

BACTERIAL PATHOGENICITY

Profiling of bacterial flora in gastric biopsies from patients with *Helicobacter pylori*-associated gastritis and histologically normal control individuals by temperature gradient gel electrophoresis and 16S rDNA sequence analysis

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The aim of this study was to establish bacterial profiles in gastric biopsy specimens from patients with *Helicobacter pylori*-associated gastritis by means of temporal temperature gradient gel electrophoresis (TTGE) of PCR-amplified 16S rDNA fragments. Specimens from eight patients with asymptomatic gastritis and five histologically normal controls revealed a *Helicobacter*-specific band in the TTGE profile with increased amounts of *Helicobacter*-specific DNA in the biopsies from most of the gastritis patients. DNA from other genera including *Enterococcus*, *Pseudomonas*, *Streptococcus*, *Staphylococcus* and *Stomatococcus* was also found in the stomach. In the absence of gastric inflammation, *Helicobacter* spp. appeared to be part of a complex, presumably indigenous microbial flora found in the biopsy specimens from the stomach.

Introduction

Generally, very few bacteria are able to survive in the strongly acidic environment of the stomach. The gram-negative bacterium *Helicobacter pylori*, which is the major cause of chronic gastritis [1], peptic ulcer disease [2], gastric cancer and gastric mucosa-associated lymphoid tissue (MALT) lymphoma of the stomach [3], is an exception to this rule. During infection associated with gastritis, the majority of *H. pylori* are found as bacilli attached to the surface of the epithelial cells of the gastric mucosa [4, 5]. Coccoid forms, not culturable *in vitro*, have also been observed in biopsy specimens from the human stomach [6–9]. These could be identified by broad-range PCR (BR-PCR) of 16S rRNA genes, which has been used as a powerful tool to identify uncultured bacteria [9–13]. The 16S rRNA molecule can be described in terms of structure motifs. Highly conserved (universal) nucleotide sequences (U1–U8), interrupted with variable regions (V1–V9), are fairly evenly distributed along the c. 1500 nucleotide long molecule [14]. The universal sequence motifs can conveniently be used

for design of PCR primers (broad-range primers). The variable sequence motifs are suitable for genus and species identification and phylogenetic studies. By partial 16S rDNA sequence analysis, this group and others have found that some *Helicobacter* strains present in *H. pylori*-associated gastritis biopsy specimens differ from the *H. pylori* type strain in the variable regions V3 and V4 [9, 15]. It was proposed that different strains might be associated with different types of gastritis [9]. However, they might also represent other closely related species. For example, *H. heilmannii* has recently been identified in the human stomach [16] and it was proposed that this strain might be less pathogenic than *H. pylori* and might allow other eubacteria to colonise the stomach mucosa [17].

Recently, methods relying on sequence-specific separation of equal sized 16S rDNA PCR amplified fragments (obtained with conserved primers flanking variable regions) have been used successfully for the analysis of mixed microbial communities [18–20]. The aim of the present study was to evaluate the potential of a new method, temporal temperature gradient gel electrophoresis (TTGE), that could be used for profiling and identification of eubacteria in gastric biopsy specimens. For that purpose, PCR-amplified variable V6 regions (flanked by universal primers as described by Muyzer and co-workers [20]) of 16S rRNA were analysed. The

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present data demonstrate the feasibility of using TTGE for the description in molecular terms of a complex, presumably indigenous microbial flora that was found in the gastric biopsy specimens from patients and controls.

Materials and methods

Gastric biopsy specimens and bacterial reference strain

The selection criteria used to enrol the patients and controls, and methods for obtaining the 13 gastric biopsy specimens used in this study have been described in detail [9]. The *Helicobacter* infection status of biopsies was determined by means of a rapid urease test and histological, microscopic and serological analysis with classification according to the Sydney system. The results of these analyses have been described previously [9]. Eight biopsies were from patients with asymptomatic gastritis (subject nos. 410, 418, 422, 430, 453, 457, 479, 483) and five were from matched control individuals with normal gastric mucosal morphology (subject nos. 436, 450, 451, 454, 456). *H. pylori* CCUG 17874^T (ATCC 43504^T, obtained from the Culture Collection of the University of Gothenburg, Sweden) was used as a reference.

