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Bactericidal Activity of a New Antiulcer Agent, Ecabet Sodium, against *Helicobacter pylori* under Acidic Conditions

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***Helicobacter pylori* NCTC 11637, which is nonviable at pH 3.0, became viable after addition of 10 mM urea owing to ammonia production by urease. In a buffer supplemented with urea, ecabet sodium decreased both the production of ammonia and the number of viable cells of *H. pylori* NCTC 11637 and changed the bacteria from the bacilliform to the horseshoe or doughnut shape in a concentration-dependent manner. In particular, ecabet sodium (2 and 4 mg/ml) decreased the number of viable cells below the control level. Benzohydroxamic acid, a urease inhibitor, also caused a decrease in ammonia production accompanied by a decrease in the number of viable cells and changed the morphological form at pH 3.0, but the number of viable cells was not lowered below the control level. In buffers at various pHs without urea, ecabet sodium showed a concentration-dependent bactericidal effect on *H. pylori* at pHs 4.0 and 5.0 but not at pHs 6.0 and 7.0 while benzohydroxamic acid caused only a slight decrease in the number of viable cells at pH 4.0. These results suggest that ecabet sodium has strong bactericidal activity in addition to its urease-inhibiting activity under acidic conditions.**

Helicobacter pylori, first isolated from the gastric mucosa of gastritic patients (32), has been associated with various upper gastrointestinal tract disorders, such as chronic gastritis, peptic ulceration, duodenal ulceration, and gastric cancer (7, 8, 27, 28, 34). It has been reported that urease, protease, phospholipase A2, and vacuolating toxin are virulence factors of *H. pylori* (3). In particular, ammonia produced from urea by the action of urease enables *H. pylori* to survive in gastric acid by neutralization of hydrogen ions around the organisms (20) and injures gastric mucosal epithelial cells (3, 20, 29). It has been shown that ammonia may affect the mucosal charge gradient, paracellular permeability, and epithelial cell Na^+/K^+ ATPase, leading to back diffusion of hydrogen ions and resulting in gastric ulcer formation (10).

Various antibiotics have antimicrobial activity against *H. pylori* in vitro (33). However, clinical trials with such an agent alone have failed to eradicate *H. pylori* (9). This discrepancy between in vitro antimicrobial activity and clinical efficacy may be due to the instability of antibiotics in gastric acid, insufficient drug concentration in the gastric mucosa, and acquired resistance of *H. pylori* to antibiotics (9, 21). Although therapies with antibiotics in combination with antiprotozoal agents or antiulcer agents such as proton pump inhibitors are generally more effective than monotherapies with antibiotics alone (5, 6, 26), they do not always eradicate *H. pylori* (2, 30).

Ecabet sodium [(+)-(1R,4aS,10aR)-1,2,3,4,4a,9,10,10a-octahydro-1,4a-dimethyl-7-(1-methylethyl)-6-sulfo-1-phenanthrenecarboxylic acid 6-sodium salt pentahydrate; Fig. 1), a new locally acting antiulcer agent, has been shown to prevent the development of various experimental ulcers and accelerate healing of chronic gastric ulcers in rats (13, 14, 23–25). The mechanism of the antiulcer effect of ecabet sodium includes antipepsin activity (13, 14) and enhancement of gastroduodenal mucosal defensive factors (24, 25). Ecabet sodium is distributed mainly in the gastrointestinal tract, and the intestinal absorption rate is estimated to be only 3.4 to 7.0% of the dose administered in rats (11). Furthermore, the high affinity of ecabet sodium for the stomach wall (12) seems to contribute to its antiulcer effect. In regard to *H. pylori*, we have found that ecabet sodium inhibits the urease activity of a crude enzyme preparation from *H. pylori* in a time- and concentration-dependent manner at acidic pH (15). Therefore, we presumed that ecabet sodium might decrease the viability of *H. pylori* cells surviving on the surface of gastric mucosa or in gastric acid and that the drug might have potential as a new locally acting therapeutic regimen against *H. pylori* infection.

