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Comparison of the Nucleic Acids of Helical and Coccoid Forms of *Helicobacter pylori*

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The nucleic acids of the helical and coccoid forms of *Helicobacter pylori* were studied to determine if the coccoid forms are "viable (capable of growing) but nonculturable." Using a reference strain (NCTC 11638) and five clinical strains, the nucleic acid contents, DNA integrity, and results of PCR and reverse transcription-PCR (RT-PCR) were compared for helical *H. pylori* and coccoid forms induced using glycochenodeoxycholic acid or bismuth citrate. The DNA and RNA contents of the coccoid forms were respectively 6.8- and 8.1-fold lower than those of helical *H. pylori* after 3 days of induction and 11.5- and 14.7-fold lower after 7 days. Agarose gel electrophoresis of DNA extracted from the coccoid forms after 3 days of induction showed a smear pattern indicating DNA cleavage, whereas DNA from helical *H. pylori* showed a single band with a high molecular mass. After 12 days of induction, all RNA samples from 100% coccoid cultures were negative for the mRNA of urease A or the 26-kDa species-specific protein by RT-PCR. However, most RNA samples obtained after 3 or 7 days of induction were positive at low levels despite the lack of recovery from these cultures. These results suggest that the coccoid form of *H. pylori* has impaired genomic DNA and is in the process of cellular degeneration, thus being still alive but nonincreasable.

Helicobacter pylori is a gram-negative bacterium that can be cultured on artificial media under microaerobic conditions (12, 29). *H. pylori* from fresh cultures is a curved or helical rod, but physical or chemical stress transforms it into a basally respiring but nonculturable coccoid form (2, 3). Other helical bacteria that have coccoid forms include certain species of *Vibrio* (1, 10, 13, 16), *Desulfovibrio* (22), *Campylobacter* (4, 25, 26), *Aquaspirillum* (19), *Oceanospirillum* (19), and *Spirillum* (19, 39). These coccoid forms resemble penicillin-induced culturable spheroplasts (22), but the cell walls of the spheroplasts are usually thinner (19). Coccoid forms can also be discriminated from spheroplasts by the retention of sufficient peptidoglycan to resist lysis (19).

H. pylori colonizes the mucosa of the human stomach and causes nonspecific chronic gastritis as well as, probably, gastroduodenal ulcers and carcinoma (20, 21). In developed countries, *H. pylori* seropositivity is found in 40 to 50% of the population aged 20 to 60 years (21), but the mode of spread remains unclear (3). While oral-oral and/or fecal-oral routes have been postulated for the transmission of *H. pylori*, isolation from saliva and stool has been unsuccessful (36, 37), with rare exceptions (11, 27). However, PCR analysis of saliva and stool specimens usually detects *H. pylori* DNA (23, 30). This had led to the hypothesis that coccoid forms are the origin of such DNA and are ingested orally, after which they change into helical bacteria and colonize the stomach (3). This hypothesis has been strengthened by findings that the coccoid forms retain urease activity (31), reduce tetrazolium salts (13), exhibit a protein pattern similar to that of the helical bacteria in sodium dodecyl sulfate-polyacrylamide gel electrophoresis (31), and synthesize DNA (3). The recovery of coccoid forms from animals fed *Campylobacter jejuni* (17) and *H. pylori* (6) has been reported by several authors, and use of the temperature upshift technique achieves the same result with *Vibrio parahaemolyticus* and *Vibrio vulnificus* (16). However, the germination or multiplication of coccoid forms has never been documented morphologically except by Williams and Rittenberg for an organism of uncertain genus, "*Spirillum lunatum*" (19, 39).

Since nucleic acids contain the information needed to control cellular multiplication (24), the nucleic acids of the helical and coccoid forms of *H. pylori* were compared in the present study to determine if the coccoid forms are "viable (capable of growing, as defined in this paper) but nonculturable" (13).