Precautions taken to avoid bacterial contamination of instruments and tools

Separately sterilised biopsy forceps were used for each location in the stomach. Between gastroscopies, the forceps were treated in a Branson 3200 ultraware machine (Branson Europa BV). Both forceps and gastroscopes were washed for 30 min with EDT cleaner (ethylenebutylether 8–10%, 1,2-propyleneglycol 30–50%, proteolytic enzymes 0.5–2% and sodium alkylsulphonate 3%) (Olympus Optical AB) and disinfected for 5 min with EDT disinfectant (glutaraldehyde 20–22% and ethanol 7–10%) in an Olympus Optical dishwasher for endoscopes.

DNA isolation

DNA from the 13 biopsy specimens and from the *H. pylori* type strain was extracted with a DNA extraction kit (QIAmp tissue kit, Qiagen-Hilden, Germany); 2–3 μ l of DNA extract was then used in each PCR reaction. Before PCR amplification, the integrity of the DNA preparations was tested by broad-range 16S rDNA PCR amplification with full-length 16S rDNA primers pBR-5'/se 5'-GAAGAGTTTGATCATGGCT CAG-3' and p13B 5'-GTGTACTAGCCCGGGAA CGTATTC-3. PCR conditions were as described previously [9, 12].

Primers and TTGE-PCR amplification

Primers TGGE-2: 5'-ATTACCGCGGCTGCTGG-3' and TGGE-gc3: 5'-CGCCCGCCGCGCGGCGGG

CGGGGCGGGGGCACGGGGGGCCTACGGGAGGC AGCAG-3', targeting adjacent conserved regions of the variable V6 regions, were chosen for TTGE-PCR amplification (nomenclature according to Gray and co-workers [14]). The same TTGE primers have been used in profiling complex microbial populations by denaturing gradient gel electrophoresis [20]. Full-length 16S rDNA amplicons were used as DNA templates (see above). The first 40 nucleotides of TGGE-gc3 comprise the GC-clamp. The PCR product was *c.* 200 bp (between positions 341 and 534 in the numbering system of 16S rRNA of *Escherichia coli* [14]). PCR amplifications were performed in a final volume of 50 μ l [9] and a final concentration of 1.5 mM MgCl₂ in the reaction mix. Modified thermostable DNA polymerase AmpliTaq-Gold and thin-walled reaction tubes (Perkin-Elmer, Stockholm, Sweden) were used in a Perkin-Elmer TC-1 thermocycler as follows: initial activation of Taq-polymerase at 94°C for 10 min, denaturation at 94°C for 30 s, annealing at 56°C for 30 s, extension at 72°C for 1 min (30 cycles) and a final extension step at 72°C for 10 min. Negative PCR controls containing only buffers and PCR primers were run in parallel. Before TTGE analysis, all PCR products were checked on an agarose 1.2% (w/v) gel.

TTGE analysis

A DCode universal mutation detection kit (BioRad Laboratories, Sundbyberg) was used for sequence-specific separation of PCR-amplified products. Electrophoresis was performed in a polyacrylamide:bisacrylamide (19:1) 9% (w/v) gel, and 6 M urea with 1.25 \times TAE buffer (1 \times TAE = 40 mM Tris acetate, 20 mM acetic acid, 1 mM EDTA, pH 8.3) at a constant voltage of 90 V. A temperature gradient from 60°C to 70°C and a ramp temperature of 1°C/h was applied. The samples were loaded on to the gels and the temperature was adjusted to 60°C at a constant voltage of 15 V before the temperature gradient was built up. The gels were stained with ethidium bromide and photographed under UV light (Gel Doc 2000, BioRad Laboratories). All chemicals used were of molecular biology grade and purchased from BioRad.

Cloning and DNA sequence analysis

To characterise the bacterial flora present in gastric biopsy specimens, 10 individual bands were selected and cut out from the TTGE gel (Fig. 1). Gel slices were homogenised in sterile TE buffer (10 mM Tris-HCl, pH 7.5; 1 mM EDTA-Na₂) and DNA was concentrated by ethanol precipitation. Finally, the DNA pellets were dissolved in 20 μ l of sterile ultrapure water. Appropriate samples were re-amplified with primers TGGE-2: 5'-ATTACCGCGGCTGCTGG-3' and TGGE-3: 5'-CCTACGGGAGGCAGCAG-3' (amplifying the V6 variable region of the 16S rDNA). PCR amplification conditions were as described above. Re-amplified PCR products derived from bands I and II