In the present study, we investigated effects of ecabet sodium on the viability, ammonia production, and morphology of *H. pylori* under acidic conditions in vitro.

MATERIALS AND METHODS

Antiulcer agents and reagents. Ecabet sodium (ecabet) (31), plaunotol, sofalcon, omeprazole, and lansoprazole were synthesized in the Organic Chemistry Research Laboratory of Tanabe Seiyaku Co., Ltd. Benzohydroxamic acid (BHA), a urease inhibitor, and bismuth citrate were purchased from Sigma Chemical Co. (St. Louis, Mo.) and Nacalai Tesque (Kyoto, Japan), respectively.

Bacterial strain. *H. pylori* NCTC 11637 was kindly provided by Takeshi Itoh (Tokyo Metropolitan Research Laboratory of Public Health, Tokyo, Japan). Twenty-five clinical isolates of *H. pylori* were kindly provided by T. Nishino (Kyoto Pharmaceutical University, Kyoto, Japan).

In vitro antibacterial activity. MICs were determined by the agar dilution method. The test strains were cultured on brain heart infusion agar (Eiken Kagaku, Tokyo, Japan) supplemented with 10% horse blood at 37°C for 3 days in GasPak jars (BBL Microbiology Systems, Cockeysville, Md.) with CampyPak Plus (BBL) and then suspended in brain heart infusion broth (Eiken Kagaku) to a concentration of about 10^6 CFU/ml. The bacterial suspensions (5 μl) were inoculated on brain heart infusion blood agar plates containing serial twofold dilutions of agents with a Microplanter (Sakuma Seisakusyo, Kanagawa, Japan). The plates were incubated at 37°C for 3 days in a microaerobic atmosphere with CampyPak Plus. MICs were determined as the lowest concentrations of the agents that visibly inhibited bacterial growth.

Measurements of bactericidal activity and urease activity at pH 3.0. The buffer solution used was 130 mM citrate buffer alone or supplemented with 10 mM urea (Nacalai Tesque) at pH 3.0. A modification of the liquid cultivation method of Morgan et al. (22) was used. *H. pylori* NCTC 11637 was inoculated in 5 ml of brain heart infusion broth supplemented with 10% heat-inactivated fetal bovine serum and incubated at 37°C for 48 h in an atmosphere of 5% O_2–10% CO_2–85% N_2 with gyration. To obtain large inocula, the culture was transferred into a 300-ml flask containing 40 ml of the same medium and incubated at 37°C for 24 h under microaerobic conditions with gyration. After rinsing with saline,

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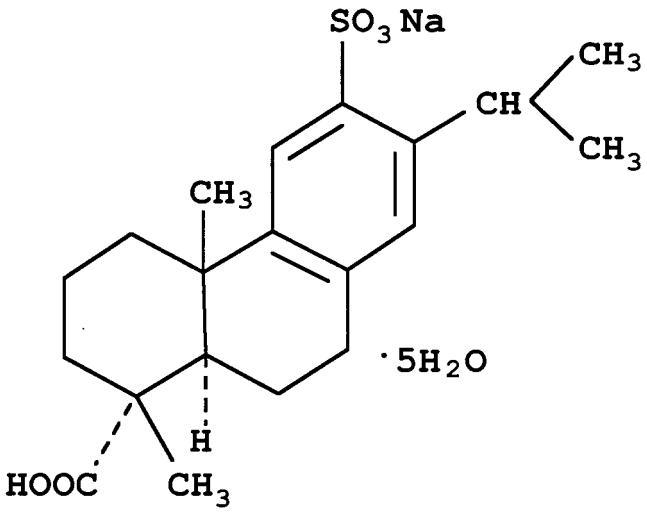

FIG. 1. Chemical structure of ecabet sodium.

