

The Action of Bismuth against *Helicobacter pylori* Mimics but Is Not Caused by Intracellular Iron Deprivation

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***Helicobacter pylori* is highly susceptible to bismuth, a heavy metal with antimicrobial activity linked to its effect on bacterial iron uptake. Three strains of *H. pylori* were analyzed for indicators of iron limitation following exposure to the MIC of colloidal bismuth subcitrate (MIC_{CBS}). Similar morphologic and outer membrane changes were observed following growth in iron-limiting medium and at the MIC_{CBS} that inhibited the growth of all three strains. These changes, which were also observed for iron-limited bacteria, were alleviated by the addition of iron to the cultures. *H. pylori* ATP levels, reduced in iron-limiting medium, were below the limits of detection in two of the three strains following exposure to bismuth. The addition of iron partially restored bacterial ATP levels in these two strains, although not to normal concentrations. In contrast, exposure of the same strains to the MIC_{CBS} failed to deplete intracellular levels of iron, which were significantly reduced by culturing in iron-limiting medium. Thus, the antimicrobial effect of bismuth and of iron limitation on *H. pylori* may be similar. However, the respective mechanisms of intracellular action would appear to be mediated by different pathways within the cell.**

Prior to the discovery of the link between *Helicobacter pylori* and peptic ulcer disease (10), ulcers were thought to result from abnormal levels of gastric acid secretion. Bismuth compounds, commonly prescribed for ulcers, were initially thought to act as a barrier to the digestive effects of stomach acid by coating the ulcer site (31). Instead, it appears that *H. pylori* is highly susceptible to bismuth compounds (39), and treatment with colloidal bismuth subcitrate (CBS) is associated with a reduction in bacterial numbers and a concurrent reduction in gastritis in vivo (37). Despite these findings, bismuth monotherapy often fails to completely eradicate these bacteria (13, 43), an observation that correlates with increasing ulcer relapse rates over time (34).

The oldest regimen, an extremely effective one for clearing *H. pylori* infection, uses bismuth in combination with certain antibiotics (2, 51). Bismuth “triple therapy” consists of a bismuth compound (usually CBS or bismuth subsalicylate) in combination with metronidazole and tetracycline or amoxicillin (16) and reportedly cures 87.9% of patients within 1 week of treatment and 89.2% of patients within 2 weeks of treatment (49). *H. pylori* strains resistant to bismuth have not been reported and presumably arise at a lower frequency than strains resistant to antimicrobial agents such as nitroimidazoles, macrolides, and tetracycline (40). Bismuth compounds may reduce the development of resistance to coadministered antibiotics (27) and are also effective at treating *H. pylori* strains with established resistance to other antibiotics (3, 40).

How bismuth is toxic to *H. pylori* is not known. A number of studies have linked the antimicrobial activities of many heavy metals, including bismuth, to their effects on iron uptake by

bacteria (1, 21, 23, 24). Iron is required for growth because it is a cofactor for many essential enzymatic processes (42), and many of the observed effects of bismuth on bacteria could be the result of iron limitation. These effects include a reduction in intracellular ATP levels (44), inhibition of protein and cell wall synthesis and of membrane function (32), and a reduction in capsular polysaccharide production (19, 20, 22, 28).

A reduction in outer membrane lipopolysaccharide (LPS) expression occurs when *H. pylori* is deprived of iron (J. Keenan, unpublished data), whereas several iron-repressible outer membrane proteins are concomitantly up-regulated (18, 29, 52). These observations led us to hypothesize that the antimicrobial action of bismuth could be due to it competitively inhibiting iron uptake. To test this hypothesis, we searched for indicators of iron limitation in bismuth-exposed *H. pylori* and investigated whether iron could protect these bacteria from the antimicrobial effect of bismuth.