MATERIALS AND METHODS

Bacterial strains. The reference strain used was NCTC 11638, which was kindly provided by T. Itoh (Tokyo Metropolitan Research Laboratory of Public Health) (15). Five representative clinical strains (SM 6, SM 7, SM 8, SM 14, and SM 17) were obtained from antral biopsy specimens of patients at St. Marianna University Hospital. They were isolated on Skirrow's medium (Eiken, Tokyo, Japan) by microaerobic techniques with a CampyPak (BBL, Cockeysville, Md.) and were identified by the method of Itoh et al. (15).

Culture conditions. Each strain was cultured on brain-heart infusion agar (Difco, Detroit, Mich.) plates with 10% horse serum (Gibco BRL, Gaithersburg, Md.) and 0.25% yeast extract (Difco) (12) at 37°C for 3 days under microaerobic conditions with a CampyPak envelope (BBL) equipped with a palladium catalyst (29).

Induction of coccoid forms. Coccoid forms were induced by the method of Nilius et al. (31). An inoculum of 20 mg of fresh wet cells (2×10^9 CFU as determined by the usual spread plate technique) (29) was suspended in brain-heart infusion (Difco) broth with 10% horse serum and 0.25% yeast extract and then incubated at 37°C with glycochenodeoxycholic acid (Sigma, St. Louis, Mo.) at a final concentration of 1 mM (472 μg of sodium salt/ml) or with 0.1 mM (32-μg/ml) bismuth citrate (Nacalai Tesque, Inc., Kyoto, Japan). Drug-free control cultures were incubated under anaerobic conditions (28) to arrest multiplication of the bacteria and were designated "aging" cultures (31). Incubation with or without drugs was done for 3, 7, and 12 days. After removal of residual helical bacteria and clumps of organisms by centrifugation at $600 \times g$ for 5 min, the induced coccoid forms were sedimented by centrifugation at $11,000 \times g$. One loopful of cells (1 mg [wet weight]) taken from a prepared sample served as

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inoculum for detecting helical bacteria and was cultured for 7 days under the conditions described above.

Morphology. Bacterial morphology was determined by light microscopy after half-Gram staining (38). The percentage of coccoid forms was estimated by counting 600 bacteria in three fields per smear (26).

Estimation of genomic DNA and total RNA. Bacteria were added to a 1.5-ml microcentrifuge tube, rinsed once with phosphate-buffered saline (pH 7.2), and pelleted by centrifugation at 11,000 × *g*. Genomic DNA was extracted by the guanidium thiocyanate method (35) using IsoQuick (MicroProbe Corp., Garden Grove, Calif.). The DNA was further purified by treatment with 10 μg of DNase-free RNase (bovine pancreatic RNase boiled for 15 min; Sigma) per ml for 1 h to remove residual RNA. Purified DNA was precipitated by removing added RNase with a reagent of IsoQuick and then adding isopropanol. The resulting DNA pellet was suspended in TE buffer (10 mM Tris hydrochloride, 1 mM EDTA; pH 8.0). Proteinase K was not used in the extraction process, as it is reported to have a potential inhibitory effect on the PCR (9). Total RNA was isolated by acid guanidinium thiocyanate-phenol-chloroform extraction (7) using RNAzol (Biotecx Laboratories, Inc., Houston, Tex.) and was stored at −80°C. The RNA pellet was dissolved in TE buffer. The relative amounts of nucleic acids extracted from *H. pylori* cells and from the culture fluid were estimated by determining the ratio of A_{260} and A_{280} with a spectrophotometer (UV-3100S; Shimadzu, Kyoto, Japan). Salmon sperm DNA (Wako Pure Chemical Industries, Ltd., Osaka, Japan) and yeast RNA (Wako) were used as the standards (1). One optical density unit at 260 nm was assumed to be equivalent to 50 μg of double-stranded DNA and 40 μg of RNA/ml.

