JOURNAL OF BACTERIOLOGY, Nov. 1996, p. 6487–6495 Vol. 178, No. 22
0021-9193/96/$04.00+0

A Bifunctional Urease Enhances Survival of Pathogenic *Yersinia enterocolitica* and *Morganella morganii* at Low pH

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Received 13 May 1996/Accepted 13 September 1996

To infect a susceptible host, the gastrointestinal pathogen *Yersinia enterocolitica* must survive passage through the acid environment of the stomach. In this study, we showed that *Y. enterocolitica* serotype O8 survives buffered acidic conditions as low as pH 1.5 for long periods of time provided urea is available. Acid tolerance required an unusual cytoplasmically located urease that was activated 780-fold by low-pH conditions. Acid tolerance of *Helicobacter* species has also been attributed to urease activity, but in that case urease was not specifically activated by low-pH conditions. A *ure* mutant strain of *Y. enterocolitica* was constructed which was hypersensitive to acidic conditions when urea was available and, unlike the parental strain, was unable to grow when urea was the sole nitrogen source. Examination of other urease-producing gram-negative bacteria indicated that *Morganella morganii* survives in acidic conditions but *Escherichia coli* 1021, *Klebsiella pneumoniae*, *Proteus mirabilis*, *Providencia stuartii*, and *Pseudomonas aeruginosa* do not. Consistent with these results, biochemical evidence demonstrated that *Y. enterocolitica* and *M. morganii* ureases were activated in vitro by low pH with an unusually low activity optimum of pH 5.5. In whole cells activation occurred as medium values decreased below pH 3.0 for *Y. enterocolitica* and pH 5.5 for *M. morganii*, suggesting that in vivo activation occurs as a result of cytoplasmic acidification. DNA sequence analysis of portions of the *M. morganii ure* locus showed that the predicted primary structure of the enzyme structural subunits is most similar to those of *Y. enterocolitica* urease. One region of similarity between these two ureases located near the active site is distinct from most other ureases but is present in the urease of *Lactobacillus fermentum*. This region of similarity may be responsible for the unique properties of the *Y. enterocolitica* and *M. morganii* ureases since the *L. fermentum* urease also has been shown to have a low pH optimum for activity.

Bacterial survival depends on the ability to tolerate changes in the environment such as temperature, pH, osmolarity, and nutrient availability. Bacteria are able to coordinate appropriate physiological responses to non-life-threatening or gradual environmental changes. The life style of the gram-negative bacterium *Yersinia enterocolitica* is a good example of how survival depends upon the ability of the bacteria to adapt to environmental changes. It survives as a saprophyte at temperatures ranging from 4 to 28°C, free-living in environments where the availability of many essential nutrients can be limited (2, 8). It is also a facultative intracellular bacterium, pathogenic for humans and other mammals where the temperature is increased to 37°C. In a host, *Y. enterocolitica* assumes a parasitic life style characterized by a coordinate change in the expression of several genes. Increase of temperature results in repression of genes involved in motility and biosynthesis of several amino acids and cofactors that are presumably readily available. Expression increases for other genes that encode proteins such as the Yops which are believed to be necessary for evading a host immune response (8). While most environmental changes experienced by *Y. enterocolitica* going from the free-living environment to the host environment allow for a progressive coordinated bacterial response, some conditions, such as changes in pH, occur rapidly and are potentially lethal, requiring the bacteria to maintain preemptive mechanisms for survival. Environments in a host where *Y. enterocolitica* experiences rapid changes in pH during the course of a normal infection are the stomach and an acidified phagosome of a host cell (16).