(Fig. 1), respectively, were sequenced directly with a Cy5.5 cycle sequencing kit. Re-amplified PCR products from bands 1–8 (Fig. 1) were cloned into a T/A cloning vector, with a TOPO-T/A cloning kit (Invitrogen, Groningen, The Netherlands). Positive colonies were selected by the blue/white colony screening procedure. Subsequently, inserts were analysed by PCR with primers directed against the SP6 and T7 phage promoter sequences. Ten positive clones were selected from each band which was cut out from the TTGE gel, and appropriate nucleotide sequences were established with a 5'-biotinylated SP6 primer, a dye terminator Cy5.5 cycle-sequencing kit, and a SEQ4 × 4 Personal Sequencing System (Amersham-Pharmacia Biotech). The results were compared with published DNA sequences through a BLAST database search (EMBL and GenBank databases).

Results and discussion

TTGE analysis

The present study explored the potential use of TTGE of PCR amplified 16S rRNA gene fragments for the diagnosis of *H. pylori*-associated gastric inflammation. It compared TTGE profiles from gastric biopsy specimens obtained from eight patients with gastritis and five histologically normal control patients (specimens

410, 418, 422, 430, 453, 457, 479, 483 and 436, 450, 451, 454, 456, respectively) (Fig. 1). As shown in Fig. 1, a distinct band corresponding to the migration of the *H. pylori* ATCC 43504^T variable V6 region of the 16S rDNA was observed in all gastric tissue samples, although the relative abundance was minimal in specimens 436 and 450 (histologically normal controls). In an earlier study, it had not been possible to identify *Helicobacter* DNA in gastric biopsy specimen 451 [9]. Uneven distribution of *Helicobacter* might explain why biopsy specimen 451 was *Helicobacter*-positive in this study but not in the previous one, as each specimen consisted of multiple small biopsies which were taken at the same site. DNA was extracted from each piece separately. These findings would indicate that the prevalence of bacteria belonging to the genus *Helicobacter* was 100%, which confirms the results obtained with the same specimens in the previous study with a conventional PCR technique combined with DNA sequencing to assure the specificity of the reactions [9]. However, as the number of biopsy specimens examined in the present study was rather limited, conclusions cannot be extrapolated to the general population.

As judged from the intensity of the *Helicobacter*-specific band in relation to the rest of the TTGE profile, gastric inflammation was associated with the