Gel electrophoresis of native DNA. The integrity of the DNA (10-μl samples) was examined by horizontal gel electrophoresis (12 V/cm) on 1% agarose containing ethidium bromide (0.1 μg/ml) in TBE buffer (89 mM Tris, 89 mM boric acid, 2 mM EDTA; pH 8.3) (35). The gels were photographed on Polaroid P/N type 667 film by UV transillumination (TDM-40; Ultra Violet Products, Inc., Upland, Calif.).

Samples for PCR amplification. Tenfold serial dilutions of *H. pylori* DNA were prepared at concentrations from 50 ng/μl to 5 fg/μl and used as PCR templates. cDNAs were also amplified after preparation from mRNAs as described below. The negative and positive controls contained TE buffer alone and 50 ng of DNA from the reference strain, respectively.

Synthetic primers. A single primer pair was used to amplify a 314-bp sequence of the urease A (*ureA*) gene or the cDNA by the method of Kawamata et al. (18). Primers designated A-2F2 (5′-ATATTATGGAAGAAGCGAGAGC-3′) and A-2R (5′-ATGGAAGTGTGAGCCGATTTG-3′) were identical to residues 2993 to 3014 and 3306 to 3286, respectively, of the published sequence. Another pair of primers was designed and selected on the basis of computer-assisted analysis of the published sequence of a major species-specific protein (ssp) antigen of 26 kDa (32). These primers, designated SSPF (5′-AAGCGCCTGCC GTTTTAG-3′) and SSPR (5′-CGATACCGCCTTTTTCCACA-3′), were identical to residues 293 to 310 and 544 to 525, respectively. The ssp primers were used to amplify a 252-bp sequence for mRNA detection, as described below.

PCR amplification. PCR was performed in a 50-μl reaction mixture in 0.6-ml thin-walled tubes in an automatic thermal cycler (PJ2000; Perkin-Elmer Cetus, Norwalk, Conn.). The PCR mixture contained 5 μl of 10× PCR buffer (N808-0160; Perkin-Elmer Cetus), 4 μl of 2.5 mM deoxynucleoside triphosphate, 2 μl of 10 μM oligonucleotide primers, 1 U of AmpliTaq polymerase (Perkin-Elmer Cetus), 37.8 μl of molecular-biology-grade distilled water, and 1 μl of sample DNA. Prior to the addition of sample DNA, each reaction mixture was overlaid with a drop of mineral oil (Sigma) (20). The mixtures were incubated for 5 min at 94°C for initial denaturation of the target DNA and then subjected to 35 cycles of denaturation at 94°C for 1 min, annealing at 55°C (*ureA*) or 53°C (ssp) for 1 min, and extension at 72°C for 1 min (18). After amplification was completed, extension was continued for another 7 min. The amplified products (10 μl) were analyzed by electrophoresis on 3% agarose gel (NuSieve 3:1 agarose; FMC BioProducts, Rockland, Maine) containing 0.1 μg of ethidium bromide per ml in TBE buffer. The PCR product was visualized under UV light and photographed as described above.

Preparation of cDNA from mRNA. Reverse transcription was performed with total RNA extracted from 100% coccoid cultures from which no bacteria could be isolated. Total RNAs from helical *H. pylori* and from the corresponding aging cultures were used as the positive controls. Two cDNAs synthesized with antisense primers A-2R and SSPR were included in the reaction mixture. Total RNA (1 μg) was dissolved in 10 μl of the reaction buffer, which consisted of 2 μl of 5× reverse transcription reaction buffer (Gibco BRL) and 8 μl of diethyl pyrocarbonate (DEPC)-treated distilled water (33). The RNA solutions were treated with 1 U of RNase-free DNase I (amplification grade; Gibco BRL), 1 μl of 25 mM EDTA was added, and the DNase I was heat inactivated for 10 min at 65°C. The RNA sample (12 μl) was mixed with 2 μl of 5× reverse transcription reaction buffer, 2 μl of 100 mM dithiothreitol (Gibco BRL), 1 μl of 10 mM deoxynucleoside triphosphate, 1 μl of 10 mM antisense primer, 5 U of RNase inhibitor (Takara, Tokyo, Japan), and 1.8 μl of DEPC-treated distilled water and then incubated at 42°C for 90 min with 20 U of Moloney murine leukemia virus reverse transcriptase (Gibco BRL) (30).