This study focuses on a mechanism used by *Y. enterocolitica* to survive rapid decreases in environmental pH and the role of the enzyme urease for acid survival. Urease is a multisubunit metalloenzyme that converts urea to ammonia and carbonate. Ureases have been described primarily in terms of their contribution to nitrogen metabolism. However, more recent studies have implicated urease as a factor that is necessary for survival and pathogenesis of some bacteria (30, 32). Previous studies have shown that urease activity is necessary for *Helicobacter pylori* to survive the acidic conditions of the stomach, where it causes disease (12, 26, 37, 46, 48). More recently, urease activity was shown to affect survival of *Y. enterocolitica* O9 under acidic conditions both in vitro and in vivo (10). For *Y. enterocolitica* O8 and *Morganella morganii*, we describe how this enzyme contributes to survival. In addition, the contribution of urease to acid tolerance was determined for other gram-negative bacteria that survive both free-living and in a susceptible host.

MATERIALS AND METHODS

Strains, media, and chemicals. Strains used in this study are listed in Table 1. Cultures were normally maintained on Luria-Bertani (LB) agar plates and grown in LB broth (28). *Y. enterocolitica* strains were grown at 26°C, and all other bacteria were grown at 37°C. Medium used for bacterial conjugation was minimal 63 supplemented with 2% glucose and 0.2% $MgSO_4$ (28). Defined medium containing urea as the sole nitrogen source was prepared as described previously (42). Defined media containing limited sources of nitrogen for induction of urease expression by some bacteria were prepared as described previously (7) with the following supplements: 0.2% glucose, 0.2% thiamine, 0.02% vitamin-free Casamino Acids, 0.01% biotin, 35 mM citrate, 0.3 mM glutamine, and 0.2 mM arginine. Where appropriate, antibiotics were included in media as follows: 100 μg of ampicillin, 20 μg of gentamicin, 20 μg of nalidixic acid, and 50 μg of

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TABLE 1. Bacterial strains and plasmids

Bacterium or plasmid	Relevant characteristics	Source
Bacteria		
Yersinia enterocolitica		
JB580	Δ*yenR* of 8081v^{Nal}	22
DAV1	JB580 *ure*::pGY3	This study
Escherichia coli K-12		
DH5α	*recA1 endA1 hsdR17 gyrA96 relA1* Δ(*lacZYA-argF*) (ϕ80 *lacZ*Δm15)	Lab collection
S17-1λ*pir*	λ(*pir*) *hsdR pro thi*, chromosomal integrated RP4-2 Tc::Mu Km::Tn*7*	41
Aeromonas hydrophila 2439 4668		Clinical isolate
Escherichia coli 1021		6
Klebsiella pneumoniae		7
Morganella morganii		Lab collection
Pleisiomonas species 84OA 4668		Clinical isolate
Proteus mirabilis PM1		C. Collins
Providencia stuartii		C. Collins
Pseudomonas aeruginosa PAO1		Lab collection
Vibrio parahaemolyticus		Clinical isolate
Plasmids		
pIVET8	*pir*-dependent *ori*R6K, Mob$^+$ Amp^r	25
pEGZ-H	*ori*ColE1, Mob$^+$ Amp^r Gen^r	J. F. Miller
pYURI	pBR322 with *ure* locus cloned into *Eco*RI-*Hin*dIII sites; from serotype O5,27 strain 637-83	C. Collins
pGY2	*Sph*I (*trpA-lacZY*) fragment of pIVET8 replaced with *Sph*I (*aadA*) fragment, Str^r	This study
pGY3	pGY2 with 0.3-kb *ureC Bam*HI fragment cloned into the *Bgl*II site	This study
pGY5	*Eco*RI-*Hin*dIII *ure* fragment from pYURI cloned into *Eco*RI-*Hin*dIII sites of pEGZ-H	This study

streptomycin per ml. Except where indicated, chemicals were purchased from Sigma Chemical (St. Louis, Mo.).

