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Long-Term Colonization with Single and Multiple Strains of *Helicobacter pylori* Assessed by DNA Fingerprinting

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The gastric pathogen *Helicobacter pylori* establishes long-term chronic infections that can lead to gastritis, peptic ulcers, and cancer. The species is so diverse that distinctly different strains are generally recovered from each patient. To better understand the dynamics of long-term carriage, we characterized *H. pylori* isolates from initial and follow-up biopsy specimens from a patient population at high risk of *H. pylori* infection and gastric cancer. Eighty-five isolates were obtained from 23 patients and were analyzed by genomic restriction enzyme analysis, arbitrarily primed PCR fingerprinting, (random amplified polymorphic DNA analysis), and/or restriction of specific PCR-amplified genes (restriction fragment length polymorphism analysis). A single strain was found in sequential biopsy specimens from 12 of 15 patients (80%) receiving sucralfate. In the remaining three patients treated with sucralfate, two strains were identified in two patients and three strains were identified in the third patient. In contrast, a single strain was found in sequential biopsy specimens from only three of eight patients (37%) receiving bismuth, metronidazole, and nitrofurantoin. Two strains were identified in five other patients receiving bismuth-antibiotic (63%). Immunoglobulin G antibodies to *H. pylori* were present in the sera of all patients. Thus, *H. pylori* colonization can persist for long periods (up to at least 4 years), despite high titers of immunoglobulin G antibodies in serum. Resistance to metronidazole was noted in some strains before and/or after treatment, but all strains remained susceptible to amoxicillin, tetracycline, and nitrofurantoin. We conclude that *H. pylori* genotypes, as measured by several sensitive DNA fingerprinting methods, can remain stable for years in vivo, despite the acquisition or loss of drug resistance, circulating antibody, or exposure to antibiotics or sucralfate.

The pathogen *Helicobacter pylori* colonizes the human gastric mucosa and causes chronic bacterial gastritis and ulcers in humans and is a major risk factor for the development of gastric cancer (5, 20, 24, 34). Early trials attempting to eradicate *H. pylori* were often unsuccessful, even with drugs that had seemed useful in tests in culture. We now recognize that there is a great diversity among *H. pylori* strains in many traits including drug resistance (31, 37), adherence specificity (6, 10, 19, 35), and cytotoxin production (9, 11, 12). Even greater diversity has been documented by various typing methods, including ribotyping, restriction digests of genomic DNA (restriction enzyme analysis [REA]), random amplified polymorphic DNA (RAPD) analysis, and restriction of PCR-amplified gene segments (PCR-restriction fragment length polymorphism [PCR-RFLP] analysis) (2–4, 8, 14, 15, 26–28, 30, 32, 33, 36). In general, any two independent clinical isolates are distinguishable from each other by such DNA fingerprinting techniques. In some studies in which antibiotic treatment failed to eradicate *H. pylori*, the isolates obtained in a second biopsy specimen seemed to be closely related to the original isolate (2, 4, 15). Cases of patients colonized with more than one *H. pylori* strain have also been reported (15, 18, 30).

We have been studying strains of *H. pylori* from a patient population of low socioeconomic strata with an historically high prevalence of both gastric cancer and *H. pylori* infection (12, 13). The purpose of this report is to characterize the *H. pylori* strains obtained from sequential gastric biopsy specimens from representative patients who had participated in one of the early unsuccessful clinical trials. In particular, we compared strains of *H. pylori* from patients with recurring infection obtained before and after treatment with antibiotic or sucralfate using the complementary DNA fingerprinting methods of REA of genomic DNA and PCR-RFLP and RAPD. Cases of long-term colonization with a single strain and cases of colonization with multiple strains were documented.