RESULTS

Light microscopy findings. Three-day plate cultures of the test strains consisted of almost 100% helical bacteria (Fig. 1A). Induction culture of the NCTC 11638 strain with glycochenodeoxycholic acid or bismuth citrate for 3 days generated a high proportion of coccoid forms. Forms obtained after removal of bacterial clumps and amorphous debris by centrifugation at 600 × *g* for 5 min (Fig. 1B) were nearly 100% coccoid. Aging cultures of NCTC 11638 contained an average of 30% coccoid and 70% helical forms after 3 days (Fig. 1C). The percentage of coccoid forms increased with prolongation of the incubation period, but the aging cultures still included helical bacteria after 12 days. There was some variation between strains in the rate of transformation from helical to coccoid form.

Nucleic acid content. All test strains were lysed within 1 min of adding the DNA and RNA extraction reagents. Subsequent extraction steps were also performed successfully. Table 1 summarizes the results of nucleic acid determinations. The DNA and RNA contents of the 100% coccoid cultures were lower than those of helical cultures of the reference strain (NCTC 11638) and all clinical isolates tested. After 3 days of induction with glycochenodeoxycholic acid or bismuth citrate, the coccoid forms had 6.8- and 8.1-fold-lower average DNA and RNA contents, respectively, than the helical bacteria. After 7 days of induction, coccoid forms had 11.5- and 14.7-fold-lower average DNA and RNA contents, respectively, than helical *H. pylori*. Coccoid forms induced from any of the strains tested using the two agents showed no significant difference in the loss of nucleic acids. *H. pylori* in all aging cultures showed higher nucleic acid levels than the same strains in drug-containing cultures.

Integrity of the extracted DNA. Agarose gel electrophoresis of native DNA obtained from helical bacteria gave a single band with a high molecular mass, whereas native DNA from pelleted coccoid forms showed a smear pattern indicating DNA cleavage (Fig. 2). Both the discrete band and the smear were observed when DNA was obtained from aging cultures consisting of both helical and coccoid forms (Fig. 2).

Sensitivity and species specificity of PCR. The lower limit of detection by the single-step PCR with *ureA* or ssp primers was 50 to 500 fg of genomic DNA, theoretically corresponding to about 3×10^1 to 3×10^2 helical bacteria containing 1.8 fg of DNA/cell (20). Specific PCR products were not obtained when DNAs from 36 other species of bacteria, including *Helicobacter fennelliae* and *Helicobacter cinaedi*, were tested as described by Kawamata et al. (18). The lower limit of detection for the coccoid forms was in the same range as that for helical *H. pylori.*

Detection of mRNA. All samples of total RNA (50 ng) were positive by PCR with both primer pairs because of contamination with genomic DNA at a level of less than 0.01% (5 pg) as computed from the sensitivity of PCR detection. After residual DNA had been eliminated by treatment with DNase I, all samples were negative in both PCR assays. Table 2 shows the results of semiquantitative determination of *ureA* and ssp mRNA. The lower limits of detection by reverse transcription-PCR (RT-PCR) assays with antisense primers A-2R and SSPR were 50 to 500 and 5 to 50 pg, respectively, of total RNA from helical *H. pylori*. These RNA amounts theoretically corresponded to about 5×10^4 to 5×10^5 and 5×10^3 to 5×10^4 cells, respectively, as computed from the DNA/RNA ratio of the reference strain. After 12 days of induction, all RNA samples from coccoid forms were negative for *ureA* or ssp mRNA (Table 2). RNA samples from coccoid forms of the reference strain were also negative for mRNA after 7 days of induction