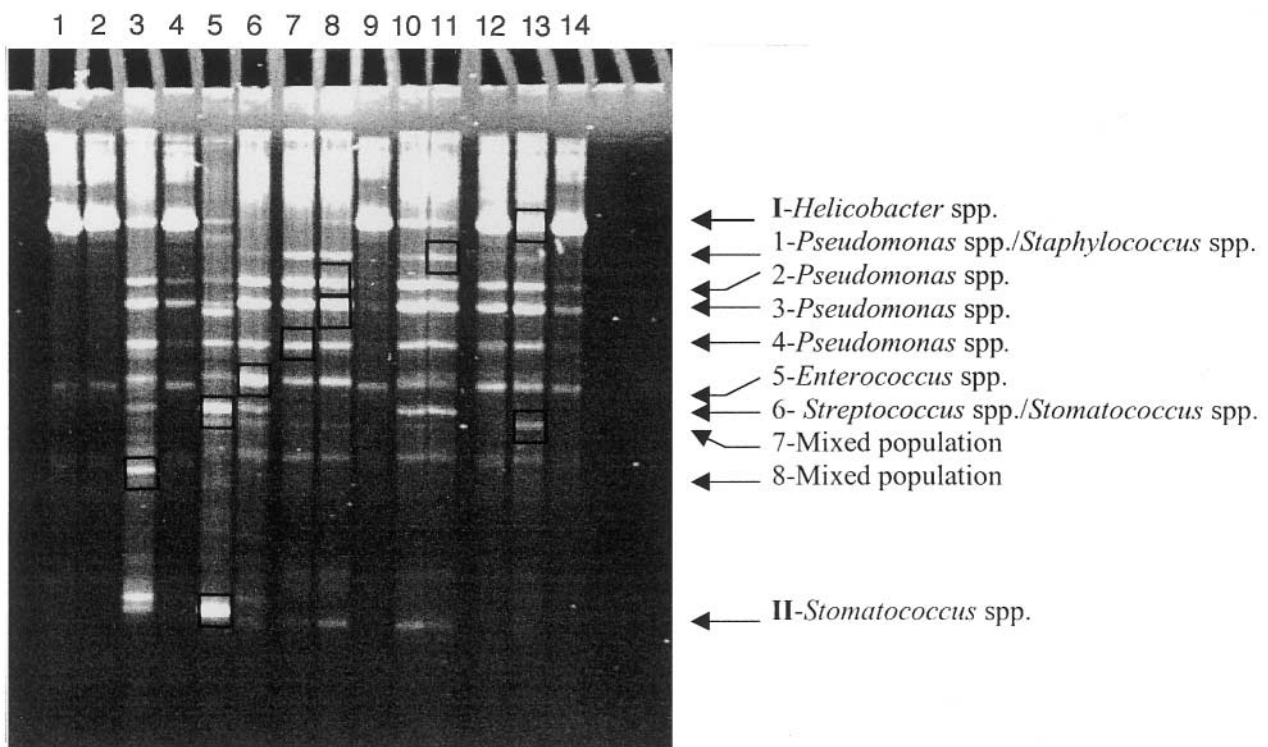


Fig. 1. TTGE analysis of 16S rDNA variable V6 regions in biopsies obtained from eight gastritis – GAS 410 (lane 2; R), 418 (3;R), 422 (4; R), 430 (5; C), 453 (9; R), 457 (12; R), 479 (13; R), 483 (14; R) – and five histologically normal control patients – HNC 436 (6; C), 450 (7), 451 (8), 454 (10) and 456 (11; C). *H. pylori* ATCC 43504^T (1) was included as a positive control. Open rectangles denote the bands excised from the TTGE gel. Arrows I and II denote genera identified by direct DNA sequence analysis, and arrows 1–8 denote genera identified by cloning and DNA sequence analysis of the PCR amplified products. The presence of rod-shaped (R) and coccoid (C) forms of *H. pylori* in the gastric biopsy specimens has been described elsewhere [9].

presence of significantly increased amounts of *Helicobacter* DNA in the biopsy. Nevertheless, gastritis specimen no. 430 showed a distinctly different TTGE profile with a prominent band corresponding to *Stomatococcus* and only a minor band corresponding to *Helicobacter* (Fig. 1). The biopsy contained predominantly coccoid bacteria, which previously had been interpreted as coccoid *H. pylori*. In view of the present findings it might be reasonable to question the assignment of these bacteria as coccoid *H. pylori*. In all other *H. pylori*-associated gastritis biopsy specimens analysed, microscopy revealed the presence of rod-shaped bacteria [9].

H. pylori has been demonstrated to undergo autolysis *in vivo* [21]. It has been suggested that *H. pylori* lysed in the stomach could release antibacterial activity against faster growing micro-organisms [22, 23]. The increased amounts of *H. pylori* and its possible release of antibacterial activity in gastric inflammation could perhaps explain the different bacterial profiles in gastric biopsy specimens obtained by TTGE analysis (Fig. 1).

Cloning and DNA sequence analysis

It is generally believed that helicobacters are unique amongst bacteria in the ability to colonise the stomach. However, based on the results obtained from broad-range 16S rDNA PCR on gastric biopsy specimens combined with Southern blot analysis of the PCR amplified products, an earlier study suggested that other bacterial species may also be present in the stomach [9]. The profiles obtained in the present work based upon TTGE analysis indicate that other bacteria were relatively abundant in the gastric biopsy specimens. To identify these bacteria, 10 TTGE bands were selected and eluted for DNA sequence analysis. Direct DNA sequencing of eluted and re-amplified TTGE-PCR products (with conserved primers flanking the variable V6 region) yielded unique sequences in two cases (nos. 479 and 430). Thus, *Helicobacter* spp. (no.