MATERIALS AND METHODS

Patient population. The patient population has been described previously (13). Briefly, beginning in the late 1980s, 96 patients attending the Gastroenterology Clinic at Charity Hospital in New Orleans, La., were entered into a therapeutic *H. pylori* trial approved by the Human Studies Committee at Louisiana State University. The patients in this trial were predominantly black, from lower socioeconomic strata, and at high risk of *H. pylori* infection (12, 13). Patients were randomly assigned to treatment groups. In addition to clinical evaluation, patients underwent gastroscopy and biopsy specimens were taken from the antrum and corpus for histologic examination. Tissue from the antrum was cultured for *H. pylori*. Patients were biopsied on their initial visit (visit 1), and treatment commenced with either antibiotic therapy (group 1; 48 patients) or sucralfate (group 2; 48 patients). Patients were biopsied on subsequent visits: at 1 month (visit 2), 3 months (visit 3), 6 months (visit 4), 12 months (visit 5), and yearly thereafter. Serum for *H. pylori* enzyme-linked immunosorbent assay (ELISA) was collected at each visit. Of 48 patients receiving sucralfate, 4 had one or more follow-up visits with negative culture results. Of the 48 patients receiving antibiotics, 15 had one or more follow-up visits with negative culture for *H. pylori.*

Treatment groups. Strains of *H. pylori* from 23 patients selected randomly from the two treatment groups were studied. Patients in treatment group 1 (n = 8) received bismuth subsalicylate (262 mg four times per day for 28 days), nitrofurantoin (four 100-mg doses four times per day on days 8 to 17), and

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metronidazole (500 mg four times per day on days 18 to 28). Patients in treatment group 2 ($n = 15$) received sucralfate (1 g four times per day for 28 days).

***H. pylori* strains.** *H. pylori* were cultured from biopsy specimens in the following manner. The biopsy specimens were homogenized aseptically in a sterile tissue grinder with phosphate-buffered saline (PBS) as the diluent. Homogenates were plated onto 5% sheep blood agar plates (Remel Laboratories, Lenexa, Kans.) and were incubated microaerophilically for 5 to 7 days at 37°C. All strains of *H. pylori* were identified by Gram stain morphology and by positive urease, catalase, and oxidase reactions.

Growth of *H. pylori*. Broth cultures were grown in standard 150-mm-diameter petri dishes containing 12 ml of brucella broth (BBL, Cockeyesville, Md.) and 5% fetal calf serum (FCS; Hyclone Laboratories, Logan, Utah). Inoculum was obtained from 3- to 5-day-old blood agar plates incubated at 37°C under microaerophilic conditions. Plates were stacked in GasPak jars (BBL) and gassed with an anaerobic mixture (90% N_2, 5% CO_2, 5% H_2), and the jar was placed in a 37°C incubator on a rotary shaker (New Brunswick Scientific, Edison, N.J.) at 60 rpm. Cells were harvested by centrifugation after 2 to 3 days of incubation. Alternately, isolates were grown on 5% sheep blood agar plates, and the growth after 3 to 5 days was scraped into tubes containing PBS. Pellets were washed three times in PBS.

Preparation of chromosomal DNA. Washed pellets were suspended in STET buffer (8% sucrose, 50 mM EDTA, 0.1% Triton X-100, 50 mM Tris-HCl [pH 8.0]). Lysozyme (hen egg white; Boehringer Mannheim Biochemicals, Indianapolis, Ind.) was added to a final concentration of 3 mg/ml. The suspension was incubated for 12 min at 37°C and was then lysed with sodium deodecyl sulfate (final concentration, 1%). RNase A (bovine pancreas; Boehringer Mannheim) was added to a final concentration of 0.05 mg/ml, and the solution was incubated for 1 h at 37°C. Pronase and proteinase K (final concentrations, 0.8 and 0.5 mg/ml respectively; Boehringer Mannheim) were added and the suspension was incubated overnight at 37°C. A 1:10 volume of 5% CTAB–0.5 M NaCl solution (Sigma Chemical Company, St. Louis, Mo.) was added, the solution was gently mixed and then incubated at 65°C for 10 min. DNA was extracted with an equal volume of phenol-chloroform (1:1; vol/vol), precipitated overnight in 0.3 M sodium acetate with 2:1 volumes of absolute ethanol at −20°C, and pelleted by centrifugation at 13,000 × *g* for 1 h at 4°C. The ethanol was decanted, and the pellet was air dried and suspended in sterile distilled water. DNA concentrations were assessed spectrophotometrically at wavelengths of A_{260} and A_{280}.