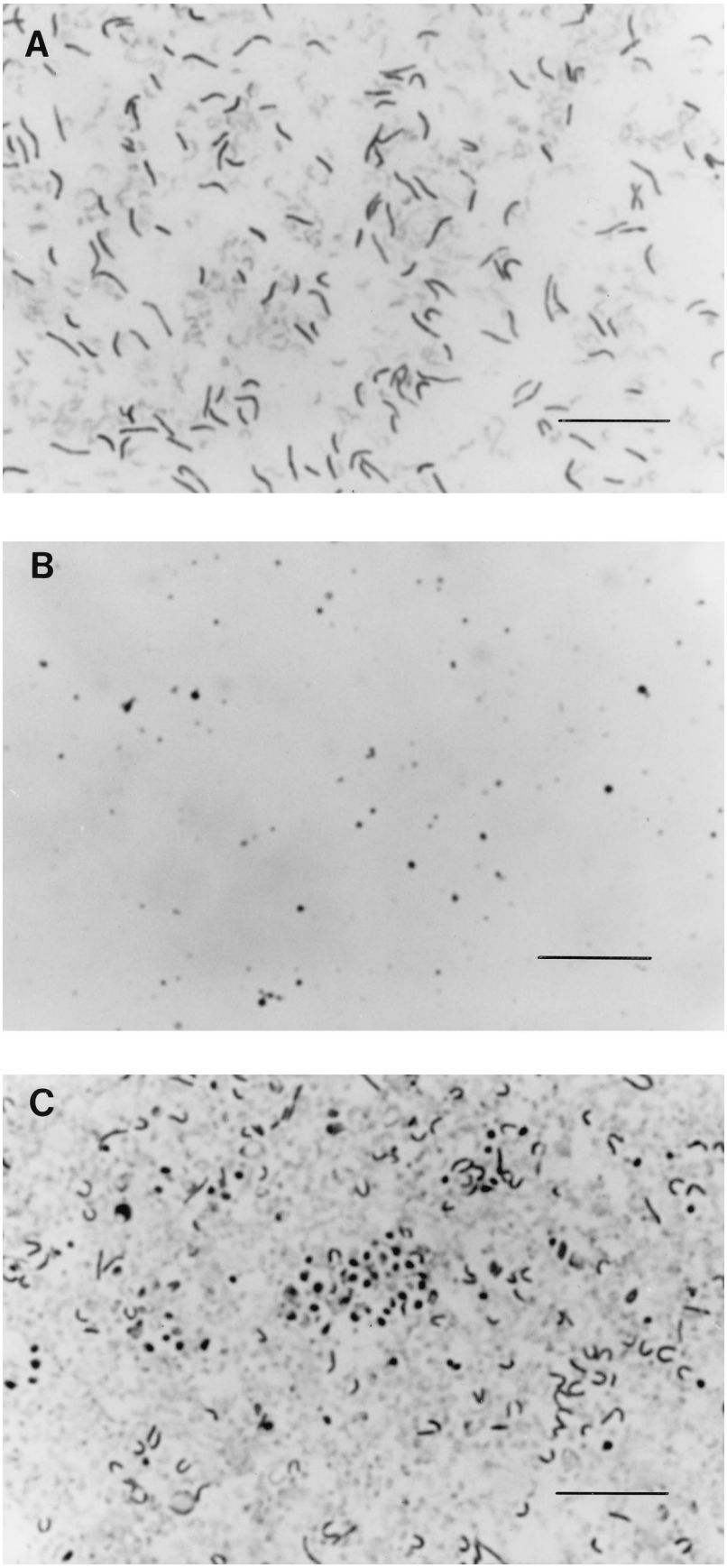

FIG. 1. Photomicrographs of helical and coccoid forms of *H. pylori* NCTC 11638. (A) Three-day plate cultures of the test strain consisted of almost 100% helical bacteria. (B) Nearly pure coccoid forms were obtained by 3 days of incubation with glycochenodeoxycholic acid and subsequent removal of bacterial clumps and amorphous debris by centrifugation at 600 × *g* for 5 min. (C) Drug-free control (aging) cultures contained an average of 30% coccoid and 70% helical forms after 3 days. Half-Gram stain. Bars, 10 μm.

