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Coccoid Forms of *Helicobacter pylori* Are the Morphologic Manifestation of Cell Death

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Helicobacter pylori **can transform from its normal helical bacillary morphology to a coccoid morphology. Since this coccoid form cannot be cultured in vitro, it has been speculated that it is a dormant form potentially involved in the transmission of *H. pylori* and in a patient's relapse after antibiotic therapy. In this study we determined the effects of aging, temperature, aerobiosis, starvation, and antibiotics on the morphologic conversion rate and culturability of *H. pylori*. Aerobiosis and the addition of a bactericidal antibiotic to the culture medium resulted in the highest conversion rate. During the conversion to coccoid forms, the cultures always lost culturability at the stage where 50% of the organisms were still in bacillary form; this result indicated that culturability and coccoid morphology are two separate but related entities. Independent of the conditions used to induce the conversion into coccoids, the morphological conversion was accompanied by several marked antigenic and ultrastructural changes. Also, both the total amounts and the integrity of RNA and DNA were significantly reduced in coccoid forms. With the potential-sensitive probe $diOC_5$-3, a clear loss of membrane potential in coccoid forms was observed. Inhibition of protein or RNA synthesis by the addition of bacteriostatic antibiotics did not prevent the conversion to coccoid forms but resulted in an increased conversion rate. Hence, we conclude that conversion of *H. pylori* from the bacillary to the coccoid form is a passive process that does not require protein synthesis. Our data suggest that the coccoid form of *H. pylori* is the morphologic manifestation of bacterial cell death.**

Helicobacter pylori is now recognized as the main etiologic agent of gastritis in humans. Infection with this microorganism also increases the risk of peptic ulcer disease and gastric cancer (1, 8). During infection the majority of the bacteria are present as spiral-shaped gram-negative bacilli in the gastric mucosal layer of the host (22, 30), but coccoid forms have also been found in the human stomach (14). Also, when cultured under favorable conditions in vitro, the majority of *H. pylori* bacteria have a bacillary appearance, but aging or exposure to unfavorable conditions results in the conversion of this bacillary form to a coccoid form (2–5, 9–14, 20, 21, 31). A similar phenomenon has been described for the closely related *Campylobacter* spp. (6, 18, 23, 24). The coccoid forms are not culturable by standard laboratory methods, but there are indications that these forms may be viable and possibly even infectious (2–5, 13, 15, 26, 29, 31). It has been postulated that the coccoid forms are a dormant stage of *H. pylori* and that they play a role in the survival of the bacterium in a hostile environment (2–5, 10–14, 17, 20, 21, 25, 31). Since *H. pylori* has been recovered only occasionally from sources other than the human stomach, it is generally accepted that every environment outside the human stomach is unfavorable to *H. pylori* and hence stimulates its conversion to the coccoid, nonculturable form.

The possibility that the coccoid form plays a role in transmission of *H. pylori* and in relapse of infection after antimicrobial therapy is still a matter of debate. For other microorganisms, morphologic transition to nonculturable but viable forms has been described as a mechanism to survive harsh environmental conditions. With these microorganisms, however, the conversion appears to be reversible upon improvement of these conditions. For *H. pylori*, reversion of the coccoid form to the bacillary form does not seem to occur easily. In this respect the morphologic conversion of *H. pylori* is analogous to that of the closely related *Campylobacter* spp. For *Campylobacter* it is now proposed that the coccoid form is a degenerate morphologic phase associated with dead bacteria that does not pose an infectious risk (18, 23, 24).

We studied the effects of different incubation conditions on the morphologic conversion rate of *H. pylori* in order to establish to what extent adverse incubation conditions stimulate the conversion to coccoids. In addition, we investigated whether morphologic conversion is an active process requiring de novo protein synthesis by comparing conversion rates in the presence and absence of protein and of RNA synthesis inhibitors. We also determined the effects of various conditions and antibiotics on the bacterial ultrastructures, the protein profiles, the RNA and DNA compositions, and the membrane potentials of bacillary and coccoid forms of *H. pylori.*

MATERIALS AND METHODS

Bacterial strains and culture conditions. The following *H. pylori* strains were used: ATCC 43504, NCTC 11638, HP 1280, and HP 1075. The last two are recent clinical isolates from two unrelated patients with duodenal ulcers (28). The MICs for all the strains were 2.0 mg/liter for kanamycin, 1.0 mg/liter for chloramphenicol, 0.38 mg/liter for rifampin, and 0.75 mg/liter for tetracycline.

All strains were maintained at −80°C in brain heart infusion broth (Difco, Detroit, Mich.) with 20% (vol/vol) glycerol (Merck, Darmstadt, Germany). Bacteria were cultured either on Columbia agar plates (Oxoid, Basingstoke, Hampshire, England) supplemented with 7% (vol/vol) lysed, defibrinated horse blood (BioTrading Benelux BV, Mijdrecht, The Netherlands), 0.004% (wt/vol) tetrazolium chloride (Sigma, St. Louis, Mo.), and 0.0025% (wt/vol) Dent supplement (*H. pylori* selective supplement; Oxoid), resulting in final concentrations of 10 μg/ml for vancomycin, 5 μg/ml for trimethoprim, 5 μg/ml for cefsulodin, and 5 μg/ml for amphotericin B (CA^+ plates) or in brucella broth (Difco) supplemented with 2% (vol/vol) newborn calf serum (Gibco, Paisley, Scotland) and 0.0025% (wt/vol) Dent supplement (BB^+). All cultures were incubated mi-

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croaerobically at 37°C in jars flushed with 5% O_2, 10% CO_2, and 85% N_2, unless stated otherwise.