Genomic REA. Ten to 20 μg of genomic DNA from each isolate was digested to completion with an excess of a restriction enzyme as described previously (33). DNA from each strain was cut by at least two enzymes. The enzymes used were *Hin*dIII, *Pvu*II, *Bgl*II, and *Hae*III (Boehringer Mannheim). The reaction was terminated by the addition of gel loading buffer (40% sucrose, 0.25% bromophenol blue). Restricted DNA was electrophoresed in a 0.8% horizontal agarose gel for 16 h at 30 V in TAE buffer (40 mM Tris-HCl, 20 mM sodium acetate, 2 mM EDTA [pH 7.6]). The gels were stained with a solution of ethidium bromide (1 mg/ml) for 30 min and were destained for 1 h.

RAPD fingerprinting. In the RAPD DNA fingerprinting method, oligonucleotides of arbitrarily chosen sequences were used as primers in low-stringency PCR amplification, which allows DNA synthesis to initiate from sites to which the primer is fortuitously matched. It generally yields arrays of some 4 to 20 prominent DNA fragments which are strain specific and reproducible (3, 4). In brief, PCR for RAPD tests was carried out in a volume of 25 μl containing 20 ng of *H. pylori* genomic DNA, 3 mM $MgCl_2$, 20 pmol of primer, 1 U of AmpliTaq DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.), and 250 μM (each) dCTP, dGTP, dATP, and dTTP (Boehringer Mannheim) in 10 mM Tris-HCl (pH 8.3)–50 mM KCl under a drop of mineral oil. A Perkin-Elmer TC480 thermal cycler was used for amplification. The cycling program was 45 cycles of 94°C for 1 min, 36°C for 1 min, and 72°C for 2 min) and then 72°C for 10 min (3). Ten-microliter aliquots of products were electrophoresed in 2% agarose gels containing 0.5 μg of ethidium bromide per ml in the gel and 1× Tris acetate running buffer (0.04 M Tris acetate, 0.001 M EDTA, 0.02 M acetic acid [pH 8.4]). The 1-kb DNA ladder (Gibco BRL, Grand Island, N.Y.) was used as a size marker in all gels. Tests with two to four RAPD primers were used to assess whether strains were identical.

PCR-RFLP fingerprinting. The PCR-RFLP fingerprinting method exploits restriction site polymorphisms in specific gene segments and entails PCR amplification of the gene or segment of interest and then restriction to detect sequence diversity (2). The oligonucleotides used for PCR amplification of specific fragments were 5′-AGGAGAATGAGATGA and 5′-ACTTTATTGGCTGGT for a 2.4-kb *ureA-ureB* fragment, 5′-TGGGACTGATGGCGTGAGGG and 5′-ATCATGACATCAGCGAAGTTAAAAATGG for a 1.7-kb *ureC-ureD* fragment, and 5′-ATGGCTTTTCAGGTCAATAC and 5′-GCTTAAGATATTTTGTTGAACG for a 1.5-kb *flaA* fragment (2). PCR amplification was carried out in a volume of 100 μl containing 0.2 U of AmpliTaq polymerase, standard PCR buffer (Cetus), and 20 pmol of each primer. The reaction was carried out in a Perkin-Elmer 480 thermal cycler programmed for 35 cycles as follows: 94°C for 1 min at 50°C (*ureA-ureB*) or 60°C (*flaA* or *ureC-ureD*) and then 72°C for 2 min. After amplification the DNA products were ethanol precipitated and resuspended in 25 μl of sterile distilled water, and 3 μl of product was electrophoresed on a 1% agarose gel to ensure homogeneity and to assess the yield. Ten microliters of product was digested with 5 to 10 U of restriction enzyme for 1 h in the buffer recommended by the supplier (New England Biolabs) and was electrophoresed in a gel consisting of 3.5% NuSieve GTG agarose and 1.5% SeaKem LG agarose (FMC Bioproducts, Rockland, Maine). PCR-RFLP was carried out with two and in some cases three gene segments.