bacterial cells were resuspended in saline to give about 10^9 CFU/ml. Reaction mixtures were prepared by adding 0.25 ml of an ecabet or BHA solution in water to 4.65 ml of each buffer and incubated in a water bath at 37°C. The bacterial suspension (0.1 ml) was added to the mixtures. The cultures were further incubated at 37°C with gyration. Samples for viability (0.1 ml) and urease activity (0.5 ml) measurements were taken at 0, 15, 30, and 60 min. Viability was measured by the plate colony count technique. After serial 10-fold dilution with saline, 0.1 ml of each sample was spread onto Mueller-Hinton agar (Oxoid, Basingstoke, England) supplemented with 8% horse blood. After cultivation at 37°C for 3 days in a microaerobic atmosphere with CampyPak Plus, the colonies were counted and expressed in CFU per milliliter. Urease activity was assayed by measurement of the ammonia produced in the samples. The activity was stopped by addition of 1 N sulfuric acid (0.125 ml). After addition of 10% sodium tungstate (VI) (0.125 ml), the samples were centrifuged and the ammonia in the supernatant was measured by the indophenol method (1).

Measurement of bactericidal activity at various pHs. The buffer solutions used were 130 mM citrate buffer (pHs 4.0 and 5.0), 10 mM phosphate buffer (pH 6.0), and 67 mM Sorensen phosphate buffer (pH 7.0). Ecabet or BHA in 0.25 ml of a water solution was added to 2.15 ml of each buffer, and the mixture was incubated at 37°C. The bacterial suspension of *H. pylori* NCTC 11637 used (about 10^7 CFU/ml) was prepared by the above-described method. The bacterial suspension (0.1 ml) was added to the mixture. The cultures were further incubated at 37°C with gyration. Samples (0.1 ml) were taken at 0, 15, 30, and 60 min. Viability was measured by the plate colony count technique.

Electron microscopy. *H. pylori* cells, after exposure to ecabet or BHA for 15 min at 37°C in 130 mM citrate buffer (pH 3.0) supplemented with 10 mM urea, were prefixed with 2.5% glutaraldehyde (TAAB). The prefixed cells were dropped onto an SEM plate (Ouken Syoji, Tokyo, Japan) precoated with 10% poly-L-lysine (Sigma). After rinsing of the plate with 100 mM phosphate buffer (pH 7.2), the cells were postfixed with 1% osmium tetroxide (TAAB). The fixed cells were dehydrated with a graded series of ethanol and replaced with isoamyl acetate. Critical-point drying and coating with platinum were performed in order. The cells were observed with a JEOL T-220 scanning electron microscope.

RESULTS

Antibacterial activity against *H. pylori*. MICs of ecabet and reference drugs against the 25 *H. pylori* isolates tested and strain NCTC 11637 at neutral pH are shown in Table 1. The MICs of ecabet against all of the strains were >1,600 μg/ml, indicating that ecabet has no antibacterial activity against *H. pylori* in vitro. The MICs of the reference drugs were consistent with those previously reported (17). The MICs of ecabet and BHA against *H. pylori* NCTC 11637 were >6,400 and 25 μg/ml, respectively.

Effect of ecabet on the viability and urease activity of *H. pylori* in pH 3.0 buffer supplemented with urea. The number of viable *H. pylori* NCTC 11637 cells decreased time dependently in 130 mM citrate buffer (pH 3.0). After addition of 10 mM urea, ammonia was produced and the organism survived for at least 60 min (closed circles in Fig. 2 and 3). Ecabet and BHA decreased the number of viable cells in a concentration- and time-dependent manner (Fig. 2 and 3). Ecabet and BHA also inhibited production of ammonia in a concentration- and time-dependent manner (Fig. 2 and 3). Although ammonia production plateaued within 15 and 30 min after exposure to 4 and 2 mg of ecabet per ml, respectively, the number of viable cells was lowered below the assay limit within 60 min. BHA inhibited production of ammonia over a concentration range of 0.032 to 0.5 mg/ml but inhibited bacterial survival only at 0.5 mg/ml. BHA did not decrease the number of viable cells below the control level.