DNA manipulations and analysis. Enzymes and buffers were purchased from New England Biolabs (Beverly, Mass.) except where indicated. Plasmid pGY3 consists of a 300-bp fragment internal to *ureC* cloned into pGY2; plasmid pGY2 is a derivative of plasmid pIVET8 (25) where the *Sph*I *lac* fragment was replaced with the *aadA* gene originating from transposon Tn*1721* (39), conferring a dominant form of streptomycin resistance. To construct plasmid pGY3, a 2.6-kb DNA fragment containing a portion of the *ure* operon was generated by PCR amplification (Perkin-Elmer, Norwalk, Conn.) using primers 5′-GCAGCCGTT TGGTCACGG-3′ and 5′-CTATGCCACGCATCCCGACC-3′, corresponding to bp 458 to 475 and 3017 to 3037 of the published *ure* sequence (9). This fragment was cloned into plasmid pCRII (Invitrogen, San Diego, Calif.). Subsequently, the 300-bp *Bam*HI fragment internal to *ureC* was subcloned into the *Bgl*II site of plasmid pGY2. Plasmid pGY5 consists of an 8.0-kb fragment containing the entire *ure* operon cloned into pEGZ-H; plasmid pEGZ-H (provided by J. F. Miller, University of California, Los Angeles) is a medium-copy-number mobilizable cloning vector. To construct pGY5, an 8.0-kb *Eco*RI-*Hin*dIII fragment was subcloned from pYuri (provided by C. Collins, University of Miami) into the *Eco*RI-*Hin*dIII sites of pEGZ-H. Plasmid conjugation was performed by filter mating essentially as described elsewhere (36). Southern hybridization analysis was performed essentially as described previously (43) using the 2.6-kb PCR-generated *ure* fragment as a probe.

The nucleotide sequence of the *M. morganii ure* locus was obtained by the dideoxynucleotide method (40) according to the manufacturer's instructions (United States Biochemical, Cleveland, Ohio) with the plasmid clone pMON203 as the template (18). The initial primer used for DNA sequencing was 5′-GTC GGTATCCGTGAT-3′, corresponding to bp 1472 to 1488 of the published *Y. enterocolitica ureA* sequence (9). Subsequently, all oligonucleotide primers were derived from *M. morganii ure* sequences. Sequence was determined for both DNA strands of *ureC* for the region compared by phylogenetic analysis. Other *M. morganii* DNA sequence was determined for only a single DNA strand.

Phenol red urease test. Qualitative urease tests were performed by resuspending bacterial colonies from an agar plate or harvesting from liquid media by centrifugation in 100 μl of urease test buffer and incubating at 37°C. Urease test buffer contained (per liter) 5 g NaCl, 2 g KH_2PO_4, 2 g urea, and 20 mg of phenol red (solubilized in 1 ml of 80% methanol); the concentration of urea in these assays was 33.3 mM. Urease-positive bacteria hydrolyze the urea to ammonia, causing an increase in the pH of the buffer which is detected by a change in color of the buffer. A positive test is scored as a change in buffer color from yellow to red within 2 h.

Quantitative urease assay. Urease activity was quantitated by determining the rate of ammonia produced from the hydrolysis of urea. The colorimetric assay described previously was used with modifications (6). Cultures were pelleted by centrifugation and washed twice in double-distilled H_2O (ddH_2O), then resuspended in ddH_2O. Assay buffers were the same as described for acid survival assays (see below) containing freshly prepared urea at indicated concentrations. For assays where bacteria were permeabilized, buffers contained 0.1% cetyldimethylammonium bromide (CTAB). Assays were initiated by pipetting 5 μl of the bacterial suspension into 200 μl of buffer prewarmed to 37°C, mixed and incubated with shaking. Assays were terminated by the addition of 0.5 ml of phenol-nitroprusside (Sigma reagent 640-1), then 0.5 ml of alkaline hypochlorite (Sigma reagent 640-3). After 30 min at ambient temperature, absorbance at 635 nm was measured for each assay. Ammonia concentrations were determined by constructing a standard curve using defined concentrations of NH_4Cl prepared fresh in the same buffer used for the assay. Assays were done in duplicate for at least two time points to determine the rate of ammonia produced per minute. Standard deviation for all assays was less than 10%. Concentration of protein was determined by a detergent-compatible Lowry method using bovine serum albumin as a standard according to the manufacturer's instructions (Bio-Rad, Hercules, Calif.; product 500-0116). Bacteria were pretreated for protein concentration assays by solubilizing in 1% sodium dodecyl sulfate and heating for 10 min at 65°C. Urease activity was expressed as micromoles of ammonia produced per minute per milligram of protein.