Antibiotic susceptibility assay. The *H. pylori* isolates were tested for their resistance to metronidazole, tetracycline, amoxicillin, and nitrofurantoin by a plate dilution method reported previously (25). All antibiotics were obtained from Sigma Chemical Company. Brucella agar plates (Difco Laboratories) containing 5% FCS and various concentrations of antibiotic were prepared. *H. pylori* was grown under microaerophilic conditions on blood agar plates for 3 to 4 days. The growth was scraped from the plates into sterile PBS, and the optical density (OD) was determined by using a Beckman model DU 640 spectrophotometer (Beckman Instruments, Fullerton, Calif.). The suspensions were adjusted to an OD_{660} of 0.01. By using a Steers replicator, the isolates were inoculated onto plates containing antibiotic. Plates were incubated microaerophilically at 37°C for 5 days. The endpoint was determined visually as that dilution of antibiotic which inhibited 90% growth of the isolates (MIC_{90}) compared with the growth on an antibiotic-free control plate. Strains were considered susceptible to metronidazole if the MIC_{90} was 6.4 μg/ml or less (31, 37). Strains were considered susceptible to the other antibiotics tested if the MIC_{90}s fell within the following reported limits: for amoxicillin, 0.006 to 0.25 μg/ml (17, 29); for tetracycline, 0.015 to 0.61 μg/ml (17, 29); and for nitrofurantoin, <0.03 to 4.0 μg/ml (16, 21).

ELISA. The levels of immunoglobulin G (IgG) antibody to *H. pylori* in serum were determined by an ELISA as described previously (12). Briefly, whole-cell antigen prepared from a pool of three strains of *H. pylori* was coated onto 96-well microtiter plates. After a blocking step, dilutions of the patient serum were incubated in the wells for 1 h. After washing, alkaline phosphatase-labeled goat anti-human IgG was incubated in the wells for 1 h. Following washing, ONPP (orthonitrophenyl phosphate) substrate was added to the wells, and the color was allowed to develop. The plates were read on an automated ELISA reader (Dynatech, Chantilly, Va.). The titer was determined to be the dilution of serum which gave an OD equal to the mean plus 3 standard deviations of the negative controls.

Figure preparation. Polaroid photographs of the gels were processed for presentation by scanning them with a Microtek Scanmaker 600ZS by using Adobe Photoshop 2.0 software (Adobe Systems, Inc., Mountain View, Calif.) and were imported into Canvas 3.0.2 software (Deneba Software, Miami, Fla.) running on a Macintosh Quadra 700 computer.

RESULTS

We studied *H. pylori* isolates from 15 patients receiving sucralfate, an agent that protects the gastric mucosa (Table 1), and 8 patients receiving a combination of bismuth, metronidazole, and nitrofurantoin (Table 2). This combination had seemed promising from in vitro susceptibility tests, but it proved to be ineffective in eradicating *H. pylori* infection (22). Comparison of results of REA carried out at the Massachusetts Institute of Technology with RAPD or PCR-RFLP tests performed at Washington University showed 97% agreement among the methods. The only exception was that of isolates 3 and 4 from patient 2016, which were distinguished from isolate 1 by REA but not by PCR-RFLP. Isolates from different biopsy specimens were considered to match only if their profiles were matched in each fingerprinting test. Conversely, any result suggesting differences among isolates from a given patient were verified by repetitions of the RAPD or PCR-RFLP tests that had indicated divergence. Of the 15 patients receiving sucralfate, 12 (80%) appeared to be colonized with a single strain throughout the study, as assessed by DNA fingerprinting (Table 1). In addition, 2 of the 15 patients (13%) (patients 1074 and 2007) were colonized with two different strains and patient 1010 (7%) was colonized with three different strains (Figures 1, 2, and 3). In patients infected with multiple strains, only one strain of *H. pylori* was isolated from each biopsy specimen. Although metronidazole was never administered to this group of patients for the treatment of *H. pylori* infection, 10 of the 29 isolates tested were resistant to metronidazole (Table 3). In particular, each of the three isolates from patient 1010 was metronidazole resistant. In one patient (patient 2003), isolates that seemed to be uniform by DNA fingerprinting were mixed with respect to metronidazole-resistant and -susceptible phenotypes.