TABLE 1. Estimated nucleic acid contents of helical and coccoid forms of *H. pylori*

Strain	Induction method	Cell form	% Dry wt of cells after incubation					
			DNA			RNA		
			Day 0	Day 3	Day 7	Day 0	Day 3	Day 7
NCTC 11638	Nil	Helical	15.4			9.5		
	Bile salt[a]	Coccoid		2.0	0.9		0.8	0.4
	Bismuth salt[b]	Coccoid		1.9	0.9		1.4	0.5
	Aging only	Both[c]		4.8	3.1		1.9	0.7
SM 6	Nil	Helical	3.3			2.7		
	Bile salt	Coccoid		0.5	0.1		0.3	0.1
	Bismuth salt	Coccoid		0.5	0.2		0.3	0.2
	Aging only	Both		1.0	0.5		1.0	0.6
SM 7	Nil	Helical	2.5			2.9		
	Bile salt	Coccoid		0.3	0.2		0.4	0.2
	Bismuth salt	Coccoid		0.5	0.4		0.5	0.3
	Aging only	Both		1.4	0.8		1.2	0.4
SM 8	Nil	Helical	7.2			5.6		
	Bile salt	Coccoid		0.9	0.6		0.5	0.3
	Bismuth salt	Coccoid		1.0	0.8		1.1	0.9
	Aging only	Both		3.0	1.7		1.3	1.0
SM 14	Nil	Helical	4.4			17.0		
	Bile salt	Coccoid		0.8	0.7		1.6	0.6
	Bismuth salt	Coccoid		0.9	0.7		1.7	0.8
	Aging only	Both		2.2	1.9		5.0	1.5
SM 17	Nil	Helical	3.6			2.1		
	Bile salt	Coccoid		0.4	0.1		0.2	0.1
	Bismuth salt	Coccoid		0.5	0.2		0.3	0.2
	Aging only	Both		1.1	0.4		0.8	0.3

[a] Glycochenodeoxycholic acid (1 mM).
[b] Bismuth citrate (0.1 mM).
[c] Helical and coccoid forms.

culture but were positive after 3 days, while strains SM 8 and SM 17 were still positive for mRNA after 7 days of induction (Table 2) despite the lack of growth from these cultures. The lower limit of detection for mRNA from the coccoid forms was at least 10^2-fold higher than that for mRNA from helical *H. pylori* (Table 2). Fig. 3 shows the examples of semiquantitative determination of ssp mRNA in strain SM 17.

DISCUSSION

Phadnis et al. reported that 3-day-old *H. pylori* cells spontaneously underwent autolysis and that the cytoplasmic components released by this process were adsorbed to the surfaces of intact bacteria (34). Those authors also suggested that autolysis might be followed by the development of coccoid forms on day 4. Development of coccoid forms is accelerated under conditions unfavorable for growth (2, 3). Bile and bismuth salts, aging, aerobic incubation (excess oxygen), and antibiotics have previously been used for the induction of coccoid *H. pylori* (31). We used glycochenodeoxycholic acid and bismuth citrate, both of which are recommended by Nilius et al. for the induction of viable coccoid forms with only slight structural or degenerative changes in the cell wall (31). We found that crude preparations of the coccoid forms always contained amorphous debris, which could be sedimented by centrifugation at 600 × *g*. The sediment contained no coccoid forms, whereas the supernatant contained only coccoid organisms, all of which could be sedimented by centrifugation at 11,000 × *g* or more. This difference might have been due to the lower density of coccoid forms relative to that of helical bacteria, as described by Baker and Park for a *Vibrio* sp. (1) and by Moran and Upton for *C. jejuni* (25).