TABLE 1. In vitro antimicrobial activities of ecabet sodium and reference drugs against clinical isolates of 26 *H. pylori* strains

Drug	MIC (μg/ml)[a]		
	Range	For 50% of strains	For 90% of strains
Ecabet sodium	>1,600	>1,600	>1,600
Bismuth citrate	6.25–25	12.5	25
Plaunotol	12.5–25	12.5	25
Sofalcon	>25	>25	>25
Omeprazole	25–50	25	50
Lansoprazole	3.13–6.25	3.13	6.25

[a] MICs were determined by the agar dilution method with brain heart infusion agar supplemented with 10% horse blood. Test strains were inoculated with a multipoint inoculater at 10^6 CFU per ml.

Effect of ecabet on the viability of *H. pylori* in buffers at various pHs. As shown in Fig. 4 and 5, *H. pylori* NCTC 11637 survived for 60 min in all of the buffers used at pHs 4.0 to 7.0. Ecabet showed concentration-dependent bactericidal effects at pHs 4.0 and 5.0 but not at pHs 6.0 and 7.0. Ecabet was more effective at pH 4.0 than at pH 5.0. In particular, no cells were grown at 60 min after exposure to 2 and 4 mg of ecabet per ml at pH 4.0 (Fig. 4). On the other hand, BHA (1 and 2 mg/ml) showed a slight bactericidal effect only at pH 4.0 (Fig. 5).

Effect of ecabet on morphological changes in *H. pylori*. Figure 6 shows scanning electron micrographs of *H. pylori* NCTC 11637 after exposure to ecabet for 15 min. Nonviable control

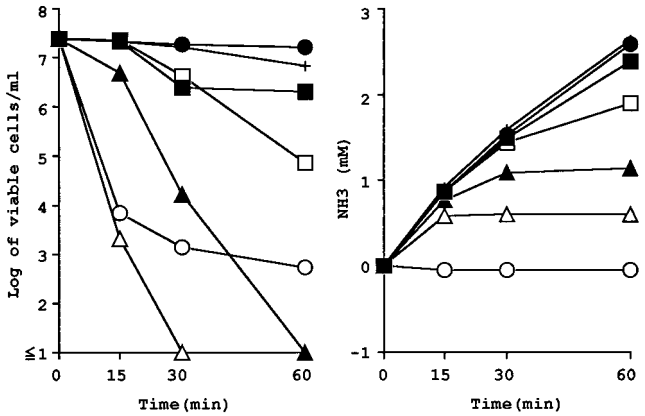

FIG. 2. Effect of ecabet sodium on the viability and urease activity of *H. pylori* in pH 3.0 buffer supplemented with urea. *H. pylori* NCTC 11637 cells were exposed to ecabet sodium at concentrations of 4 (△), 2 (▲), 1 (□), 0.5 (■), 0.25 (+), and 0 (●) mg/ml in the presence of 10 mM urea and at 0 mg/ml (○) in the absence of urea in a starting volume of 5 ml of 130 mM citrate buffer at pH 3.0 and 37°C. Samples for viability (0.1 ml) and urease activity (0.5 ml) measurements were taken from the reaction mixture at the times indicated. Viability was measured by the plate colony count technique, and urease activity was assayed as described in the text, by measurement of the ammonia produced.

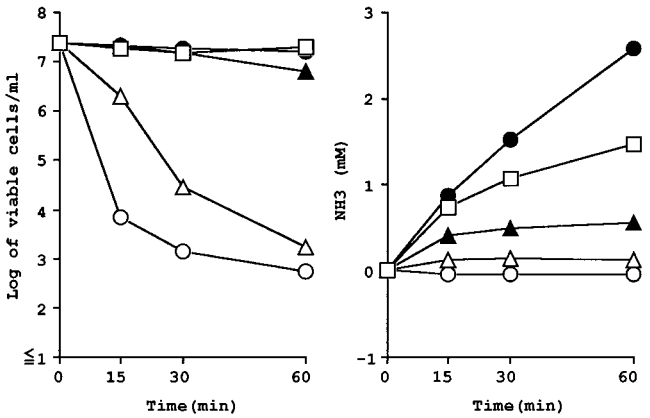

FIG. 3. Effect of BHA on the viability and urease activity of *H. pylori* in pH 3.0 buffer supplemented with urea. *H. pylori* NCTC 11637 cells were exposed to BHA at concentrations of 0.5 (△), 0.125 (▲), 0.032 (□), and 0 (●) mg/ml in the presence of 10 mM urea and at 0 mg/ml (○) in the absence of urea. See the legend to Fig. 2 for details of the measurement of viability and urease activity.