Determination of conversion rates under various environmental conditions. Bacteria harvested from freshly grown, almost confluent CA^+ plates were inoculated in BB^+ (one plate per 100 ml) and incubated overnight with gentle agitation at 37°C in a microaerobic atmosphere. At the onset of the experiment (time zero), this overnight culture was split into 25-ml portions that were further incubated with agitation under various conditions. The incubation conditions that were varied were temperature (22°C), nutrient availability (the bacteria were harvested by centrifugation and subsequently resuspended in the original volume of phosphate-buffered saline [PBS]), and use of aerobiosis (incubation in air). To study the effects of antibiotics on the conversion rate, either kanamycin monosulfate (100 μg/ml; Sigma), chloramphenicol (20 μg/ml; Sigma), rifampin (150 μg/ml; Serva), or tetracycline hydrochloride (15 μg/ml; Boehringer, Mannheim, Germany) was added to the BB^+ medium immediately after the initial overnight culture was split into 25-ml portions. In order to determine the total number of bacteria per milliliter and the number of viable bacteria (CFU per milliliter) and to study bacterial morphology, 1-ml samples were taken from these cultures at regular intervals and analyzed as described below. All experiments were performed in triplicate, and each experiment was repeated three times.

Bacterial counts. The total number of bacteria was determined with a counting chamber (Thoma, Weber, England). The counting chamber was filled with 3 μl of bacterial culture (or an appropriate dilution thereof in PBS), and the culture was examined at a magnification of ×400 with an Axioskop phase-contrast microscope (Zeiss, Oberkochen, Germany).

The number of viable bacteria was determined by plating 100 μl of the appropriate 10-fold dilutions in PBS on CA^+ plates. Plates were incubated at 37°C under microaerobic conditions. After 80 h of incubation, colonies were counted; results are expressed as CFU per milliliter. When bacteria were incubated in media that contained antibiotics, the bacterial cells were washed once with PBS to remove the antibiotic before being diluted in PBS and plated on CA^+ plates.

Bacterial morphology. Bacterial morphology was studied in Gram-stained preparations with a Zeiss standard microscope at a magnification of ×1,000. The ratio of bacillary to coccoid forms was determined by counting a minimum of 100 bacteria on each of three triplicate samples obtained from a single culture. Although the analysis of the morphology was not performed in a double-blind fashion, an unprejudiced determination of the morphology was confirmed by a double-blind recount of organisms of each form from a random selection of the samples by an independent investigator.

Electron microscopy. Bacteria were harvested from the BB^+ by centrifugation, resuspended in PBS, and fixed for 60 min by the addition of glutaraldehyde to a final concentration of 2.5%. Bacteria were then washed three times in PBS, postfixed with osmium tetroxide for 60 min, dehydrated with a series of ethanol, and embedded in epoxy medium. From the epoxy, 70-nm-thick sections were cut with a Reichert III ultramicrotome and applied to Formvac-coated copper grids. The grids were contrasted with uranylacetate and lead citrate and examined on a Philips model CM100 transmission electron microscope. Control experiments showed that the PBS washes and centrifugation steps had no effect on the viability, morphology, and ultrastructure of the cells.

Analysis of cell composition of bacillary and coccoid forms of *H. pylori*. Bacteria were incubated under various environmental conditions and assayed at regular intervals for the total number of bacteria and CFU and for bacterial morphology as described above. At the same time, samples were obtained for the analysis of protein and nucleic acid compositions.

For the determination of protein profiles bacteria were harvested from 2-ml samples by centrifugation (2 min, 19,000 × *g*), immediately resuspended in 100 μl of 2× Laemmli sample buffer, and incubated at 100°C for 10 min. Thirty-microliter aliquots of appropriate dilutions of these samples (containing approximately 5×10^8 bacteria) were resolved by sodium dodecyl sulfate–12% polyacrylamide gel electrophoresis (SDS–12% PAGE) (19) and visualized with Coomassie brilliant blue stain (Bio-Rad, Hercules, Calif.).

RNA and DNA were isolated from 5-ml samples in a single procedure with an RNeasy total RNA kit (Qiagen, Westburg BV, Leusden, The Netherlands) according to the manufacturer's instructions. The concentrations of RNA and DNA were determined optically with a GeneQuant apparatus (Pharmacia, Uppsala, Sweden). The integrity of the RNA and DNA was analyzed by resolving 25-μl aliquots of the obtained RNA and DNA samples in a 0.7% agarose gel containing 0.5× Tris-borate-EDTA. Nucleic acids were stained with ethidium bromide and visualized under a UV light source.

Membrane potential. A 25 μM stock solution of the probe $diOC_5$-3 (3,3′-dipentyloxacarbocyanine iodide; Molecular Probes Inc., Eugene, Oreg.) was prepared in ethanol and stored at −20°C. Bacteria were inoculated in BB^+ and subsequently grown under various conditions as described above. Samples from these liquid cultures were adjusted with BB^+ to a final concentration of approximately 10^6 bacteria per ml. Bacterial samples were subsequently incubated for 10 min at 37°C in the presence of 150 nM $diOC_5$-3 and measured in a FACScan flow cytometer (Becton Dickinson, Mountain View, Calif.). The extracellular probe is nonfluorescent and therefore does not disturb the reading. Bacteria were distinguished from debris and background noise on the basis of their forward- and sideward-scatter characteristics (both in a logarithmic setting). The green fluorescence of 10,000 bacteria was analyzed with LYSIS software (Becton Dickinson) and plotted as fluorescence histograms.