MATERIALS AND METHODS

Bacterial strains and cultures. Three well-characterized *H. pylori* strains were used in this study: *H. pylori* 60190 (ATCC 49503), a *cag* pathogenicity island (PAI)-positive toxigenic strain (5, 14); *cag* PAI-negative *H. pylori* strain Tx-30a (ATCC 51932) (14), which fails to produce detectable cytotoxin activity in vitro (35); and mouse-adapted *H. pylori* Sydney strain 1 (SS1) (33). Individual strains were grown in the base medium, brucella broth (BB; Difco, Detroit, Mich.) supplemented with 5% fetal bovine serum (FBS; Gibco BRL, Auckland, New Zealand), for 72 h at 37°C under microaerophilic conditions and with constant rotation (120 rpm).

Iron limitation and bismuth inhibition were achieved by growing the strains in BB–5% FBS with the addition of an iron chelator (deferoxamine mesylate; Sigma, St. Louis, Mo.) at a final concentration of 50 μM or CBS at the MIC (MIC_{CBS}) determined for each strain (see below). Iron salts (ferrous ammonium sulfate; Sigma) at final concentrations of 50 to 1,000 μM were added to the base medium containing MIC_{CBS} to determine the amount of iron required to alleviate the effects of bismuth. The deferoxamine, CBS, and iron salts solutions were prepared immediately before use. Deferoxamine and iron salts solutions were filter sterilized prior to addition to the medium.

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Morphologic changes. The morphologic changes in the bacteria were assessed by transmission electron microscopy (TEM) over the 72-h period. Washed bacteria were placed on carbon-colloidal-coated mesh grids and negatively stained with 1% aqueous phosphotungstic acid (pH 7.0). Photographs were taken by using a CM12 transmission electron microscope (Philips).

Determining MIC_{CBS} values for individual *H. pylori* strains. MIC_{CBS} values were determined by using 12-well tissue culture plates inoculated with CBS stock solution to a final volume of 2 ml/well. CBS, prepared from bismuth citrate powder and ammonia (26), was combined with 2×10^7 *H. pylori* organisms per ml at final concentrations of 0, 1, 2, 4, 8, 16, 32, and 64 mg/liter in the base medium, and the mixture was incubated for 72 h in a microaerophilic environment with constant rotation. *H. pylori* growth assessed by measuring the optical density at 650 nm in each well (corrected by using uninoculated controls) was confirmed as the growth of gram-negative, urease-positive colonies on blood agar plates. Each strain was tested six times per experiment, performed on three separate occasions, and controls containing ammonia without bismuth were included to ensure that any inhibitory effects were due to bismuth alone.

OMV. Bacteria were removed from 72-h broth cultures by centrifugation ($10,000 \times g$, 15 min, 4°C), and the supernatants were ultracentrifuged ($100,000 \times g$, 2 h, 4°C) to recover outer membrane vesicles (OMV) as described previously (30). The OMV pellet was washed twice with phosphate-buffered saline (PBS) and assayed for protein content (36). OMV components separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis under reducing conditions were silver stained to visualize protein and LPS profiles (30). OMV-associated protease activity was detected by zymography (29). Briefly, OMV were electrophoresed under nonreducing conditions through an acrylamide gel containing a copolymerized substrate (gelatin). After extensive washing to remove sodium dodecyl sulfate, the gel was incubated in 50 mM Tris buffer (pH 7.4) with 10 mM calcium chloride for 4 h at 37°C. OMV-associated proteolytic activity was visualized as clear bands (indicative of substrate lysis) against the blue background of the gels following Coomassie blue staining.

Collection of samples for analysis of ATP and intracellular iron levels. *H. pylori* organisms were grown in BB-5% FBS for 24 h before being adjusted to the conditions described above for the remaining 48 h of culturing. At 72 h, a 1-ml aliquot was removed to determine the effects of the various culture conditions on bacterial numbers by viable colony counting. A second aliquot was snap-frozen (in liquid nitrogen) and stored at -80°C prior to analysis of ATP levels. The remaining bacteria were harvested ($10,000 \times g$, 20 min) to determine intracellular iron levels. ATP and iron levels were determined in relation to bacterial dry weight.