Acid survival assays. Acid tolerance assays were performed with cultures grown for 18 h in LB and, where appropriate for induction of urease activity in LB, containing 100 mM urea or in low-nitrogen media. Assay solutions contained 0.6% NaCl buffered with 20 mM phosphate (pH 1.5, 2.0, and 7.0), 20 mM 2-(*N*-morpholino)ethanesulfonic acid (MES) (pH 6.5) or 100 mM citrate (pH 3.0, 4.0, and 5.5). To assay for acid survival, bacteria were collected by centrifugation, washed twice in ddH_2O, and resuspended in ddH_2O. At the beginning of the assay, 5 μl of the bacterial suspension was added to 0.5 ml of buffer prewarmed to 37°C, mixed, and incubated with shaking. At the indicated time points samples were removed, immediately diluted in buffer at pH 7.0, and plated on LB agar plates or LB containing antibiotics when appropriate. The number of CFU recovered reflected the number of viable bacteria remaining in each sample. At the conclusion of each assay the pH of the buffer was determined and was found to remain constant. All assays were repeated at least twice on different days. Representative results from a single set of experiments are shown for clarity.

Nucleotide sequence accession number. The DNA sequence of the *ureC* region examined here by phylogenetic analysis has been submitted to GenBank under accession number U69175.

RESULTS

***Y. enterocolitica* O8 survives extreme acidity when urea is available.** Pathogenic *Y. enterocolitica* O8, which produces urease, can persist both free-living and in a susceptible host where

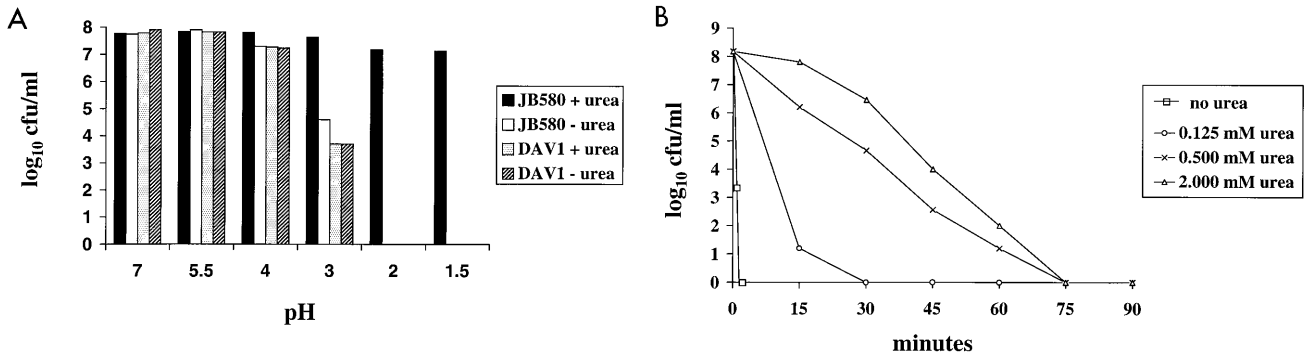

FIG. 1. Urease-dependent survival of *Y. enterocolitica* under acidic conditions. (A) Survival in buffers at different pH values. The number of viable bacteria was determined after 30 min in buffers with and without 2 mM urea. (B) For strain JB580, viability was measured over time in buffer at pH 2.0 containing different initial concentrations of urea.