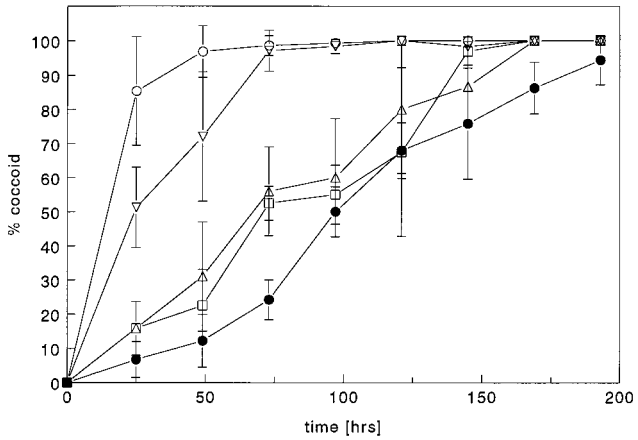

FIG. 1. Conversion rates of *H. pylori* ATCC 43504 incubated under various conditions. Conversion is represented as the percentage that coccoid bacteria comprise of the total number of bacteria present in the culture. Cultures were grown under the following conditions: 37°C microaerobiosis (aging), ●; 37°C aerobiosis, ○; 22°C microaerobiosis, □; 22°C aerobiosis, ▽; and treatment with PBS (starvation), △. Data are means (± standard errors) of three independent experiments performed in triplicate.

RESULTS

Effects of aging, temperature, aerobiosis, and starvation on the viability and morphology of *H. pylori*. The exposure of *H. pylori* ATCC 43504 to different temperatures, starvation, and aerobiosis resulted in changes in the morphologic transition rate of the bacterium as shown in Fig. 1. Exposure to an aerobic environment had the greatest effect on both the viability of the organism and the rate at which helical forms of *H. pylori* transformed into coccoid forms (Fig. 1). Also, while cells incubated for 24 h under standard culture conditions for *H. pylori* (37°C in a microaerobic atmosphere) showed a stable number of CFU per milliliter over a 50-h period (Fig. 2), the number of CFU of cells exposed to aerobic conditions rapidly dropped below the detection limit (Fig. 2).

Bacterial growth (multiplication) was observed exclusively when the bacteria were incubated under the normal microaerobic conditions (Fig. 2). Even when only a single parameter was changed, i.e., the temperature was decreased to 22°C, the BB^+ medium was replaced by PBS, or the bacteria were incubated under aerobic conditions, no bacterial growth was observed. In spite of their negative effects on bacterial growth, changes in temperature and nutrient availability resulted in only minor effects on the rate of conversion from helical to coccoid forms (Fig. 1). Combinations of any of the above-listed conditions resulted in a conversion rate equal to that of the condition with the fastest conversion, i.e., no additive or inhibitory effects were observed (data not shown).

For all conditions tested, a complete loss of culturability had already occurred at the stage where approximately half of the bacteria were transformed into the coccoid form (Fig. 2, in which only aerobic and microaerobic growth is shown).

Effects of RNA and protein synthesis inhibition. To test if de novo RNA or protein synthesis is a prerequisite for the morphologic conversion, one of the following bacteriostatic antibiotics was added to the cultures: chloramphenicol or tetracycline for inhibition of protein synthesis or rifampin for inhibition of RNA synthesis. In addition, we used the bactericidal

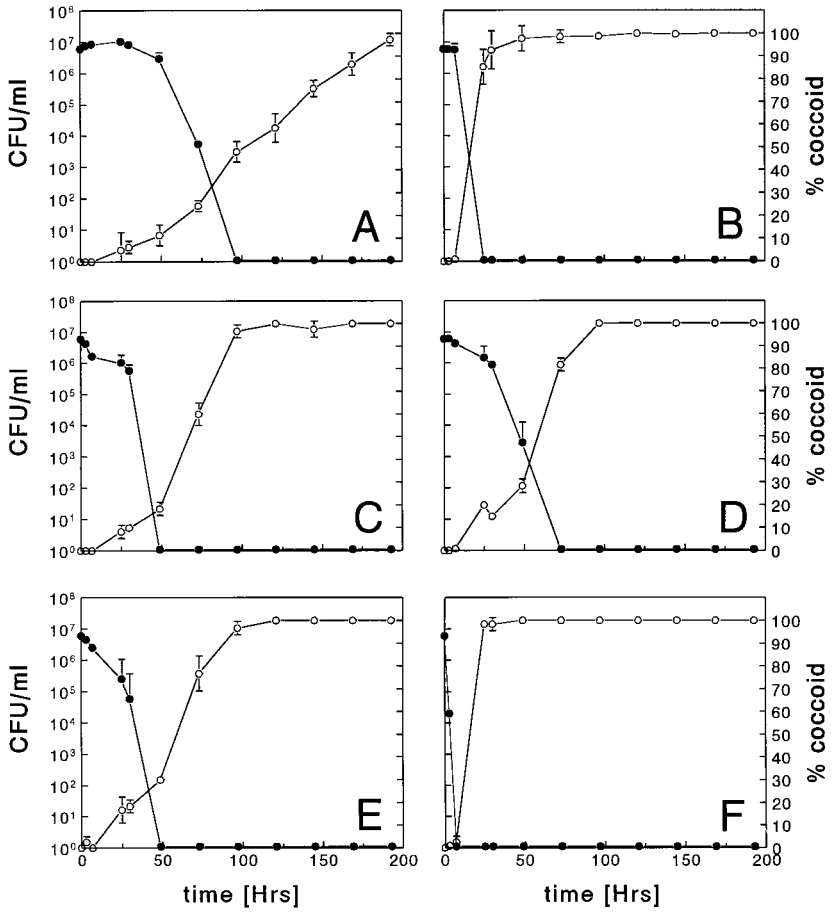

FIG. 2. Relationships between morphologic transition and viability of *H. pylori* ATCC 43504 incubated under the following conditions: microaerobiosis (aging) (A), aerobiosis (B), presence of chloramphenicol (C), presence of tetracycline (D), presence of rifampin (E), and presence of kanamycin (F). The filled circles correspond to the left *y* axis of each panel and represent CFU per milliliter, and the open circles correspond to the right *y* axis and represent percentages of coccoid bacteria. Data are means (± standard errors) of three independent experiments performed in triplicate.

