  | 3                     |          | 1 Y                  | Esophagitis    |
| 257   | 52  | 3                     |          | >1 Y                 | Esophagitis    |
| 249   | 52  | 3                     |          | 1 M                  | Esophagitis    |
| 243   | 53  | 3                     |          | >1 Y                 | Esophagitis    |
| 255   | 66  | 3                     |          | 2-3 Y                | Barrett’s esophagus |
| 258   | 68  | 3                     |          | 2-3 M                | Esophagitis    |
| 260   | 75  | 3                     |          | 2-3 M                | Esophagitis    |
| 253   | 71  | 5                     |          | 4 Y                  | Esophagitis    |
| Esophagitis (n = 12) |     |                       |          |                      |                |
| 242   | 69  | 0                     |          | NA                   | BE             |
| 245   | 70  | 0                     |          | NA                   | BE             |
| 246   | 66  | 3                     |          | >1 Y                 | BE             |
| Barrett’s esophagus (n = 3) |     |                       |          |                      |                |
| 242   | 69  | 0                     |          | NA                   | BE             |
| 245   | 70  | 0                     |          | NA                   | BE             |
| 246   | 66  | 3                     |          | >1 Y                 | BE             |

1 None of the 24 patients had a history of recent use of antibiotics, previous gastric/esophageal surgery, active infection of the oral cavity[23].
2 All 24 patients are males, reflecting the veterans population.
3 NA, not available; Y, year; M, month.
4 NP: no pathologic changes; BE: Barrett’s esophagus; EO: eosinophils; CI: chronic inflammation; AI: acute inflammation.
from each of the 24 studied patients. From each of the 24 biopsies, 2 clones of PCR products were randomly picked and sequenced. The 48 samples yielded 36 unique sequences belonging to 24 different species (97% identical), as established through GenBank BLAST searches. Of the 24 species identified, 14 represented known cultivation-defined bacterial species by sharing 97% identity; 5 shared 97% identity to 5 noncultured/identified bacterial species, and 5 did not share significant homology (<97% identity) with any existing bacterial 16S rDNA sequences in the GenBank (Table 2).

Of the 48 clones sampled, unidentified oral bacterium SH66 was the most prevalent species amplified, accounting for 22.9% (11 clones from 9 patients), followed by *Prevotella veroralis* (10.4%, 5 clones from 5 patients), members of the *Streptococcus* genus (10.4%, 5 clones from 5 patients), and *H pylori* (6.3%, 3 clones from 2 patients) (Table 2). None of the remaining species constituted more than 5% of

![Image of bacterial cells](image)

**Figure 1** Microscopic examination of bacterial cells in the esophagus. Esophageal biopsies were fixed in formalin, paraffin-embedded, sectioned, and examined by using Gram-Twort stain. A: In the biopsy from patient #265 with a normal esophagus, Gram-negative cocci and coccobacilli were tightly associated with the surface of squamous epithelial cells. B: In the biopsy from patient #246 with Barrett’s esophagus, Gram-positive cocci were highly concentrated within the lumen of an intestinal-type gland.