479) and *Stomatococcus* spp. (no. 430) were (most likely) the only bacterial DNA present in band I and II, respectively (Fig. 1, arrows I and II). In all other cases, mixed DNA sequence profiles were produced, indicating the presence of more than one bacterial species. Therefore, shotgun cloning and automatic DNA sequence analysis were applied to these other eight bands. This procedure yielded a unique sequence from each clone, which could subsequently be analysed. Results from a database Blast-search, with a cut-off value of 97% identity at the genus level, revealed *Enterococcus*, *Pseudomonas*, *Staphylococcus*, *Stomatococcus* and *Streptococcus* as dominant members of the TTGE profile (Fig. 1, arrows 1–8; Table 1). Furthermore, *Acinetobacter*, *Brevundimonas*, *Enterobacter*, *Helicobacter* (other than *H. pylori*), *Propionibacterium*, *Rhizobium*, and so far unidentified bacteria (which showed <97% identity in database searches) were also found (Table 1). Most of the bacteria identified are normal inhabitants of the respiratory tract and oral cavity and, therefore, it might not be surprising if swallowed bacteria are found in the stomach. Moreover, these results are in good agreement with a recent study revealing the presence and distribution of a gastric mucosal microflora in dyspeptic patients with *H. pylori* infection [24].

However, several TTGE bands represented different *Pseudomonas* spp., which are not dominant members of the flora of the respiratory tract and oral cavity except for *P. aeruginosa* in cystic fibrosis patients. Although *Pseudomonas* spp. are frequently found in tap water and have also been demonstrated in contaminated endoscopes [25] it seems very unlikely that the present results represent such contamination. A comparison of the 16S rDNA sequences of the variable V6 regions displayed clusters of sequence variations, which explains the different *Pseudomonas* spp. migration patterns seen on the TTGE gel (Fig. 1, arrows 1–4). For statistical reasons, considering the precautions taken in this study to avoid carry-over of bacterial

Table 1. Bacterial genera characterised by 16S rDNA sequencing of cloned V6 regions

Bacteria	TTGE bands (n = total) from gastric biopsy specimen no.							
	456 (10)	451 (10)	451 (10)	450 (7)	436 (10)	430 (8)	479 (5)	418 (8)
<i>Acinetobacter</i> spp.			1				1	1
<i>Brevundimonas</i> spp.								1
<i>Enterobacter</i> spp.				1				
<i>Enterococcus</i> spp.		1		1	6			
<i>Helicobacter</i> spp.					2			
<i>Propionibacterium</i> spp.			1				1	
<i>Pseudomonas</i> spp.	5	8	6	3	1			2
<i>Rhizobium</i> spp.					1		1	
<i>Staphylococcus</i> spp.	5	1						
<i>Stomatococcus</i> spp.						3		
<i>Streptococcus</i> spp.				1		4		1
Unidentified genera*			2	1		1	2	3

Dominating bacterial genera are indicated in bold text.

* < 97% identity in database searches.

DNA between specimens, the simultaneous presence of multiple *Pseudomonas* spp. due to contamination should be improbable. Interestingly, *Pseudomonas* spp., *Streptococcus* spp., *Staphylococcus* spp. and gram-positive bacilli have been grown from gastric aspirates from patients with reflux gastritis syndrome. These authors concluded that the microbial flora might be an association factor and might play a role in certain patients [26].

In the present study, no efforts were made to culture the bacteria; therefore, there is no evidence as to whether the identified bacteria were metabolically active or not. Nevertheless, the fact that it was possible to amplify and identify bacterial DNA at the genus level indicates that a mixed population of bacterial DNA was present in the gastric biopsy specimens.

Are helicobacters part of an indigenous microflora in the stomach?

The present findings suggest that, in the absence of gastric inflammation, helicobacters could be part of an indigenous flora of the stomach. DNA corresponding to the genus *Helicobacter* was identified in all 13 human gastric biopsies. Five of the gastric biopsies were obtained from histologically normal controls with no evidence of *H. pylori*-associated gastritis. Interestingly, DNA sequences assigned to *Helicobacter* included some variation. Partial 16S rDNA sequence analysis of variable regions V3 and V4 of specimens 430, 436, and 450, respectively, revealed DNA sequences identical with the type strain *H. pylori* ATCC 43504^T. In contrast, DNA sequence variations in 16S rDNA of the variable regions V3 and V4 were observed in gastritis specimen nos. 410, 422, 453, 457 and 479 [9]. It is assumed that *H. pylori* is the prototypical bacterium residing in the mucosal layer of the stomach, but its role as an obligate pathogen has been questioned [27]. Recently, it was proposed that helicobacters are indigenous biota of the human stomach and that *H. pylori* have both pathogenic and symbiotic features [28, 29]. The different TTGE profiles described here, obtained from patients with asymptomatic *H. pylori*-associated gastritis and histologically normal control specimens (Fig. 1), might lend some support to these hypotheses.

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