it causes disease. Urease activity could be maintained for utilizing urea as a nitrogen source in either of these environments. Additionally, urease could be necessary for survival in acidic environments where urea is available such as in the stomach or, potentially, an acidified phagosome of a macrophage, polymorphonuclear leukocyte or other host cell. At either location, the production of ammonia could buffer the bacteria from the lethal effect of an extreme acid assault. To investigate this possibility, the capacity of *Y. enterocolitica* O8 strain JB580 to survive conditions of decreased pH in the presence and absence of urea was determined. The number of viable bacteria recovered decreased considerably (>2 logs) at values below pH 4.0 when no urea was available (Fig. 1A). Below pH 2.0 no viable bacteria were recovered. In contrast, viability was not drastically affected even by the most extreme acid assault of pH 1.5 when urea was included in the buffer at a physiologically relevant concentration (5, 26). Survival of *Y. enterocolitica* O8 required urea that was, presumably, converted to ammonia. However, survival was not due to an increase in buffer pH, since measurements at the completion of the assays showed that no pH change occurred.

To further characterize the conditions necessary for *Y. enterocolitica* O8 to survive 30 min at pH 2.0, bacteria were incubated in buffer with varying initial urea concentrations. These results showed that a minimum of 1 mM initial urea concentration was required to maintain complete viability of the bacteria for 30 min (data not shown). Survival of *Y. enterocolitica* O8 over time in acid at pH 2.0 containing an initial concentration of 0, 0.125, 0.5, or 2 mM urea was also determined (Fig. 1B). Under these conditions the rate of decline of bacterial viability over time was clearly dependent on urea availability. Higher initial concentrations of urea promoted viability, but even the highest concentration of 2 mM did not extend viability beyond 75 min. These results suggest that survival under buffered acidic conditions lasts only as long as urea remains available.

Construction of a urease mutant. To define the role of urease in the urea-dependent mechanism of acid tolerance, a *Y. enterocolitica* Ure$^-$ strain was constructed. Bacteria not able to produce urease were initially identified phenotypically as strains that were negative for the phenol red urease test. For one strain, DAV1, the *ure* mutation was confirmed by Southern hybridization analysis (data not shown). Survival of the urease mutant was examined in the presence and absence of urea. In the absence of urea, survival of the mutant was not affected compared to the parent strain. In the presence of urea the mutant was hypersensitive to changes in acidity, compared to the wild type, showing no capacity to survive below pH 3.0 (Fig. 1A). Introduction of plasmid pGY5 containing the complete *ure* operon into DAV1 completely restored urease activity and the capacity for survival at low pH (Table 2). Thus, the presence of the wild-type copy of the *ure* gene cluster in *trans* complemented the chromosomal mutation and restored the ability to survive acidic conditions. Additionally, introduction of pGY5 into *E. coli* DH5α, but not the vector control, conferred urease activity and the capacity for tolerating extremely low pH conditions (Table 2), suggesting that the *Y. enterocolitica ure* locus is sufficient for extreme acid tolerance.

Urease-dependent acid tolerance of other gram-negative, pathogenic bacteria. Ureases from many bacteria have been characterized biochemically and genetically. It has already been shown that *H. pylori* survives acidic conditions when urea is available but that another urease-producing bacterium, *Proteus mirabilis*, does not survive under the same conditions (26). The possibility that other urease-producing bacteria have the capacity to survive under extreme acidic conditions was evaluated. For these experiments, survival of *Proteus mirabilis* was reassessed and several other urease-producing bacteria were measured at pH 2.0 in buffer without or with 2 mM urea. Cultures were grown under conditions known to result in urease expression to increase the potential for urease-dependent acid tolerance. Prior to each assay, urease expression was confirmed by a qualitative urease test. For those bacteria that showed no survival capacity the experiment was repeated with an initial urea concentration of 100 mM. Urea availability should not be limited under these conditions, since it can diffuse across biological membranes and the concentration is well above the K_m value for all characterized ureases (32). The results showed that *Proteus mirabilis*, ureolytic *E. coli* 1021, *Klebsiella pneumoniae*, *Providencia stuartii*, and *Pseudomonas aeruginosa* did not survive (Table 2). Other bacteria tested that did not have the ability to survive acid assault included *Aeromonas hydrophila*, *Pleisiomonas* species, and *Vibrio parahaemolyticus*. Interestingly, *M. morganii* remained viable at levels similar to *Y. enterocolitica*, suggesting that the ureases of these two organisms may be distinct from other ureases.