Viable colony counting. After serial dilution in PBS, bacterial titers were inferred from CFU per milliliter, determined by spreading bacterial suspensions over the surface of blood agar plates containing 5% defibrinated sheep blood. The agar plates were incubated for 5 days at 37°C under microaerophilic conditions (as described above).

Analysis of ATP levels. ATP levels were measured by using a luminescent ATP detection assay kit (ATPLite-Packard Bioscience) according to manufacturer directions. Briefly, frozen samples were quickly defrosted, and 100- μ l portions of the samples were added to the wells of 96-well tissue culture plates in duplicate. Following the addition of 50 μ l of lysis solution, the plates were shaken for 5 min (700 rpm), and then 50 μ l of substrate buffer solution was added. The plates were shaken for a further 5 min and dark adapted for 10 min before luminescence was measured with a luminometer (BMG Labtechnologies). ATP standards, prepared for each of the four conditions used to culture *H. pylori* and ranging from 10^{-5} M to the blank, were used to generate a standard curve (Graphpad-Instat) from which unknown sample concentrations were calculated.

Analysis of iron levels. PBS-washed bacterial pellets were oven dried at 80°C for approximately 18 h in 1.5-ml Eppendorf tubes and then transferred to 10-ml acid-washed flasks for the determination of dry weights. Each sample was dissolved in 1 ml of concentrated nitric acid at 55 to 65°C overnight. Dissolved samples were cooled to an ambient temperature and then brought to 10 ml with Milli-Q distilled water. Samples were examined on a Spectra-10 (Varian) atomic absorption spectrometer, and iron concentrations were determined from a standard curve that ranged from 0 to 5,000 μ g/liter (25).

Statistical analyses. The effects of each of the growth conditions on bacterial iron and ATP levels were evaluated by using a factorial analysis of variance. Significant effects indicated by this analysis were further explored by using Fisher's least-significant-difference test. The ATP measurements were natural logarithm transformed prior to analysis, so that very small values could be easily analyzed.

RESULTS

Bismuth or iron limitation induces a change in *H. pylori* morphology and outer membrane composition. The MIC_{CBS} values were found to be 4 mg/liter (10 μ M) for strains 60190 and SS1 and 8 mg/liter (20 μ M) for Tx-30a. The addition of ammonia alone (see Materials and Methods) did not inhibit the growth of any of the strains. Thus, the inhibitory effects of CBS were due to bismuth alone (results not shown).

Each of the three *H. pylori* strains was examined by TEM following 72 h of growth in base medium (control), iron-limiting medium (containing 50 μ M deferoxamine), or medium containing MIC_{CBS} (Fig. 1). Both iron limitation and exposure to MIC_{CBS} resulted in the transition of *H. pylori* from helical to coccoid morphology (Fig. 1A); a larger proportion of MIC_{CBS}-exposed bacteria had completed this transition by 72 h. Exposure to MIC_{CBS} resulted in some more distorted forms of *H. pylori* (Fig. 1B). In contrast, bacteria grown under normal culture conditions exhibited normal curved-rod morphology.

There was little observable difference in the outer membrane proteins of individual *H. pylori* strains following iron limitation or exposure to bismuth (Fig. 2). Specific markers for iron stress were difficult to identify, but a 12-kDa protein band was clearly enhanced in the presence of MIC_{CBS} or 50 μ M deferoxamine (Fig. 2). Both iron-limited and MIC_{CBS}-exposed bacteria also had reduced levels of OMV-associated LPS (Fig. 3). In addition, zymography revealed OMV-associated protease activity in each strain following growth in the presence of MIC_{CBS} or under iron-limiting conditions (Fig. 4).