| Best matched bacterial 16S rDNA | % Identity | Normal esophagus (n = 9) | Esophageal disease | All patients (n = 24) |
|--------------------------------|------------|--------------------------|--------------------|----------------------|
| Cultivation-defined species* (n = 14) |           |                          |                    |                      |
| *Prevotella veroralis* | 98.2%      | 4                        | 1                  | 5                    |
| *Streptococcus* | 100%       | 1                        | 2                  | 3                    |
| *Pseudomonas* | 100%       | 1                        | 2                  | 3                    |
| *Helicobacter pylori* | 100%       | 1                        | 2                  | 3                    |
| *Prevotella pallens* | 100%       | 1                        | 1                  | 2                    |
| *Streptococcus salivarius* | 100%       | 1                        | 1                  | 2                    |
| *Actinobacillus pleuropneumoniae* | 99.4%      | 1                        | 1                  | 2                    |
| *Acinetobacter sp.* OM-E81 | 100%       | 1                        | 1                  | 1                    |
| *Citrobacter amalonaticus* | 100%       | 1                        | 1                  | 1                    |
| *Haemophilus influenzae* | 100%       | 1                        | 1                  | 1                    |
| *Haemophilus parainfluenzae* | 99.4%      | 1                        | 1                  | 1                    |
| *Veillonella atriglutlis* | 99.4%      | 1                        | 1                  | 1                    |
| *Campylobacter fetus* | 97.9%      | 1                        | 1                  | 1                    |
| *Prevotella eutlera* | 97%        | 1                        | 1                  | 1                    |
| **Subtotal** |            | 10                       | 13                 | 4                    |
| Unidentified species* (n = 5) |           |                          |                    |                      |
| Oral bacterium SH66 | 100%       | 1                        | 6                  | 3                    |
| Oral bacterium RP55-18 | 100%       | 1                        | 2                  | 2                    |
| Oral bacterium SH13 | 100%       | 1                        | 1                  | 1                    |
| Oral bacterium SH64 | 99.4%      | 1                        | 1                  | 1                    |
| Oral bacterium AP60-12 | 98.3%      | 1                        | 1                  | 1                    |
| **Subtotal** |                      | 8                        | 6                  | 2                    |
| Unknown* (n = 5) |           |                          |                    |                      |
| (Bacterium CEC2) | 96.5%      | 1                        | 1                  | 1                    |
| (Veillonella ratti) | 95%        | 1                        | 1                  | 1                    |
| (Cytophagales) | 92.1%      | 1                        | 1                  | 1                    |
| (Marine bacterium SS1) | 89.9%      | 1                        | 1                  | 1                    |
| (Rumen bacterium RFN91) | 89.2%      | 1                        | 1                  | 1                    |
| **Subtotal** |                      | 5                        | 5                  | 5                    |

*Multiple numbers indicate that sequences were obtained from more than one clone, and that the identity to the specified best matching bacteria varied between clones.

*Unidentified species: 16S rDNA sequence with equal or greater than 97% identity with PCR-derived 16S rDNA sequence.

*Unknown: 16S rDNA sequence with <97% identity with any known 16S rDNA sequences.
the sequenced pool. Cultivation-defined and unidentified species were distributed to nearly the same extent between specimens from normal or diseased esophagus. Of the 27 clones of cultivation-defined bacterial species, 10 were from patients with normal esophagus and 17 were from the 15 patients with esophageal diseases. Of the 16 clones of unidentified/noncultured species, 8 were from the 15 patients with normal esophagus and 8 were from the 9 patients with esophageal disease. In contrast, the 5 clones of novel species were all from the 15 patients with esophageal diseases. In total, 17 species were found in reflux esophagitis, 5 in Barrett's esophagus, and 10 in the normal esophagus.

 Colonization of the bacterial populations within a single esophageal biopsy

To characterize the bacterial populations within a single esophageal biopsy in the presence of esophageal disease, we further analyzed the biota from a patient with Barrett's esophagus (case 242; Table 3). From this biopsy, 99 clones were randomly picked and sequenced to allow a more in-depth analysis of the bacterial population in this biopsy. The 99 clones contained 36 unique 16S rDNA sequences comprising 21 species, including 10 homologous to cultivation-defined bacterial species, 5 homologous to unidentified/noncultured species, and 6 without significant homology (<97% identity) to any known 16S sequences at the species level (Table 4). Unidentified oral bacterium SH66 represented the most prevalent bacterial species (50.5% of the clones sequenced), followed by Neisseria flavescens (11.1%) and Prevotella pallens (6%).

Through combining the preliminary examination of the 24 patients, and the in-depth examination of a single patient with Barrett's esophagus, 147 sequences were obtained, belonging to 39 different species (Figure 2). Twenty-two of the sequences were homologous with cultivation-defined bacterial species, 7 with uncultivated species, and 10 were not homologous with any known bacterial species. The clones belonged to four phyla: Bacteroidetes, Firmicutes, Proteobacteria, and Actinobacteria (Figure 2).