antibiotic kanamycin, an inhibitor of protein synthesis. The effects of these antibiotics on the morphology of *H. pylori* ATCC 43504 are shown in Fig. 3; the effects of aging, aerobiosis, chloramphenicol, tetracycline, rifampin, and kanamycin on growth and culturability are shown in Fig. 2.

At the concentrations used, all antibiotics inhibited bacterial cell growth immediately, indicating that they effectively blocked protein or RNA synthesis. Incubation with kanamycin resulted in loss of the ability to form colonies on agar plates within 7 h (Fig. 2). This rapid killing confirmed that this antibiotic was bactericidal. In contrast, when any of the three other antibiotics was added, CFU counts remained unchanged for 36 h (Fig. 2), which demonstrated that these antibiotics had bacteriostatic activity only. The inhibitory effect of the antibiotics on *H. pylori* was confirmed in an experiment where ^{35}S-labeled methionine was added to the cultures. When any of the three above-mentioned protein synthesis-inhibiting antibiotics was present, incorporation of this label into bacterial proteins was no longer observed (data not shown).

All bacteriostatic antibiotics increased the rate whereby bacillary *H. pylori* became coccoid to values that were intermediate between the low rate of conversion resulting from aging under optimal conditions (37°C microaerobic atmosphere) and the high rate of conversion resulting from exposure to an aerobic environment (Fig. 3). The bactericidal protein synthesis inhibitor kanamycin was the antibiotic most effective in promoting fast conversion. The presence of kanamycin was even more effective than aerobic growth: cultures became >99% coccoid within 24 h with kanamycin (Fig. 3). Although compared to results with kanamycin, the addition of bacteriostatic antibiotics resulted in much slower killing, again, significant transition was observed only when the majority of the bacteria were no longer culturable. As with the experiments in which different incubation conditions were used to induce morphologic conversion, with all four antibiotics we observed that bacteria already could no longer be cultured at the time point where approximately half of the bacteria were transformed into coccoid forms (Fig. 2).

Ultrastructural changes during the morphological conversion of *H. pylori*. In order to determine whether ultrastructural changes followed a set pattern, samples obtained at various stages of the morphologic conversion of *H. pylori* were analyzed by electron microscopy. The times of occurrence of the various ultrastructural forms were determined, and from this we inferred the temporal sequence depicted in Fig. 4. Although the ultrastructural changes occurred at different times under the different conditions used (aging, aerobiosis, temperature, and presence of antibiotics), the sequence of occurrence of the ultrastructural changes was independent of the method used to induce the conversion (not shown). At the onset of the experiments, the cultures consisted mainly of dividing, rod-shaped bacteria typical of replicating *H. pylori* (Fig. 4A). The

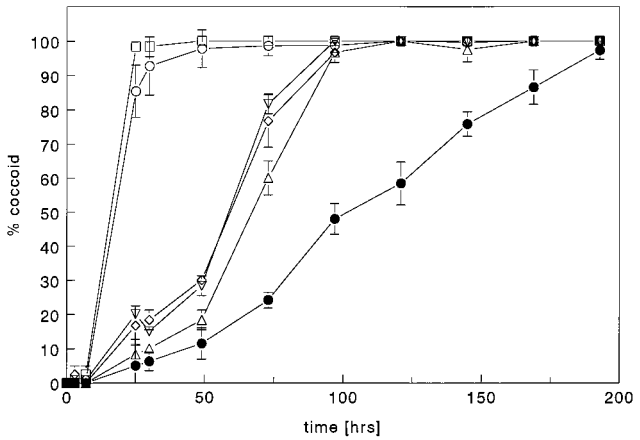

FIG. 3. Effects of antibiotics on the conversion rate of *H. pylori* ATCC 43504. The conversion rates of *H. pylori* ATCC 43504 are represented as percentages that coccoid bacteria comprise of the total number of bacteria present in the cultures (summarized from Fig. 2 for easier comparison). Cultures were incubated as described in Materials and Methods under the following conditions: microaerobiosis (aging), ●; aerobiosis, ○; presence of chloramphenicol, △; presence of tetracycline, ▽; presence of rifampin, ◇; and presence of kanamycin, □.

first ultrastructural sign of the morphological conversion was the formation of a bleb-like structure at one end of the bacterium (Fig. 4B). This bleb then increased in size and moved to the middle, thereby increasing the curvature of the bacterium (Fig. 4C). The bleb-like structure continued to grow in size, and with it the morphology of the bacterium changed from a rod, via a U-shaped form (Fig. 4D), into a fully coccoid form (Fig. 4E) where there was no longer a clear protoplasmic cylinder. Finally, the bacterium lost its membrane integrity (Fig. 4F).

We related the forms and structures as observed by electron microscopy to CFU counts, and from these calculations we determined that bacteria without blebs (Fig. 4A) were fully viable but that bacteria with structures like those shown in Fig. 4B and C were nonculturable. These forms made up the majority (if not all) of the bacillary forms observed at the first time point where no CFU could be obtained. The three forms in Fig. 4A to C were scored as bacillary forms by the light-microscopic analysis of the bacterial morphology as represented in Fig. 1 to 3. The forms shown in Fig. 4D to F were scored as coccoid forms in our light-microscopic analysis and were nonculturable forms, since no bacteria could be grown from cultures containing only these forms.