Iron protects *H. pylori* from the inhibitory effects of bismuth. The addition of 250 μ M iron protected strains 60190 and SS1 from the inhibitory effects of 10 μ M CBS. *H. pylori* Tx-30a, which required twofold more bismuth for growth inhibition (20 μ M), also required more iron (500 μ M) to achieve any protective effect. However, this amount of iron only reduced the bactericidal effect of bismuth on Tx-30a, as indicated by growth after culturing on blood agar. It did not completely reverse growth inhibition, as demonstrated by a slight increase in absorbance readings after 72 h of culturing. *H. pylori* grown in medium containing MIC_{CBS} and protective iron maintained the normal curved-rod morphology typical of these bacteria (Fig. 1D). Furthermore, this treatment of *H. pylori* 60190 (results not shown) and SS1 (Fig. 2) resulted in outer membrane protein profiles similar to those of bacteria grown in base medium. The provision of protective iron to *H. pylori* Tx-30a apparently failed to decrease the expression of the 12-kDa protein band (Fig. 2B). We speculate that this result may have been due to the visible overloading of the corresponding lane. However, it is possible that this protein is still expressed at a higher level in this strain under these conditions. Protease activity was no longer detectable in bacteria of all three strains grown in medium containing MIC_{CBS} and protective iron (Fig. 4).

ATP but not iron levels are reduced following exposure of *H. pylori* to bismuth. Intracellular iron levels of *H. pylori* exposed to MIC_{CBS} were compared with those of bacteria cultured under normal and iron-limiting conditions to assess whether bismuth inhibited iron uptake. To achieve the biomass required for this analysis, cultures were grown overnight before being exposed to bismuth or limiting iron for 48 h. Colony counts confirmed that the established MIC_{CBS} values were