**Table 3** Prevalence of specific 16S rDNA in 99 subclones from a single biopsy from patient #242 with Barrett's esophagus

| Best matched bacterial 16S rDNA | % Identity | Number of sequences |
|--------------------------------|-----------|--------------------|
| **Cultivation-defined species** (n = 10) |           |                    |
| Neisseria flavescens             | 98.8, 98.2| 11                 |
| Prevotella pallens               | 100       | 6                  |
| Porphyromonas sp oral clone CW034| 100, 99.4, 97.6| 3          |
| Gemella morbillorum              | 100       | 2                  |
| Prevotella sp oral clone BI027   | 100       | 1                  |
| Campylobacter fetus              | 99.4      | 1                  |
| Rothia mucilaginosa              | 99.4      | 1                  |
| Veillonella sp oral clone AA050  | 98.8      | 1                  |
| Veillonella paracola             | 98.2      | 1                  |
| Catonella morbi                  | 98.1      | 1                  |
| **Subtotal**                     |           | 28                 |
| **Unidentified species** (n = 5)  |           |                    |
| Oral bacterium SH66              | 100, 99.4| 50                 |
| Oral bacterium SH25              | 99.4      | 4                  |
| Oral bacterium SH13              | 100       | 1                  |
| Oral bacterium AP60-12           | 98.7      | 1                  |
| Oral bacterium AP60-35           | 98.2, 97.6| 2          |
| **Subtotal**                     |           | 58                 |
| **Unknown** (n = 7)              |           |                    |
| (Prevotella sp oral clone FO45)  | 95        | 3                  |
| (Rumen bacterium JW17)           | 93.9      | 3                  |
| (Rumen bacterium RNF91)          | 88.4, 89  | 3                  |
| (Rumen bacterium 30-15)          | 94.5, 93.9| 2                  |
| (Rumen bacterium JW17)           | 96.3      | 1                  |
| (Prevotella sp oral clone AH125) | 93.9      | 1                  |
| **Subtotal**                     |           | 13                 |

1 Multiple numbers indicate that sequences were obtained from more than one clone, and that the identity to the specified best matching bacteria varied between clones.

2 Cultivation-defined species: 16S rDNA sequence with equal or greater than 97% identity with 16S rDNA of cultivation-defined bacterial species.

3 Unidentified species: 16S rDNA sequence with equal or greater than 97% identity with PCR-derived 16S rDNA sequence.

4 Unknown: 16S rDNA sequence with <97% identity with any known 16S rDNA sequences.

**Table 4** Comparison of representation of bacterial phyla observed in studies of the esophagus and subgingival crevice

| Phylum                      | Subgingival crevice | Esophagus |                        |
|-----------------------------|---------------------|-----------|-------------------------|
|                             | Number of clones    | Number of species | Number of clones   | Number of species | Present study |
|                            | Pastor<sup>1</sup> | Pastor<sup>2</sup> | Kroes<sup>3</sup> | Pei<sup>4</sup> |   |
| Clostridium group           | 0                   | 0          | 13                     | 0                   | 0          |
| Obsidian pool OB11          | 6                   | 1          | 0                      | 0                   | 0          |
| Deferrribacteres            | 86                  | 8          | 8                      | 0                   | 0          |
| Spirochaetes                | 537                 | 58         | 0                      | 0                   | 0          |
| TM7                         | 34                  | 5          | 0                      | 13                  | 3          |
| Fusobacteria                | 353                 | 19         | 2                      | 20                  | 6          |
| Actinobacteria              | 275                 | 32         | 15                     | 39                  | 8          |
| Firmicutes                  | 659                 | 113        | 15                     | 626                 | 41         |
| Proteobacteria              | 338                 | 51         | 14                     | 20                  | 14         |
| Bacteroidetes               | 234                 | 38         | 8                      | 182                 | 23         |
| Total                       | 2522                | 347        | 75                     | 900                 | 95         |

<sup>1</sup>From Paster et al<sup>24</sup>; 2 from Kroes et al<sup>28</sup>; 3 from Pei et al<sup>30</sup>.