In comparison, of eight patients receiving antibiotic treat-

TABLE 1. Characteristics of *H. pylori* isolates obtained from patients receiving sucralfate[a]

Patient no.	Visit no.	DNA fingerprint[b]			No. of strains
		REA	RAPD	RFLP	
2002	1	a	a	a	
	4	a	a	a	
	5	a	a	a	
	7	a	a	a	
	8	a	a	a	1
2003	1	b	b	b	
	3	b	b	b	
	4	b	b	b	
	7	b	b	b	1
1017	1	c	ND	c	
	4	c	ND	c	
	5	c	ND	ND	
	6	c	ND	ND	1
3001	1	d	ND	d	
	3	d	ND	d	
	5	d	ND	d	
	6	d	ND	d	1
1016	1	e	e	e	
	2	e	e	e	
	3	e	e	e	
	4	e	e	e	
	5	e	e	e	1
1027	1	f	ND	f	
	3	f	ND	f	
	5	f	ND	f	1
1037	2	g	ND	ND	
	4	g	ND	ND	
	5	g	ND	ND	1
1054	1	h	ND	h	
	2	h	ND	h	
	4	h	ND	h	
	5	ND	ND	h	1
1062	1	i	ND	i	
	2	i	ND	i	
	3	i	ND	i	
	4	i	ND	i	1
3005	1	j	j	j	
	3	j	j	j	
	4	j	j	j	1
1009	1	k	k	k	
	2	k	k	k	
	3	k	k	k	1
1050	1	1	ND	1	
	2	l	ND	l	1
2007	2	m	m	m	
	4	n	n	n	
	5	n	n	n	2
1074	1	o	ND	ND	
	2	p	ND	ND	
	3	o	ND	ND	
	4	o	ND	ND	2
1010	1	p	p	p	
	2	q	q	q	
	3	r	r	r	3

[a] The following patients were culture negative on visit 2: 1017, 1027, 3001, and 3005. The following strains were not characterized by DNA fingerprintings: 1017-3, 1027-4, 1037-1 and -3, 1054-3, 2002-2, -3, and -7, 2003-2 and -6, 2007-1 and -3, and 3001-4.

[b] Letters indicate individual fingerprint type. ND, not done.

ment, five (62%) were colonized with two strains of *H. pylori* (Table 2), and only one strain was recovered from the other three patients (38%). Results of representative REA, RAPD, and PCR-RFLP typing of isolates from an antibiotic-treated

TABLE 2. Characteristics of *H. pylori* isolates from patients receiving antibiotic therapy[a]

Patient no.	Visit no.	DNA fingerprint[b]			No. of strains
		REA	RAPD	RFLP	
1021	1	a	a	a	
	4	a	a	a	
	6	a	a	a	
	7	a	ND	ND	1
1067	1	b	b	b	
	2	b	b	b	
	5	b	b	b	1
3009	1	c	ND	c	
	3	c	ND	c	1
1007	1	d	d	d	
	3	e	ND	ND	
	4	e	e	e	
	6	ND	e	e	2
1013	1	f	f	f	
	2	f	f	f	
	3	g	g	g	
	4	f	f	f	
	5	ND	f	f	
	6	f	f	f	2
1014	1	h	h	h	
	3	i	ND	ND	
	4	i	i	i	
	5	ND	h	h	
	6	i	ND	ND	2
1040	1	j	ND	j	
	3	j	ND	k	
	4	j	ND	j	
	5	k	ND	ND	
	6	k	ND	ND	2
2016	1	l	ND	l	
	3	m	ND	l	
	4	m	ND	l	2

[a] The following patients were culture negative on visit 2: 1014, 2016, and 3009. Patients 1007 and 1021 were culture negative on visits 2 and 5. Patient 1067 was culture negative on visit 3. The following strains were not characterized: 1021-3, 1040-2, and 1067-4.