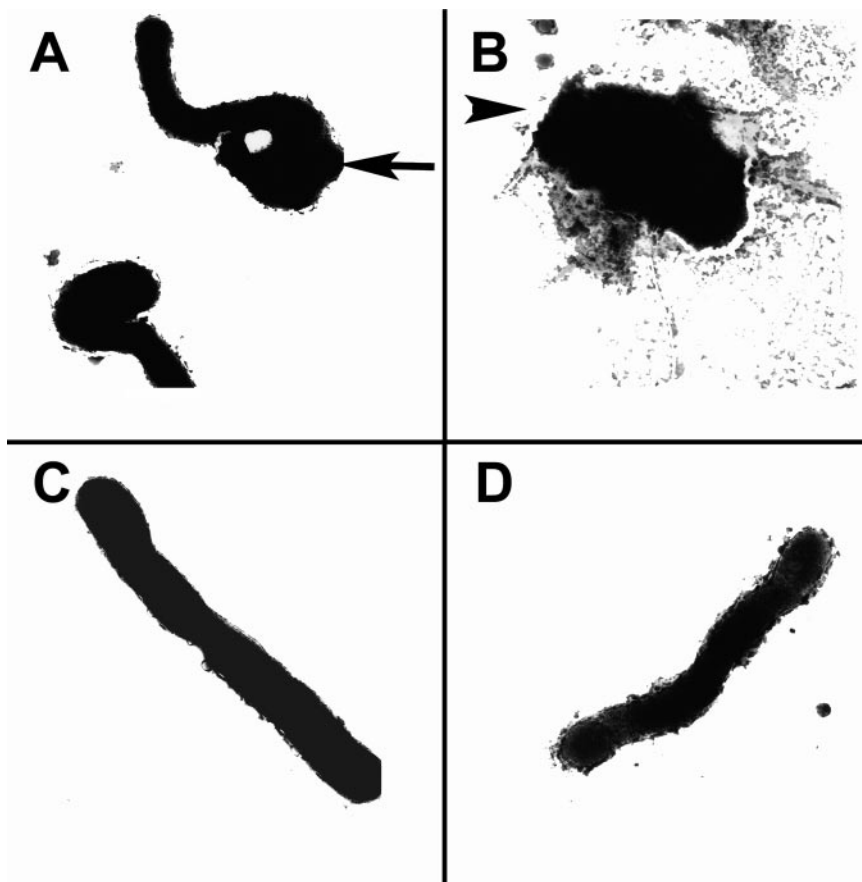


FIG. 1. Effects of bismuth exposure and iron limitation on *H. pylori* morphology. TEM images (magnification, $\times 28,900$) show strain 60190 following growth in 50 μM deferoxamine (A), MIC_{CBS} (B), MIC_{CBS} and protective iron (C), and normal culture conditions (D). The arrow in panel A indicates coccoid morphology; the arrowhead in panel B indicates distorted morphology. Similar results were obtained for strains Tx-30a and SS1 (data not shown).

sufficient to prevent growth even at these much higher concentrations of bacteria, and inhibition was still overcome by the addition of iron at protective concentrations (results not shown). PBS used to wash the bacterial pellet was assayed for iron by atomic absorption spectrometry. After three washes, the amount of iron present in the PBS supernatant was below the detectable limits of the assay, indicating this number of washes to be sufficient for removing excess unbound iron (results not shown).

H. pylori strains 60190 and SS1 grown in the presence of MIC_{CBS} had intracellular levels of iron similar to those of bacteria cultured under normal conditions, as measured by atomic absorption spectrometry. In contrast, iron levels were significantly reduced following culturing in 50 μM deferoxamine (Fig. 5). However, MIC_{CBS} had an effect similar to that of iron limitation on the intracellular iron levels of *H. pylori* Tx-30a. All three strains cultured in the presence of MIC_{CBS} and protective iron had levels of intracellular iron that were higher than those of controls but not significantly different from those of bacteria cultured with protective iron alone.

Bacterial ATP levels were only significantly reduced in strains SS1 and 60190 following growth in iron-limiting medium (Fig. 6). Exposure to MIC_{CBS} resulted in a reduction in ATP production to below the detectable limits of the assay for these two strains, whereas ATP levels in *H. pylori* Tx-30a were not

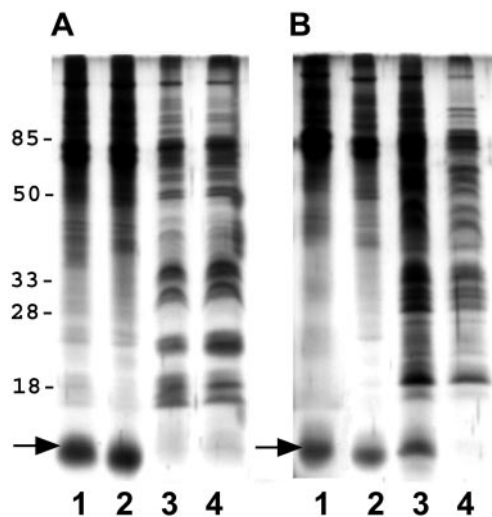


FIG. 2. Effects of bismuth exposure and iron limitation on *H. pylori* OMV protein composition. Shown is silver staining of SS1 (A) and Tx-30a (B) OMV-associated proteins following growth in 50 μM deferoxamine (lanes 1), MIC_{CBS} (lanes 2), MIC_{CBS} and protective iron (lanes 3), and normal culture conditions (lanes 4). The arrows indicate a 12-kDa protein band. Results obtained for SS1 were also representative of the 60190 OMV protein composition (data not shown).