**DISCUSSION**

Although 147 sequences from the biopsy specimens were analyzed, this study must be considered preliminary. Our strategy was to sample small populations of patients with reflux-related esophageal diseases to determine whether bacterial biota exist and ascertain any outstanding
associations. The aim of these studies was thus hypothesis-generating, to establish parameters for more definitive studies. This approach was necessary because when this study was begun there was no prior information relating to bacterial populations in reflux-related disorders. We recognize the preliminary nature of this inquiry, but believe it can serve as a first approximation that can help guide future work.

Our findings suggest the existence of highly complex bacterial populations in the distal esophagus of patients with GERD-related disorders. Because many bacteria are fastidious, slow growing, or even uncultivable, simple culture methods often overlook a large number of bacteria, such as has been documented in the oral cavity and colon [14,28-29]. These drawbacks can be overcome by universal bacterial 16S rDNA PCR, since PCR does not discriminate bacteria based on their culture properties. However, PCR cannot distinguish living bacteria from naked bacterial genomes. In organs in which major digestive activities occur, such as the stomach and small intestine, bacteria brought downstream by peristaltic movement may be lysed and genomes released. Such DNA can be falsely interpreted by PCR as representing colonizing bacteria. Using microscopy in the present study, we observed a polymorphic population of bacteria in association with the epithelium of esophageal biopsies from patients with reflux-related diseases that included Gram-positive and Gram-negative bacilli, cocci, and cocccobacilli. The variety of morphologically diversified esophageal bacteria is consistent with the highly diversified bacterial constituents identified using molecular techniques. The presence of intact bacteria closely associated with epithelial cells of the distal esophagus suggests that the 16S rDNA detected was from viable bacteria rather than from bacterial DNA only.

Because only a few PCR clones were sampled from each biopsy specimen, this study is not a quantitative comparison of the bacteria found in GERD-related disorders with those from normal esophagus or oral cavity [13,14,28-29], as the species identified may reflect chance rather than prevalence. However, the majority of bacterial species found in GERD-related disorders are shared with the previously identified bacteria in the normal esophagus [13], suggesting that certain bacterial species in the normal esophagus are resistant to the substantial environmental changes due to reflux. Finding H pylori 16S rDNA in the esophagus of two patients with GERD indicates that gastric bacteria can be brought into the distal esophagus by reflux, consistent with the previous detection of H pylori in Barrett's esophagus [30]. These observations suggest that the detected organisms (or DNA) may be transiently present, rather than persistent in the distal esophagus. Longitudinal studies of individual patients would help to address this question. Conversely, the microenvironment in the distal esophagus likely does not include all oral bacteria. Similar to our previous study of the normal esophageal biota [1-5,6-10,11-13], the most prevalent of the nine phyla identified in the subgingival crevice [14,28], *Spirochaetes*, was not found in the esophagus, consistent with the presence of endogenous bacterial populations unique to the distal esophagus.

In-depth study of a Barrett's esophagus case (Biopsy 242) revealed a single predominant species (unidentified oral bacterium SH66), representing 50.5% of the 99 clones sampled. SH66 was found in patients with or without the disease of the distal esophagus; whether there is overgrowth in Barrett's esophagus cannot be addressed without quantitative comparisons. SH66 was originally identified in the saliva by its 16S sequence, but never has been cultured [31]. Phylogenetic analysis indicates that SH66 resembles several members of the genus *Prevotella* and belongs in the phylum *Bacteroidetes* (Figure 2).