cells at pH 3.0 showed horseshoe or doughnut shapes (Fig. 6A). After addition of urea to the pH 3.0 buffer, most of the cells became viable in the bacillus form (Fig. 6B). The form of the cells was altered by ecabet in a concentration-dependent manner. After exposure to 4 (Fig. 6C) or 1 (data not shown) mg of ecabet per ml, the organisms changed from bacilliform

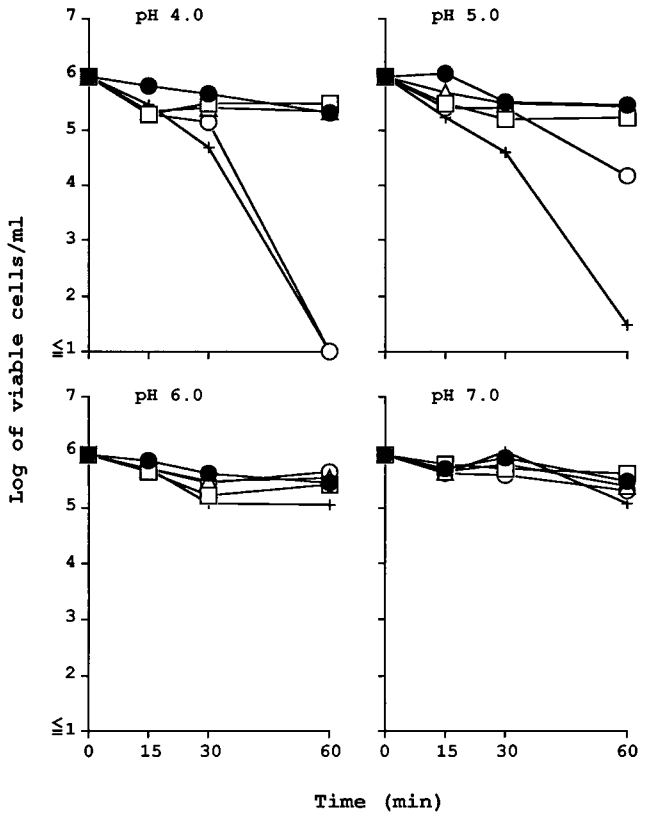

FIG. 4. Effect of ecabet sodium on the viability of *H. pylori* in buffers at various pHs. *H. pylori* NCTC 11637 cells were exposed to ecabet sodium at concentrations of 4 (+), 2 (○), 1 (△), 0.5 (□), and 0 (●) mg/ml in a starting volume of 2.5 ml at various pHs and 37°C. Samples (0.1 ml) were taken at the times indicated, and viability was measured by the plate colony count technique.