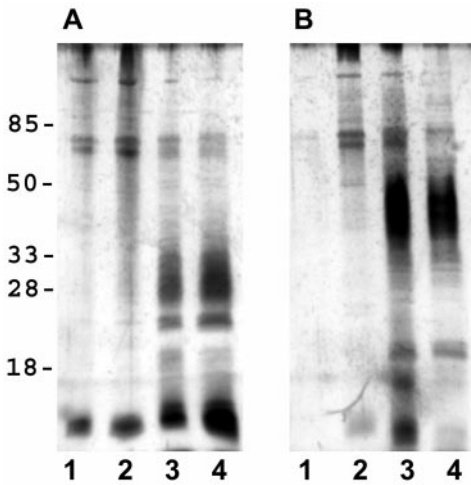


FIG. 3. Effects of bismuth exposure and iron limitation on *H. pylori* OMV LPS composition. Shown is silver staining of SS1 (A) and Tx-30a (B) OMV-associated LPS following growth in 50 μ M deferoxamine (lanes 1), MIC_{CBS} (lanes 2), MIC_{CBS} and protective iron (lanes 3), and normal culture conditions (lanes 4). Similar results were obtained for 60190 (data not shown).

significantly different from those in controls. The addition of iron to bacterial cultures containing MIC_{CBS} increased bacterial ATP concentrations in all three strains. However, protective iron failed to restore *H. pylori* 60190 ATP levels to those observed following culturing under normal or even iron-limiting conditions.

DISCUSSION

In this study, we have shown that exposure to MIC_{CBS} has the same effect on *H. pylori* as iron limitation during growth. *H. pylori* cultured in the presence of MIC_{CBS} underwent a morphologic conversion from the bacillary to the coccoid form that was associated with changes in outer membrane composition. These changes, which were also observed in iron-limited bac-

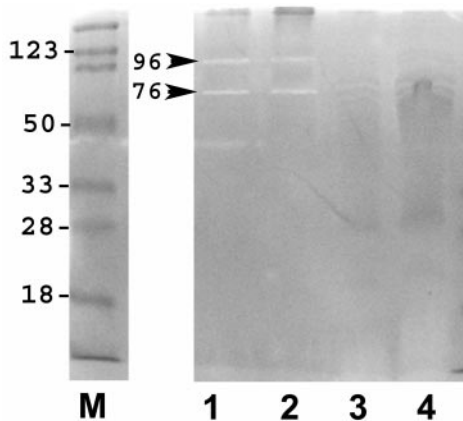


FIG. 4. Effects of bismuth exposure and iron limitation on *H. pylori* OMV protease activity. Zymography was performed on OMV harvested from SS1 following growth in 50 μ M deferoxamine (lane 1), MIC_{CBS} (lane 2), MIC_{CBS} and protective iron (lane 3), and normal culture conditions (lane 4). Protease activity is indicated (arrowheads). Similar results were obtained for 60190 and Tx-30a (data not shown). Lane M, markers.

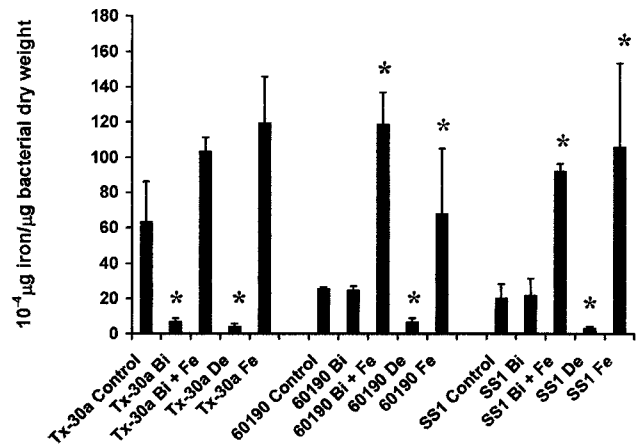


FIG. 5. Effects of bismuth exposure and iron limitation on *H. pylori* intracellular iron levels. Iron levels per microgram of bacterial dry weight were determined for *H. pylori* 60190, SS1, and Tx-30a cultured in the presence of normal growth conditions (control), iron-limiting conditions (De), MIC_{CBS} (Bi), MIC_{CBS} plus protective iron (Bi + Fe), and protective iron alone (Fe). Data are the means and standard errors of two independent experiments performed in duplicate. An asterisk indicates that the result is statistically significantly different ($P < 0.05$) from that for control (untreated) bacteria.

teria, were prevented by supplementation of the medium with iron. However, our hypothesis that the antimicrobial action of bismuth was due to the inhibition of iron uptake was challenged by unchanged intracellular iron levels in two of the three *H. pylori* strains following culturing in the presence of this bismuth compound.