[b] Letters indicate individual fingerprint types; ND, not done.

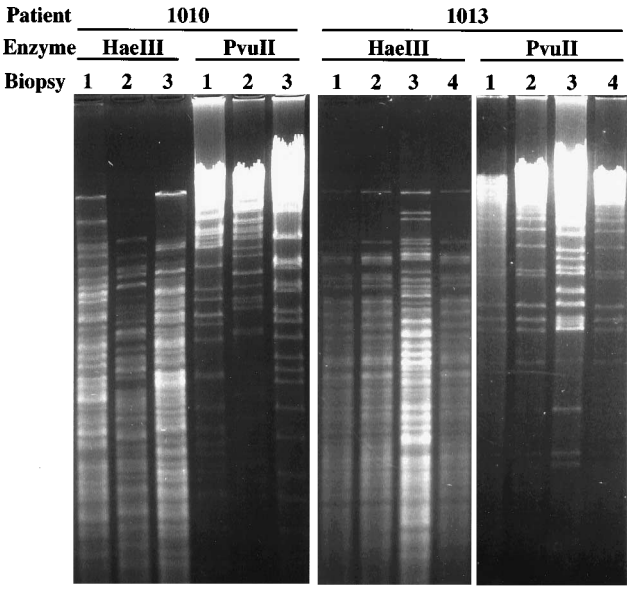

FIG. 1. Representative results of restriction nuclease digests of genomic DNA with enzymes *Hae*III and *Pvu*II. The differences in profiles of strains from patient 1010 show that a different strain was recovered from each biopsy specimen. The differences in the profiles of strains from patient 1013 show that the strain recovered from biopsy specimen 3 differed from the strains recovered from biopsy specimens 1, 2 and 4.

patient (patient 1013) are shown in Fig. 1, 2, and 3, respectively. After antibiotic treatment, four of five patients remained colonized with the strain obtained prior to treatment, but in addition, a second strain was also detected. In contrast, after apparent eradication of the organism with antibiotic treatment, as indicated by a negative culture on visit 2, patient

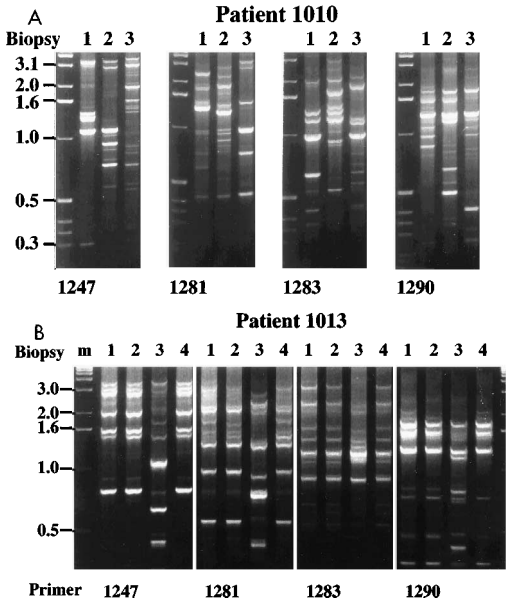

FIG. 2. Representative results of RAPD tests with four primers: 1247, 1281, 1283, and 1290. (A) The differences in RAPD profiles of strains from patient 1010 show that a different strain was recovered from each biopsy specimen. (B) The differences in RAPD profiles of strains from patient 1013 show that the strain recovered from biopsy specimen 3 differed from the strains recovered from biopsy specimens 1, 2, and 4.