Analysis of cell compositions of bacillary and coccoid forms of *H. pylori* ATCC 43504. To determine whether transition from the bacillary to the coccoid form was accompanied by changes in bacterial cell composition, bacteria in various stages of the transition process were studied. Independently of the condition used to induce coccoid forms (aging, aerobiosis, presence of chloramphenicol, or presence of kanamycin), no major changes in the total amount of protein per cell were observed (Fig. 5). However, there were several differences in the protein-banding patterns of the bacillary and coccoid *H. pylori* profiles. Results of SDS-PAGE of total bacterial protein are shown in Fig. 5. The relative intensities of several bands (Fig. 5) changed as the bacteria changed from their bacillary morphology to their coccoid morphology. These changes were observed both when bacillary forms were transformed to coccoid forms by aging or aerobiosis and when the protein synthesis inhibitor chloramphenicol or kanamycin induced transformation (Fig. 5). This result proved that the differences between the protein profiles of bacillary and coccoid forms were not the result of de novo protein synthesis but resulted from processing or degradation of existing proteins.

Figure 6 shows that the DNA (Fig. 6A) and RNA (Fig. 6B) contents of the bacteria decreased significantly when the bacillary form changed to the coccoid form. At time zero, approximately 2.6 μg of DNA was present per 5×10^8 bacteria (Fig. 6A, lane 1). With an estimated genome size of 1.7×10^6 bp for *H. pylori* (9), this figure corresponds to approximately 2.5 genome equivalents per bacterium, indicative of an actively replicating bacterial culture (7). At day 17 the bacteria incubated microaerobically, aerobically, microaerobically with chloramphenicol, and microaerobically with kanamycin contained 0.21 μg of DNA, 0.009 μg of DNA, no detectable ($<<0.001$ μg) DNA, and no detectable ($<<0.001$ μg) DNA, respectively. This corresponds to 0.23, 0.01, $<<0.001$, and $<<0.001$ genome equivalents. There were also comparable reductions in the amounts of RNA: where there was approximately 6 μg of RNA per 5×10^8 bacteria at the onset of the experiment (Fig. 6B, lane 1), at day 17 bacteria incubated microaerobically, aerobically, microaerobically with chloramphenicol, and microaerobically with kanamycin contained only 1.8, 0.75, 0.21, and <0.01 μg of RNA per 5×10^8 bacteria, respectively.

In those preparations that contained a high percentage of coccoid forms of *H. pylori*, not only was the amount of nucleic acid per bacterium significantly decreased, but degradation of DNA and RNA was also observed (Fig. 6).

Membrane potential. The existence of a membrane potential was evaluated by using the potential-sensitive probe $diOC_5$-3 and flow cytometry. Based on fluorescence patterns, cultures that contained both coccoid and bacillary bacteria consisted of two populations (Fig. 7). One population displayed a strong staining pattern (mean fluorescence, 41.1), typical for the presence of a distinct membrane potential. The other population was not stained and showed background fluorescence only (mean fluorescence, 2.4). In aged cultures that contained only nonculturable bacteria, only the background-stained population was present. In a 2-day-old microaerobic culture of *H. pylori*, 98.6% of the cells stained positive, while in a 2-day-old aerobically incubated preparation, only 6.4% of the bacteria stained positive (Fig. 7). These results correlated perfectly with the microscopic evaluation of these cultures, which revealed >99 and 5% bacillary forms, respectively.

Conversion kinetics of other *H. pylori* strains. To exclude the possibility that strain ATCC 43504 is a variant with exceptional properties, the above-described experiments on the effects of various incubation conditions and the presence and absence of the antibiotics on the morphological conversion rate and ultrastructural changes were repeated with a second reference strain, NCTC 11638, and two clinical isolates, HP 1280 and HP 1075. Results were comparable to those obtained with strain ATCC 43504 (representative data are presented in Fig. 8).

DISCUSSION

Our data show that adverse environmental conditions induced a rapid conversion of helical forms of *H. pylori* to nonculturable coccoid forms. The question whether the coccoid form of *H. pylori* represents a survival mechanism is crucial to the understanding of the epidemiology and transmission routes of this pathogen. In nature, large numbers of replicating *H. pylori* are found only in the stomachs of humans. It is therefore probable that when the bacterium leaves this natural habitat it is exposed to physical and chemical stresses that induce con-