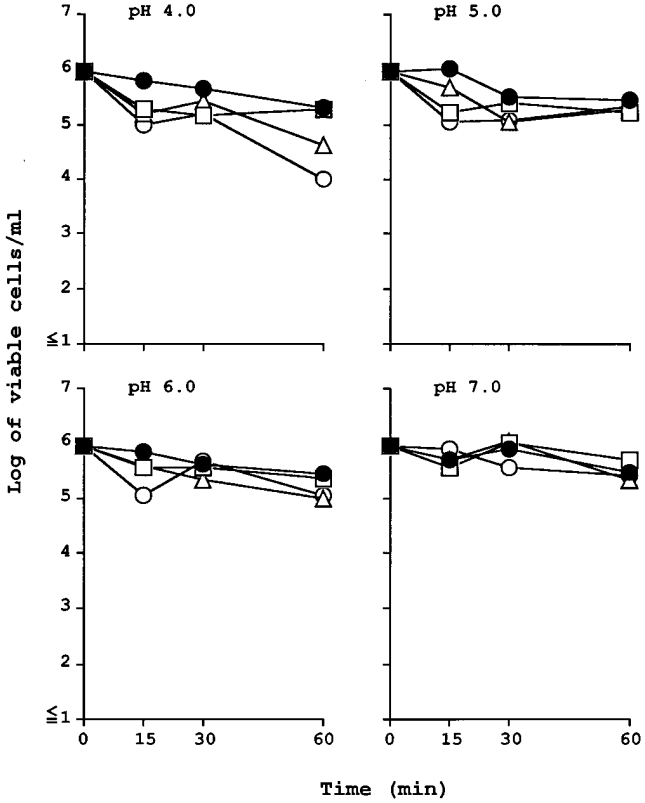

FIG. 5. Effect of BHA on the viability of *H. pylori* in buffers at various pHs. *H. pylori* NCTC 11637 cells were exposed to BHA at concentrations of 2 (○), 1 (△), 0.5 (□), and 0 (●) mg/ml. Viability was measured as described in the legend to Fig. 4.

to the horseshoe- or doughnut-shaped form. The bacterial form hardly changed at an ecabet concentration of 0.25 mg/ml (Fig. 6D). BHA also changed the form of the bacteria in a concentration-dependent manner (data not shown). Moreover, the outer envelope of the atypically shaped organisms was detached from the inner side of the bend.

DISCUSSION

The optimal pH range for growth of *H. pylori* has been reported to be 6.0 to 8.0, and the organism cannot survive at pHs below 3.0 (16, 20). However, *H. pylori* has been frequently isolated from the gastric juice of patients at pHs below 3.0 (16). Presumably, the urease of *H. pylori* generates ammonia to neutralize hydrogen ions around the cells and thus enables the organism to survive in gastric acid in the presence of urea (20).

In the present study, *H. pylori* NCTC 11637 was also unable to survive at pH 3.0 but viability was restored after addition of urea, in accordance with a previous report (20). Ecabet decreased the number of viable cells and suppressed the production of ammonia in the pH 3.0 buffer supplemented with urea in a concentration-dependent manner. This suppression of ammonia production may have resulted from inhibition of urease activity (15). In particular, ecabet (2 and 4 mg/ml) decreased the number of viable cells below the level expected from complete inhibition of urease activity (control; open circles in Fig. 2). On the other hand, BHA (0.5 mg/ml) decreased the number of viable cells only when ammonia production was almost completely inhibited. This decrease, however, did not exceed the control level (open circles in Fig. 3). Since BHA (0.125 and