Identifying complex bacterial populations in GERD-related disorders offers a new approach to understand bacterial roles as markers or as pathogenic factors in esophageal diseases. Bacterial populations in other portions of the digestive system, such as the oral cavity and colon, play important roles in the maintenance of local physiology as well as in disease pathogenesis [3-5,6-10,11-13]. The composition, transience, or stability of this complex bacterial biota in the distal esophagus and associations...
with the disease remain to be determined. The results from this study justify large-scale comparisons of bacterial biota between normal and pathological conditions in the distal esophagus.

ACKNOWLEDGMENTS

We thank Dr. Kyi T. Tham for processing the specimens for histological examination and Mr. Joseph Szmuliewicz for performing Gram-Twort stains.

REFERENCES

1. Gustafsson BE. The physiological importance of the colonic microflora. Scand J Gastroenterol Suppl 1982; 77:117-131
2. Cunningham-Rundles S, Ahrn S, Abauv-Nussbaum R, Dnistrian A. Development of immunocompetence: role of microorganisms and microenvironments. Nutr Rev 2002; 60: 568-572
3. Peek RM, Blaser MJ. Helicobacter pylori and gastrointestinal tract adenocarcinomas. Nat Rev Cancer 2002; 2: 28-37
4. Gerding DN, Gebhard RL, Sumner HW, Peterson LR. Pathology and diagnosis of Clostridium difficile disease. In: Rolfe R, Finegold SM (eds) Clostridium difficile: its role in intestinal disease. San Diego, Academic Press 1988: 259-286
5. Hopkins MJ, Macfarlane GT. Changes in predominant bacterial populations in human faeces with age and with Clostridium difficile infection. J Med Microbiol 2002; 51: 448-454
6. Hill LV, Embiy JA. Vaginitis: current microbiologic and clinical concepts. CMAJ 1986; 134: 321-331
7. Roediger WE, Lawson MJ, Radcliffe BC. Nitrite from inflammatory cells—a cancer risk factor in ulcerative colitis? Dis Colon Rectum 1990; 33: 1034-1036
8. Mueller RL, Hagel HJ, Greim G, Ruppin H, Domschke W. Endogenous synthesis of carcinogenic N-nitroso compounds: bacterial flora and nitrite formation in the healthy human stomach. Zentralbl Bakteriol Mikrobiol Hyg B 1983; 178: 297-315
9. Mowat C, Williams C, Gillen D, Hossack M, Gilmour D, Carswell A, Wirz A, Preston T, McColl KE. Omeprazole, Helicobacter pylori status, and alterations in the intragastric milieu facilitating bacterial N-nitrosation. Gastroenterology 2000; 119: 339-347
10. Calmels S, Obshima H, Henry Y, Bartsch H. Characterization of bacterial cytochrome cd(1)-nitrite reductase as one enzyme responsible for catalysis of nitrosation of secondary amines. Microb Ecol in Health and Disease 1996; 17: 533-536
11. Charrière M, Poirier S, Calmels S, De Montclous H, Dubreuil C, Poizat R, Hamidi Cherif M, de Thé G. Microflora of the nasopharynx in Caucasian and Maghrebian subjects with and without nasopharyngeal carcinoma. IARC Sci Publ 1991; 105: 158-161
12. Kado S, Uchida K, Funabashi H, Iwata S, Nagata Y, Ando M, Onoue M, Matsuoka Y, Ohwaki M, Morotomi M. Intestinal microflora are necessary for development of spontaneous adenocarcinoma of the large intestine in T-cell receptor beta chain and p53 double-knockout mice. Cancer Res 2001; 61: 2395-2398
13. Pei Z, Bini EJ, Yang L, Zhou M, Francois F, Blaser MJ. Bacterial biota in the human distal esophagus. Proc Natl Acad Sci USA 2004; 101: 4250-4255