Bacterial nucleic acids are usually extracted with guanidium thiocyanate (35), which we also used in the present study. The amounts of DNA and RNA obtained from the coccoid forms decreased as the induction period became longer, and similar results were obtained with all strains tested (the coefficient of variation of the rate of decrease during 3 days of induction was less than 30%). Light microscopy indicated that the volume of the coccoid forms (Fig. 1) was generally not larger than that of the helical bacteria, so coccoid organisms with this marked decrease in DNA may not retain enough of the genome for multiplication. RNA levels declined similarly to DNA levels in the coccoid forms, suggesting that the decreased nucleic acid content was associated with the loss of cytoplasm by autolysis, as described for *C. jejuni* (25, 26) and *H. pylori* (34).

Pitcher et al. used agarose gel electrophoresis to check the integrity of DNA extracted with guanidium thiocyanate and found that undegraded DNA migrated as a single band with a high molecular mass for the nine different bacterial species tested (35). In contrast, DNA from coccoid *H. pylori* yielded a smear pattern indicating DNA cleavage. Thus, DNA degradation appeared to have commenced in the coccoid forms by day 3 of induction culture, and this may have caused these forms to lose the ability to multiply. Virulence is apparently also lost with the development of a coccoid morphology, paralleling the decline in culturability (8).

The PCR technique was recently used for detecting *H. pylori* DNA. Our PCR assays with the two primer pairs amplified DNA from the coccoid forms, and the detection limit was the same as that for DNA from helical *H. pylori*. These data support the results of Ho et al. (14). The much lower DNA content of the coccoid forms was probably not due to the presence of fragments undetectable by PCR. Thus, clinical and environmental specimens positive for *H. pylori* by PCR but negative on culture might contain coccoid forms of this organism.

mRNA carries genetic information to the ribosomes on which protein synthesis occurs (24). Bacterial mRNA usually has a half-life of 2 to 3 min and is broken down rapidly by intracellular enzymes (5), a process that is important in the control of gene expression (5, 24). Detection of mRNA therefore suggests that a cell is alive. We tested samples from coccoid forms negative on culture and from the relevant aging controls, avoiding false-positive results by pelleting residual helical *H. pylori*. We found mRNA positivity despite culture

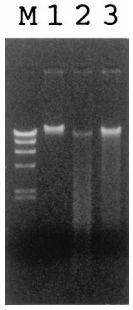

FIG. 2. Agarose gel electrophoresis of undigested DNAs from helical *H. pylori* NCTC 11638 cultured for 3 days (lane 1), coccoid forms induced by incubation with glycochenodeoxycholic acid for 3 days (lane 2), and an aging control culture (lane 3). Approximately 1 μg of DNA from washed cells was loaded on a 1% agarose gel containing 0.1 μg of ethidium bromide per ml, and electrophoresis was done in TBE buffer. Lane M denotes lambda *Hin*dIII molecular weight markers (Gibco BRL). The first through sixth bands from the top are 23.0, 9.4, 6.9, 4.3, 2.3, and 2.0 kb, respectively.

TABLE 2. RT-PCR detection of mRNA for urease A (*ureA*) and the 26-kDa ssp in *H. pylori* cells after 3, 7, and 12 days of culture for induction of coccoid forms