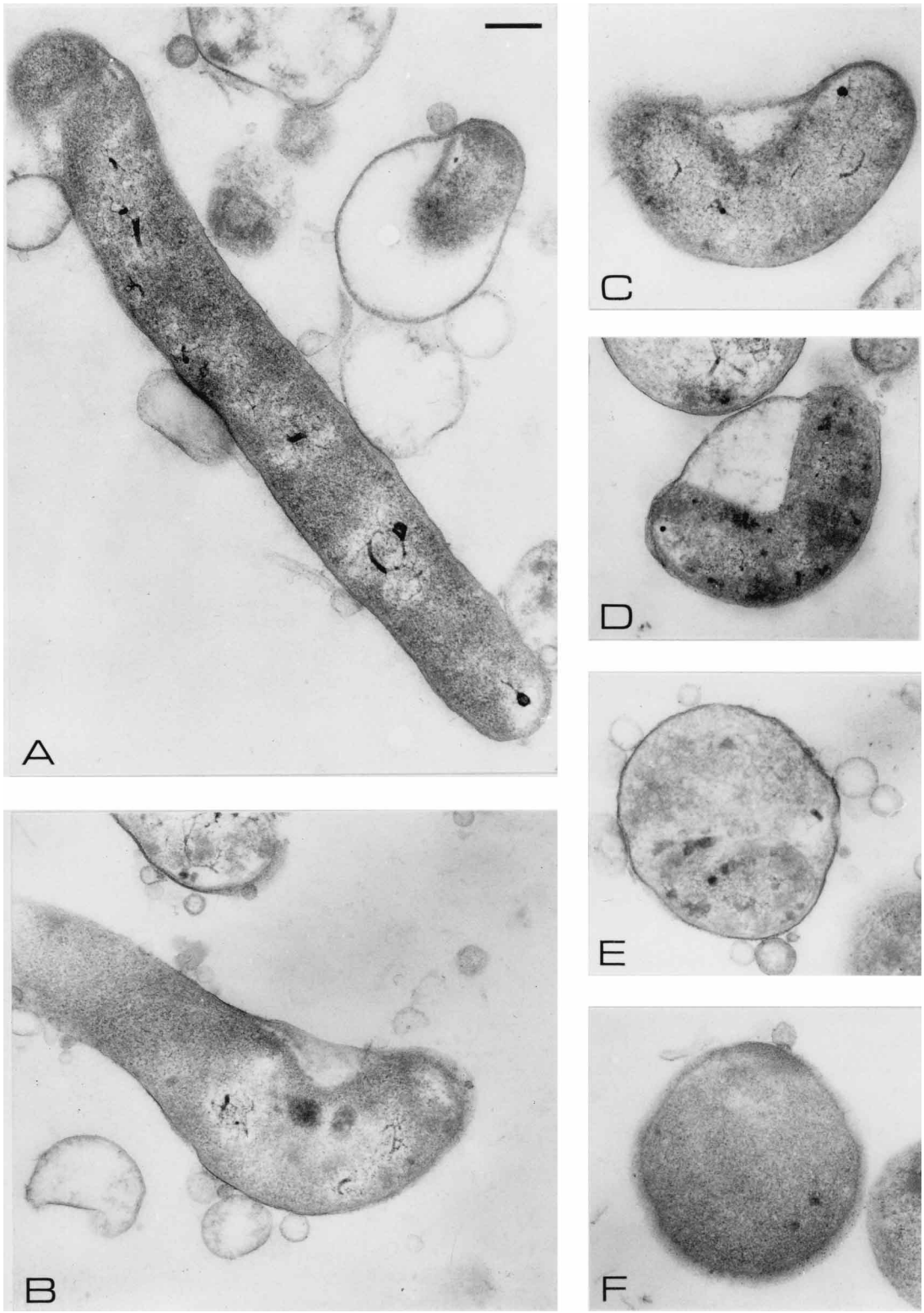

FIG. 4. Transmission electron micrographs showing the different stages in the conversion of the bacillary form of *H. pylori* to the fully coccoid form. (A) Normal bacillary form, which is the form of the majority of the bacteria present in fresh microaerobic cultures. Bar, 500 nm. (B) Initiation of the conversion occurs by the formation of a bleb at one end of the rod. (C) The bleb continues to grow and fills with electron-dense material, and the rod bends. (D) Bending of the rod results in a U-shaped bacterium inside a membrane-like structure filled with electron-dense material. (E) As more and more electron-dense material accumulates, the bacterial outline becomes difficult to discern; the fully coccoid form is achieved. (F) As the conversion proceeds, all discrete structures, including the membrane that spanned the coccoid-shaped bacterium, disappear.

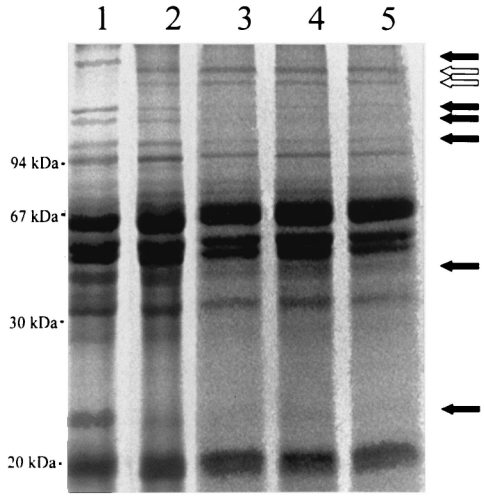

FIG. 5. Coomassie brilliant blue-stained SDS-polyacrylamide gel of whole-cell proteins of *H. pylori* ATCC 43504. Each lane contains total protein of approximately 5×10^8 bacteria. Lane 1: culture with <1% coccoid forms (incubated for 24 h in BB^+ at 37°C under microaerobic conditions with no antibiotics added); lane 2, culture with 70% coccoid forms (incubated for 5 days in BB^+ at 37°C under microaerobic conditions with no antibiotics); lane 3, culture with >99% coccoid forms (incubated for 5 days in BB^+ at 37°C under microaerobic conditions in the presence of chloramphenicol); lane 4, culture with >99% coccoid forms (incubated for 5 days in BB^+ at 37°C under aerobic conditions with no antibiotics); lane 5, culture with >99% coccoid forms (incubated for 5 days in BB^+ at 37°C under aerobic conditions in the presence of chloramphenicol). The filled arrows indicate protein bands that disappear in coccoid forms, and the open arrows indicate protein bands newly appearing in coccoid forms. Molecular masses are noted at the left.

version into nonculturable coccoid forms. If indeed the coccoid form is involved in survival under unfavorable conditions, the rapid turnover into the coccoid form implies the existence of an active switch in the metabolism of the bacterium. One might assume that, by analogy to, e.g., spore forming in *Bacillus subtilis*, this switch might involve transcription of specific sporulation genes. If so, morphologic conversion would require active transcription and translation and hence the inhibition of protein or RNA synthesis should significantly slow down or possibly even completely prevent the conversion from helical to coccoid forms. These results were not what we observed. On the contrary, inhibition of protein or RNA synthesis resulted in an even faster conversion, which implies that conversion is a passive process and that active protein and RNA synthesis is not necessary for conversion of *H. pylori* from its helical to its coccoid form.