Bismuth-induced changes in *H. pylori* morphology have been seen before (11, 45, 47), but our observations of accompanying changes in outer membrane protein and LPS profiles are new.

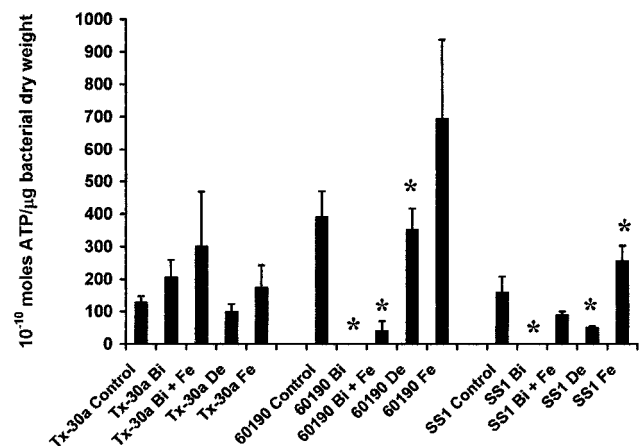


FIG. 6. Effects of bismuth exposure and iron limitation on *H. pylori* intracellular ATP levels. ATP levels per microgram of bacterial dry weight were determined for *H. pylori* 60190, SS1, and Tx-30a following growth in the presence of normal growth conditions (control), iron-limiting conditions (De), MIC_{CBS} (Bi), MIC_{CBS} plus protective iron (Bi + Fe), and protective iron alone (Fe). Data are the means and standard errors of two independent experiments performed in duplicate. An asterisk indicates that the result is statistically significantly different ($P < 0.05$) from that for control (untreated) bacteria.

The bismuth-induced changes included the up-regulation of a 12-kDa protein and two proteases and diminished LPS expression and are identical to those observed in other studies in which *H. pylori* was grown under iron-limiting conditions (9, 29). Furthermore, the addition of iron during growth mostly prevented these bismuth-induced changes. One possible exception was the continued expression of a 12-kDa protein by *H. pylori* Tx-30a grown in the presence of MIC_{CBS} and protective iron, even though TEM revealed helical forms of bacteria under these conditions. This observation may be linked to the incomplete reversal of bismuth-induced growth inhibition in the presence of protective iron. However, the apparent overloading of the corresponding lane may also be a contributing factor.

The change from helical to coccoid morphology was more rapid in the bismuth-exposed bacteria and was associated with a significant decrease in viability. ATP levels were below the limits of detection in *H. pylori* strains 60190 and SS1 following exposure to bismuth, whereas strain Tx-30a had ATP levels not significantly different from those seen under normal culture conditions, despite a rapid conversion to the coccoid form that was likewise associated with decreased viability. Strain Tx-30a was also associated, again in contrast to the other two strains, with a significant reduction in intracellular iron levels following exposure to MIC_{CBS}. Both of these results would be consistent with a loss of membrane integrity (44), leading to an accumulation of nonutilizable ATP in the extracellular medium and a concurrent loss of iron from the cell. We speculate that this effect may reflect the higher bismuth concentration needed to inhibit this strain and may also be linked to the incomplete reversal of bismuth-induced growth inhibition in the presence of protective iron.