Strain	Induction method	Cell form	Maximum dilution for detection of cDNA in RNA solutions (50 ng/μl)							
			314-bp fragment of *ureA* mRNA				252-bp fragment of ssp mRNA			
			Day 0	Day 3	Day 7	Day 12	Day 0	Day 3	Day 7	Day 12
NCTC 11638	Nil	Helical	10^{-2}				10^{-3}			
	Bile salt	Coccoid		—[a]	—	—		10^{-1}	—	—
	Bismuth salt	Coccoid		NT[b]	—	—		NT	—	—
	Aging	Both		10^{-2}	10^{-1}	10^{-1}		10^{-3}	10^{-3}	10^{-2}
SM 6	Nil	Helical	10^{-2}				10^{-4}			
	Bile salt	Coccoid		NT	NT	—		NT	NT	—
	Bismuth salt	Coccoid		NT	NT	—		NT	NT	—
	Aging	Both		NT	NT	10^{-1}		NT	NT	10^{-2}
SM 8	Nil	Helical	10^{-2}				10^{-3}			
	Bile salt	Coccoid		NT	10^{0}	—		NT	10^{-1}	—
	Bismuth salt	Coccoid		NT	10^{-1}	—		NT	10^{-1}	—
	Aging	Both		NT	10^{-2}	10^{-1}		NT	10^{-2}	10^{-2}
SM 17	Nil	Helical	10^{-3}				10^{-4}			
	Bile salt	Coccoid		NT	—	—		NT	10^{-1}	—
	Bismuth salt	Coccoid		NT	10^{-1}	—		NT	10^{-2}	—
	Aging	Both		NT	10^{-2}	10^{-2}		NT	10^{-3}	10^{-3}

[a] —, not detected in 50 ng of RNA.
[b] NT, not tested for culture recovery. If all coccoid forms induced by drugs were positive for culture recovery, aging controls were not tested either.

negativity in one NCTC 11638 sample, two SM 8 samples, and two SM 17 samples by using the ssp primers (Table 2). These findings indicate that mRNA is present in early coccoid forms, as verified by the fact that the detection limit for helical forms by culture (1 CFU/inoculating loop or 20 CFU/cell sample) was lower than that for mRNA by our RT-PCR (5 pg of total RNA/5 × 10^3 CFU/reaction or 100 pg/1 × 10^5 CFU/cell sample). Detection of some mRNA probably reflected residual life during the process of cell degradation. An additional 5 days of induction after the coccoid forms became negative on culture was required for mRNA to also become undetectable. Thus, the coccoid forms may remain alive for some days after taking on this morphology. In conclusion, our results suggest that the coccoid form of *H. pylori* is a degenerating cell with impaired genomic DNA and hence is still alive but nonincreasable.

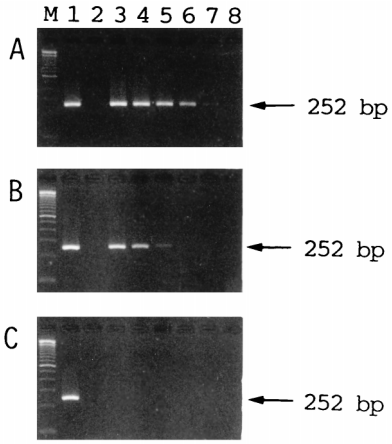

FIG. 3. Agarose gel (3%) electrophoresis of the 252-bp ssp PCR product, showing the detection of mRNA from strain SM 17 of helical *H. pylori* (A) and coccoid forms induced by incubation with bismuth citrate for 7 days (B) or 12 days (C). Lanes 1 through 8 represent crude RNA (50 ng), DNase I-treated RNA (50 ng), and 10-fold serial dilutions of reverse-transcribed RNA (50 ng, 5 ng, 500 pg, 50 pg, 5 pg, and 500 fg) pretreated with DNase I, respectively. Lane M shows a 100-bp DNA ladder (Gibco BRL). RNA samples (50 ng) were positive by PCR with ssp primers (lane 1) because of contamination with genomic DNA at a level of less than 0.01% (5 pg). After residual DNA had been eliminated by treatment with DNase I, samples were negative in PCR assays (lane 2). The lower limits of detection of cDNA in panels A, B, and C were 5 pg (10^{-4} dilution), 500 pg (10^{-2} dilution), and over 50 ng (not detected), respectively, of total RNA (lanes 3 through 8).

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