Various authors (2, 12, 25) have reported significant differences in the SDS-PAGE protein profiles of the two different morphological forms of *H. pylori*. In a recent paper, Benaïssa and coworkers (2) showed that sera from patients with *H. pylori* infection recognize some of the proteins that appear to be different in coccoid forms. We also observed differences in the protein profiles of bacillary and coccoid forms (Fig. 5), but since these changes also occurred when protein synthesis was inhibited, they must have resulted from either protein degradation or protein processing, both of which are possible in the absence of viable DNA or RNA.

Loss of culturability always preceded the complete conversion of the bacillary forms to the coccoid forms, independently of the inducing conditions used: the CFU count was already reduced to 0 when less than half of the bacteria had assumed the coccoid shape. In other words, bacteria from these cultures were already nonculturable when at least half of them were still in the bacillary form. This result suggests that it is not the conversion into coccoids that results in loss of culturability but more likely that the loss of culturability (and hence maybe loss of viability) results in conversion to the coccoid form. These kinetics of conversion and loss of culturability are slightly different from those observed by Benaïssa and coworkers. Those authors showed that no decrease in CFU counts occurs until the fraction of coccoid forms exceeds 50% (2). A major difference, however, is that Benaïssa and coworkers maintained their cultures on agar plates while we performed our experiments on bacteria kept in suspension. Bacteria on agar plates are always less synchronous in their growth phase than bacteria maintained in suspension. This fact may account for the observed differences.

The sequence of ultrastructural changes associated with the conversion from bacillary to coccoid bacteria was not affected by the conditions used to induce the conversion. During the transformation, the outer membrane appeared to detach from the inner membrane and the increasing periplasmatic space was filled with electron-dense material from the cytoplasmic cylinder. The bacteria appeared to curve more and more until they finally became coccoid in shape. Meanwhile, the cytoplasmatic cylinder and structures therein became less distinct until finally even the outer membrane disintegrated. Our observation that the outer membrane seems to stay intact during the

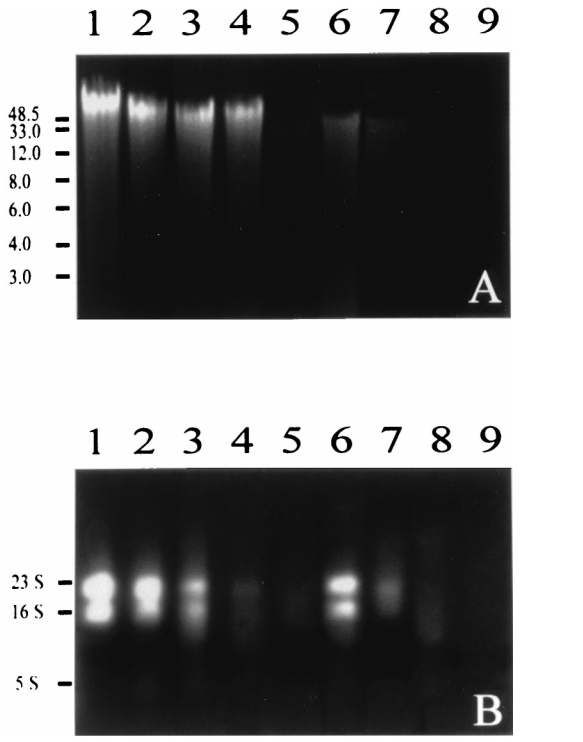

FIG. 6. Ethidium bromide-stained agarose gels demonstrating the changes in the DNA (A) and RNA (B) contents of *H. pylori* ATCC 43504 during morphologic conversion. Each lane contains the purified nucleic acids isolated from approximately 5×10^8 bacteria (the number of bacteria determined at the time of sampling). Lane 1 contains ribonucleic acids isolated from the microaerobically grown inoculum (grown in BB^+ at 37°C) at the onset of the experiment (<1% coccoid). This inoculum was split and further incubated at 37°C under various conditions: microaerobiosis (lanes 2 and 6), microaerobiosis with chloramphenicol (lanes 3 and 7), microaerobiosis with kanamycin (lanes 4 and 8), and aerobiosis (lanes 5 and 9). Samples were taken from these cultures at day 6 (lanes 2 to 5; samples contained 70, >99, >99, and >99% coccoids, respectively) and at day 17 (lanes 6 to 9; all samples contained >99% coccoids). Molecular sizes (in kilobase pairs) are noted to the left of the gel in panel A.