14. Paster BJ, Boches SK, Galvin JL, Ericson RE, Lau CN, Levanos VA, Sahasrabudhe A, Dewhirst FE. Bacterial diversity in human subgingival plaque. J Bacteriol 2001; 183: 3770-3783
15. Donowitz LG, Page MC, Mileur BL, Guenthner SH. Alteration of normal gastric flora in critical care patients receiving antacid and cimetidine therapy. Infect Control 1986; 7: 23-26
16. Peek RM, van Doorn LJ, Donahue JP, Tham KT, Figueiredo C, Blaser MJ, Miller GG. Quantitative detection of Helicobacter pylori gene expression in vivo and relationship to gastric pathology. Infect Immun 2000; 68: 5488-5495
17. Peek RM, Thompson SA, Donahue JP, Tham KT, Atherton JC, Blaser MJ, Miller GG. Adherence to gastric epithelial cells induces expression of a Helicobacter pylori gene, iceA, that is associated with clinical outcome. Proc Assoc Am Physicians 1998; 110: 531-544
18. Riddell RH. The biopsy diagnosis of gastroesophageal reflux disease, ‘carditis,’ and Barrett’s esophagus, and sequelae of therapy. Am J Surg Pathol 1996; 20 Suppl 1: S31-S50
19. Ollett WS. A method for staining both Gram positive and Gram negative bacteria in sections. J Pathol Bacteriol 1947; 59: 357
20. Novelli G, Gennarelli M, De Santis L, Angeloni P, Dallapiccola B. Inosine-containing primers in human papillomavirus detection by polymerase chain reaction. Biomed Pharmacother 1992; 46: 167-169
21. Ehlen T, Dubau L. Detection of ras point mutations by polymerase chain reaction using mutation-specific, inosine-containing oligonucleotide primers. Biochem Biophys Res Commun 1989; 160: 441-447
22. Knoth K, Robers S, Poteet C, Tamkun M. Highly degenerate, inosine-containing primers specifically amplify rare CDNA using the polymerase chain reaction. Nucleic Acids Res 1988; 16: 10932
23. Pace NR. A molecular view of microbial diversity and the biosphere. Science 1997; 276: 734-740
24. Stackebrandt E, Goebel BM. Taxonomic note: a place for DNA-DNA reassociation and 16S rRNA sequence analysis in the present species definition in bacteriology. Int J Syst Bacteriol 1994; 44: 846-849
25. Thompson JD, Higgins DG, Gibson TJ. CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, position-specific gap penalties and weight matrix choice. Nucleic Acids Res 1994; 22: 4673-4680
26. Hasegawa M, Kishino H, Yano T. Dating of the human-ape divergence by maximum likelihood of nucleotide substitution. J Mol Evol 1985; 22: 160-174
27. Dooley CP, Cohen H, Fitzgibbons PL, Bauer M, Appleman MD, Perez-Perez GI, Blaser MJ. Prevalence of Helicobacter pylori infection and histologic gastritis in asymptomatic persons. N Engl J Med 1989; 321: 1562-1566
28. Kroes I, Lepp PW, Relman DA. Bacterial diversity within the human subgingival crevice. Proc Natl Acad Sci USA 1999; 96: 14547-14552
29. Suau A, Bonnet R, Sutren M, Godon JJ, Gibson GR, Collins MD, Doré J. Direct analysis of genes encoding 16S rRNA from complex communities reveals many novel molecular species within the human gut. Appl Environ Microbiol 1999; 65: 4799-4807
30. Wright TA, Myskow M, Kingsnorth AN. Helicobacter pylori colonization of Barrett’s esophagus and its progression to cancer. Dis Esophagus 1997; 10: 196-200
31. Henihan RD, Stuart RC, Nolan N, Gorey TF, Hennessy TP, O’Morain CA. Barrett’s esophagus and the presence of Helicobacter pylori. Am J Gastroenterol 1998; 93: 542-546
32. Sakamoto M, Umada M, Ishikawa I, Benno Y. Comparison of the oral bacterial flora in saliva from a healthy subject and two periodontitis patients by sequence analysis of 16S rDNA libraries. Microbiol Immunol 2000; 44: 643-652