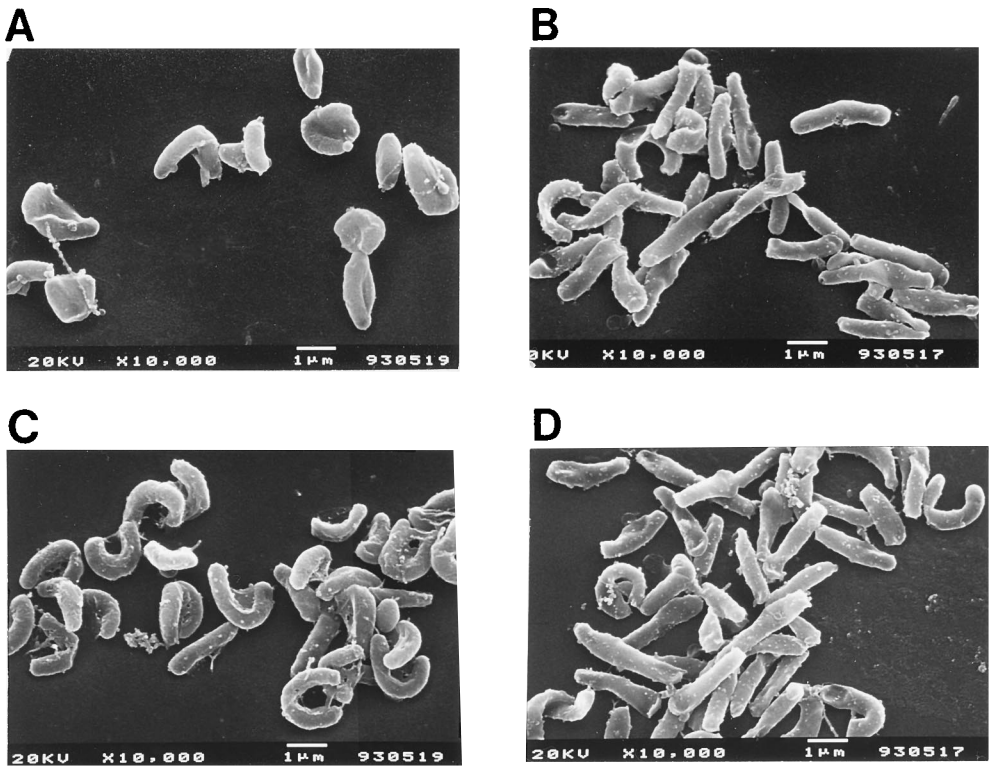

FIG. 6. Scanning electron micrographs of *H. pylori* exposed to ecabet. *H. pylori* NCTC 11637 cells were exposed for 15 min to ecabet at 0 mg/ml in the absence (A) and the presence (B) of 10 mM urea and to ecabet at 4 (C) and 0.25 (D) mg/ml in the presence of urea in 130 mM citrate buffer (pH 3.0). See the text for details of the preparation of the electron micrographs.

0.032 mg/ml) did not show a bactericidal effect despite moderate inhibition of urease activity (Fig. 3), the contribution of urease activity inhibition to the bactericidal effect may not be large.

In the morphological study, addition of 10 mM urea to pH 3.0 buffer restored the bacillus form and the organism survived. Under such conditions, in the presence of urea, addition of ecabet and BHA changed the bacterial form to a horseshoe or doughnut shape which was observable at pH 3.0 in the absence of urea. These atypical forms have been reported to appear in degenerate or nonviable cells (4, 18, 19). Thus, ecabet and BHA seem to abolish the effect of urea on the survival of *H. pylori* under acidic conditions.

The different patterns of the bactericidal effects of ecabet and BHA in pH 3.0 buffer supplemented with urea suggest that ecabet has an effect on *H. pylori* in addition to inhibition of urease activity. Therefore, we examined the effects of ecabet and BHA on the viability of *H. pylori* in buffers at various pHs without urea. *H. pylori* NCTC 11637 survived for 60 min in all of the buffers tested at pHs 4.0 to 7.0. Ecabet showed a concentration-dependent bactericidal effect at pHs 4.0 and 5.0 but not at pHs 6.0 and 7.0 (Fig. 4). In contrast, BHA produced only a slight decrease in the number of viable cells at pH 4.0 (Fig. 5). The bactericidal effect of ecabet observed at pHs 4.0 and 5.0 may contribute mainly to the bactericidal effect seen in pH 3.0 buffer supplemented with urea. The mechanism of this bactericidal effect of ecabet is not known. We presume that denaturing of the cell wall of *H. pylori* may have resulted from binding of ecabet to the surface of cells, because ecabet has the ability to bind to various proteins, such as human serum albumin or pepsin, by a nonspecific hydrophobic interaction, and the binding is dependent on pH, i.e., the lower the pH, the higher the level of binding (14). However, the possibility of the involvement of urease inhibition by ecabet is not completely excluded.

In conclusion, ecabet has a strong bactericidal effect on *H. pylori* under acidic conditions despite the absence of in vitro antibacterial activity at neutral pH. This effect of ecabet may decrease the viability of *H. pylori* surviving on the gastric mucosa or in gastric acid, thus ameliorating the damage of gastric mucosa by *H. pylori*. Ecabet may have potential as a new, locally acting therapeutic regimen against *H. pylori* infection.

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