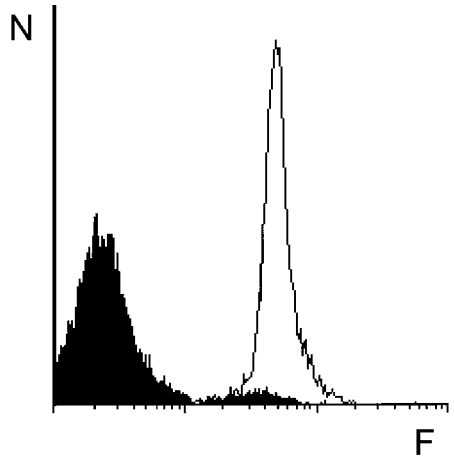

FIG. 7. Differences in membrane potential in coccoid and bacillary forms. Fluorescence histogram of $diOC_5$-3-stained *H. pylori* ATCC 43504. Green fluorescence intensity (F) is plotted on a log scale (three decades) against the number of analyzed cells (N). Representative results with a 2-day-old microaerobically incubated culture containing >95% bacillary forms and a 2-day-old aerobically incubated culture containing >95% coccoid forms are shown in white and black, respectively. From each culture, a total number of 10,000 bacteria was analyzed.

initial stages of development into a coccoid bacterium corresponds with the recent findings of Cole et al. (15), who showed that the majority of fresh coccoids were not stained by a fluorescent dye that cannot pass through intact membranes. Mai et al. suggested that two forms of coccoid *H. pylori* might exist: a viable, dormant form induced by aging and a nonviable form induced by antibiotics or nutrient deprivation (21). Our data do not support this assumption, since we find no ultrastructural differences between coccoids induced by either method.

Changes in cell morphology of bacteria resulting from starvation are well known. In *Escherichia coli*, starvation results in changes in cell morphology: the cells become smaller and almost spherical (reviewed in reference 27). Morphologically, this conversion resembles that of *H. pylori* from the helical form to the coccoid form. Starved coccoid forms of *E. coli* develop enhanced resistance to a variety of environmental assaults. These changes, however, require protein synthesis: 30 to 50 proteins are induced in response to starvation by the expression of specialized genes (27). Also, DNA remains stable in the starved cells. With the exception of undergoing similar morphologic changes, coccoid forms of *H. pylori* behave quite differently; while cultures that consisted of bacillary bacteria contained approximately 2 genome equivalents per bacterium, cultures that consisted primarily of coccoid bacteria contained almost no DNA (less than 1 genome equivalent per 1,000 bacteria). In addition, not only was hardly any DNA present in coccoids, it was also always severely degraded. Therefore, we propose that coccoid forms of *H. pylori* are the degenerate remains of dead bacteria. The loss of RNA that we observed during the conversion to coccoid *H. pylori* provides further evidence for the concept of coccoid forms being the result of cell death.

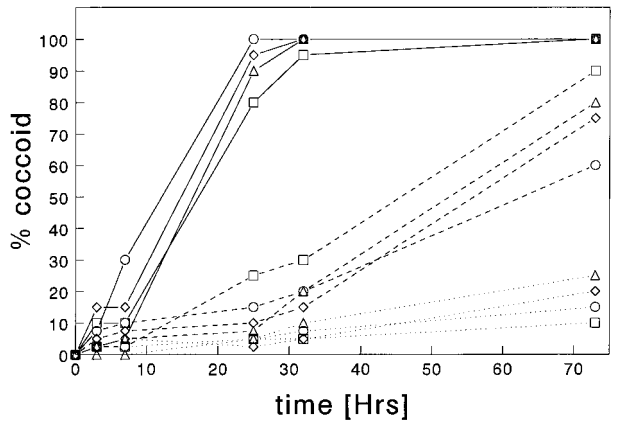

FIG. 8. Conversion rates of *H. pylori* ATCC 43502 (squares), NCTC 11638 (diamonds), HP 1280 (triangles), and HP 1075 (circles). Conversion is represented as the percentage of coccoid forms present in the culture. Only data from experiments using 37°C microaerobiosis (aging) (dotted lines), 37°C microaerobiosis with chloramphenicol (dashed lines), and 37°C aerobiosis (solid lines) are given.

Several authors have reported the presence of metabolic (enzymatic) activity in fresh coccoid cultures (5, 15, 29). Our assumption that coccoids are dead does not rule out the existence of such activity: bacterial enzymes can have extremely long half-lives, and hence enzymatic activity is no proof of viability. Evidence that coccoid forms are alive has been provided by animal inoculation studies, but these studies are contradictory. Cellini et al. showed reversion of coccoid *H. pylori* organisms in mice to a viable state (13), with histopathological changes in the gastric mucosa and a systemic antibody response. In contrast, Eaton and coworkers were not able to induce gastritis in gnotobiotic piglets inoculated with coccoid forms even though gastritis occurred after inoculation with bacillary forms (16). Cellini et al. used cultures that had aged for 20 days and assessed the number of bacteria by optical density. It is difficult to be certain that all bacteria converted to the coccoid form. In our experiments, cultures appeared to be 100% converted after 19 days, but the process of conversion was asymptotic. A few helical forms may therefore have still been present. Also, when optical density is used to adjust the number of bacteria, one must bear in mind that the optical density of coccoid forms is lower than that of bacillary forms, and hence the number of coccoid forms inoculated, compared to the number of bacillary forms, is substantially higher. Contamination of coccoid cultures by bacillary forms may then be important.

Additional evidence for our assumption that coccoid forms represent the morphological manifestation of cell death was provided by the absence of a membrane potential in these bacterial forms. The potential-sensitive probe $diOC_5$-3, which inserts itself in the cell membrane, will activate and fluoresce only if a membrane potential is present. A strong fluorescent signal was obtained only in cultures that consisted mainly of bacillary forms. In contrast, cultures that consisted of mainly coccoid forms showed no fluorescence.

In conclusion, we provide several lines of evidence indicating that the conversion of bacillary forms of *H. pylori* to coccoid forms is a passive process that results from cell death. In our opinion, therefore, coccoid forms of *H. pylori* are the degenerate remains of dead bacteria.

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