H. pylori 60190 and SS1 cultured with MIC_{CBS} and protective iron had ATP levels higher than those found in the presence of bismuth alone but significantly lower than those found under normal culture conditions. The use of protective iron and MIC_{CBS} was also associated with an increase in intracellular iron levels and partial protection from bismuth-induced growth inhibition. These observations, which would suggest that bismuth and iron were competing for uptake via the same pathway in these bacteria, were further supported by our observation that iron limitation had a similar (but less severe) effect on *H. pylori* ATP levels and viability. Unexpectedly, however, intracellular iron levels remained constant in these two strains following exposure to bismuth, whereas iron limitation over the same growth period resulted in the depletion of iron. These results would suggest that the mechanism for the antimicrobial effect of bismuth on these and other bacteria is not simply iron starvation, as suggested previously (21). Instead, the effect of bismuth only mimics the effect of iron starvation in *H. pylori*.

ATP synthesis in bacteria occurs at the cytoplasmic membrane through the action of multisubunit enzymes (F₁F₀-ATPases) that utilize the electrochemical gradient generated by respiration (48). In *H. pylori*, inhibition of these enzymes is thought to lead to ATP depletion and the associated inhibition of other pathways that are important for *H. pylori* survival (38). Other potential targets for the inhibition of ATP synthesis include the iron-sulfur clusters and cysteine-containing heme groups that act as electron carriers in the respiratory chain. The observation that bismuth complexes localize in the periplasmic space (between the cytoplasmic and outer mem-

branes) in *H. pylori* (4, 32) would be consistent with a mechanism of action of bismuth that may involve the inhibition of ATP synthesis via one or more of these pathways.

The accumulation of bismuth at the cytoplasmic membrane might have prevented iron incorporation into iron-utilizing proteins, thereby creating a functional iron limitation within the respiratory chain. Thus, the bismuth-induced production of iron-repressible outer membrane proteins could be a response to perceived iron stress, despite no change in intracellular iron levels, an effect similar to that caused by other heavy metals through competition for incorporation into iron binding proteins (8). However, bismuth is also able to inhibit the electron transport chain through binding to the sulfhydryl groups within the enzyme complex, and this mechanism might instead account for the rapid reduction in intracellular ATP levels (6, 7). Interestingly, McGowan et al. showed that F₁F₀-ATPase activity is required for the survival of *H. pylori* when the external pH is nearly neutral (38). This finding leads us to speculate that the increased efficacy of quadruple therapy, in which bismuth and a proton pump inhibitor are given in combination with two antibiotics (17), is likely to be linked to this F₁F₀-ATPase-dependent survival at a neutral pH.

Despite these subtle differences in the mechanisms for shutting down ATP synthesis, the effects are likely to be the same. Moreover, the structural and compositional changes that we observed in the *H. pylori* outer membrane are likely to have direct consequences on the survival of these bacteria in the gastric mucosa. Reduced LPS synthesis could affect the stability of the bacterial glycocalyx (46) as well as increase the exposure of surface antigens, thereby rendering *H. pylori* more susceptible to host defenses and/or hydrophilic antimicrobial agents (50).

Interestingly, considerable strain-dependent variations in the ability to preserve ATP levels were observed following culturing of the *H. pylori* strains in the presence of MIC_{CBS} and protective iron. *H. pylori* 60190 ATP levels were considerably lower than those observed under both normal and iron-limiting culture conditions, and one explanation might be an increased requirement for energy by this strain. *H. pylori* 60190 carries the complete *cag* PAI, a locus of about 37 kb containing up to 31 genes (12). Several of these genes code for a type IV secretion system (41) that includes an ATP-regulated inner membrane pore (53). Supporting this hypothesis is the observation of higher levels of ATP in the other two strains grown under the same conditions. These strains do not possess the *cag* PAI (Tx-30a) or carry only a partial *cag* PAI (SS1) (14, 15).

In summary, our results suggest that whereas the antimicrobial effects of bismuth and iron deprivation on *H. pylori* may be similar, their respective mechanisms of intracellular action would appear to be mediated by different pathways within the cell.

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