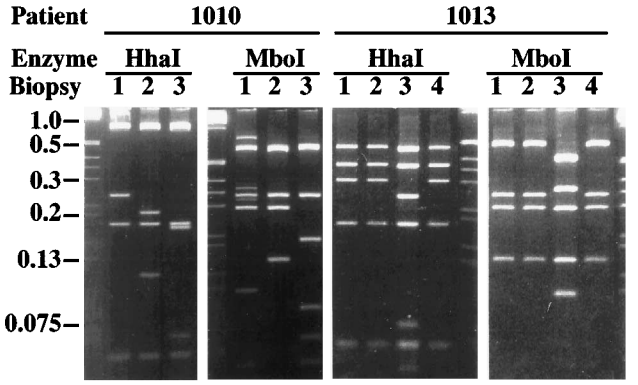

FIG. 3. Representative results of restriction analysis of PCR-amplified *flaA* DNA. A 1.5-kb *flaA* segment was PCR amplified from genomic DNA with *flaA*-specific primers. The PCR product was digested with restriction enzyme *Hha*I (GCGC) or *Mbo*I (GATC). The differences in digests of strains from patient 1010 show that a different strain was recovered from each biopsy specimen. Differences in the digests of strains from patient 1013 show that the strain recovered from biopsy specimen 3 differed from the strains recovered from biopsy specimens 1, 2, and 4.

1007 was shown to be colonized with a new strain of *H. pylori* (type e) which may have replaced the initial strain (type d) since type d was not recovered from three sequential biopsy specimens taken after antibiotic treatment (visits 3, 4, and 6). Alternatively, type e which was Met^r on visit 3, may have become the predominant strain after antibiotic therapy. In five patients treated with antibiotics, strains that were not distinguished by DNA fingerprinting were found to differ in their metronidazole susceptibility or resistance levels.

An ELISA was used to determine the levels of IgG antibody to *H. pylori* in the sera of patients at each visit. Table 4 shows the IgG antibody titer to *H. pylori* in patients colonized for longer than 1 year. All patients except one (patient 1014) who were colonized for 2, 3, and 4 years maintained high levels of IgG antibody to *H. pylori*. It was also noted that strains of *H. pylori* remained stable in vivo over 2, 3, and 4 years as monitored by DNA fingerprinting (patients 2002, 2003, 3001, 1017, and 1021).

DISCUSSION

Using three complementary DNA fingerprinting methods, we studied 85 isolates of *H. pylori* from 23 patients over a 4-year period. There was agreement among the REA, PCR-RFLP, and/or RAPD fingerprinting results for 65 of 67 isolates. The seeming discrepancy with isolates from one patient (patient 2016) may reflect a limitation of the PCR-RFLP method: specific genes from different strains may be matched fortuitously in restriction sites for one or two enzymes even if they differ at many other sites throughout the entire genome (2).

The DNA fingerprinting results reported here have shown that (i) a single strain of *H. pylori* can be recovered repeatedly over a period of at least 4 years from persons at high risk of *H. pylori* infection and gastric cancer and (ii) that such persons often carry more than one strain. Thus, our results confirm and expand earlier studies showing that a given strain can persist for months in an infected person (2, 3, 15, 23, 30) and that a person can also be colonized with two strains (15, 18, 30). Of particular interest in this context was our finding that the *H. pylori* strains in some patients were mixed with respect to their metronidazole resistance, although they were uniform with respect to their DNA fingerprints. Since some of these patients were not treated with metronidazole during our study and Met^r

TABLE 3. Metronidazole susceptibility profiles of *H. pylori* isolates

Patient no.	Visit no.	No. of strains/ REA type	Metronidazole MIC_{90} (μg/ml)/resistance[a]
1009	1	1	0.80/S
	2		3.20/S
	3		3.20/S
2003	1	1	0.20/S
	2		6.40/S
	4		12.80/R
	6		0.40/S
1016	1	1	25.60/R
	2		>25.60/R
	3		>25.60/R
	4		>25.60/R
3001	1	1	0.40/S
	3		1.60/S
1054	1	1	0.80/S
	2		25.60/R
1062	1	1	0.80/S
	2		0.80/S
1050	1	1	0.20/S
	2		12.80/R
1027	1	1	0.80/S
	3		0.80/S
	5		0.80/S
1017	1	1	0.80/S
	4		3.20/S
1037	1	1	0.20/S
	2		0.80/S
1010	1	3/a	25.60/R
	2	b	12.80/R
	3	c	12.80/R
1021[b]	1	1	25.60/R
	3		25.60/R
	4		6.40/S
	6		>25.60/R
1067[b]	1	1	1.60/S
	2		12.80/R
1007[b]	1	2/d	0.80/S
	3	e	>25.50/R
	4	e	6.40/S
1013[b]	1	2/f	25.60/R
	2	f	25.60/R
	3	g	25.60/R
	4	f	12.80/R
1014[b]	1	2/h	0.04/S
	3	i	0.04/S
	4	i	25.60/R
1040[b]	1	2/j	6.40/S
	3	j	25.60/R
	4	j	>25.60/R

[a] S, susceptible; R, resistant.
[b] Patients were treated with antibiotics and bismuth.

TABLE 4. IgG antibody response to *H. pylori* infection in sera of patients colonized for more than 1 year

Patient no.	No. of strains isolated	No. of biopsy specimens	Antibody titer (range)[a]
2002	1	8	>2,048–18,820
2003	1	7	>4,096–16,384
1017	1	6	2,048–6,654
3001	1	6	3,566–8,192
1021[b]	1	7	>2,048–5,793
1007[b]	2	6	111–1,024
1013[b]	2	6	2,048–4,098
1014[b]	2	6	128–512
1040[b]	2	6	2,048–3,822

[a] Numbers are the reciprocal of the ELISA titer.
[b] Patients treated with antibiotics and bismuth.

arises only rarely during laboratory culture, these patients may have been treated with metronidazole or an analog for some other infection at a dose sufficient to enrich for resistant strains but not completely eliminate their susceptible parents. The finding of identical DNA fingerprints of Met^s and Met^r strains from the same patient is in accord with simple mutation to resistance or interstrain transfer of (small) DNA segments that confer resistance (7). Alternatively, if DNA transfer is considered a possible contributor to mixed populations, we cannot exclude a model in which another trait was actually selected and happened to be linked to Met^r. In addition, the recovery of a Met^s and Met^r version of one strain from one metronidazole-treated patient (patient samples 1014-3 and -4) may reflect the ineffectiveness of the combined bismuth-metronidazole-nitrofuantoin therapy that was tested in this early clinical trial (and that resulted in this combination not being used currently to treat *H. pylori* infection).

Although the number of patients studied is small, it is striking that, with regard to DNA fingerprints, a larger fraction of apparently mixed infections was found among the antibiotic-treated patients relative to the fraction among sucralfate-treated patients (5 of 8 versus 3 of 15 patients, respectively). We propose that many of the *H. pylori*-infected patients actually were colonized with mixed populations, but often with a single strain being present in abundance; the antibiotic treatment used may have inhibited certain strains more than others, and thus may have changed the distribution of strains rather than eradicate *H. pylori* completely. The establishment of mixed infections, often with unequal levels of coexisting strains, has been modeled recently in gnotobiotic piglets (1).

To determine if more than one strain of *H. pylori* was present in any single biopsy specimen, we analyzed, in preliminary studies, five individual colonies grown from single biopsy specimens from two different patients. For patient 1010 a different strain was identified on each of three visits. Analysis of the colonies picked from the biopsy specimen at visit 2 showed that all strains were identical to the strain identified at visit 2. A single strain was isolated from patient 1016 over a 1-year period. Analysis of five isolates obtained from the visit 3 biopsy specimen showed that all isolates were identical to the strain identified initially (unpublished data). These data suggest that a single strain can be present at an individual biopsy site, but does not exclude the possibility that patients are infected simultaneously with more than one strain of *H. pylori* at different locations in the stomach.

Unfortunately, this and several other studies of this type by other groups were initiated before the great genetic diversity of *H. pylori* was fully appreciated. Now that it is known that *H.*

pylori has the ability to establish and maintain mixed bacterial populations, we can design experiments that more fully examine issues such as the incidence and importance of transient and persistent mixed infections, the presence of different subpopulations whose relative frequencies may change with therapy and other events, and the ability of *H. pylori* to transfer DNA horizontally. All of these factors may be of considerable significance in assessing the progression and outcome of induced disease in human populations at high risk of *H. pylori* infection, such as persons of low socioeconomic strata in developed countries (as described here) as well as residents throughout much of the Third World.

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