Activation of urease activity by acidic conditions. Acid survival by *Y. enterocolitica* and *M. morganii* is dependent on urease and urea availability. However, other ureolytic bacteria do not have the capacity to survive acidic conditions in the presence of urea, suggesting that the amount of the urease or the activity of the urease may differ in these organisms. To examine possible differences between ureases produced by acid-resistant and acid-sensitive bacteria, the rate of urea hy-

TABLE 2. Urease activity is activated at low pH for bacteria that survive acidic conditions

Strain	Acid survival[b]	Urease activity[a]			
		pH 2		pH 7	
		No CTAB	With CTAB[c]	No CTAB	With CTAB[c]
Y. enterocolitica JB580	+	12.500	0	0.016	0.010
Y. enterocolitica DAV1	−	0	0	0	0
Y. enterocolitica DAV1/pGY5	+	22.074	0	0.252	0.272
Y. enterocolitica DAV1/pEGZ-H	−	0	0	0	0
E. coli DH5α	−	0	0	0	0
E. coli DH5α/pGY5	+	0.757	0	0.256	0.058
E. coli DH5α/pEGZ-H	−	0	0	0	0
M. morganii	+	9.644	0	0.464	0.674
E. coli 1021	−	0.004	0	2.700	0.790
Klebsiella pneumoniae	−	0	0	0.043	0.041
Proteus mirabilis	−	0	0	0.182	0.132
Providencia stuartii	−	0	0	0.456	0.105
Pseudomonas aeruginosa	−	0	0	0.549	0.507
A. hydrophila	−				
Pleisiomonas species	−				
V. parahaemolyticus	−				

[a] Urease assays were performed as described in Materials and Methods in buffers containing 2 mM urea for *Y. enterocolitica*, *E. coli* DH5α, and *M. morganii*. For other bacteria, buffers contained 100 mM urea. Urease activity is expressed as micromoles of ammonia produced per minute per milligram of protein. Each strain was grown under conditions reported to result in maximal urease activity. *Proteus mirabilis* and *Providencia stuartii* were grown in LB–10 mM urea. *K. pneumoniae*, *P. aeruginosa*, *A. hydrophila*, *Pleisiomonas* species, and *V. parahaemolyticus* were grown in low-nitrogen media. All other strains were grown overnight in LB.

[b] Determined as described in Materials and Methods. Incubation time was 30 min in buffer at pH 2.0 containing 2 mM urea for *Y. enterocolitica*, *E. coli* DH5α, and *M. morganii*. For other bacteria, buffer contained 100 mM urea. +, viable bacteria were recovered when urea was present in the buffer; −, no viable bacteria were recovered.

[c] 0.1%.

drolysis was measured for all bacteria at pH 2.0 and 7.0. These experiments were done with the same buffers and under the same conditions used for survival assays. The concentration of urea in buffers was 2 mM for *Y. enterocolitica* and *M. morganii*. For other bacteria the concentration of urea was increased to 100 mM to ensure that urea availability was not limited. The rate of urea hydrolysis was also measured for bacteria that were permeabilized by the addition of detergent to the assay buffer. Results for assays at pH 7.0 showed that *Y. enterocolitica* hydrolyzed urea at a lower rate than the other bacteria tested (Table 2). *M. morganii* hydrolyzed urea at a rate similar to *Proteus mirabilis* and *P. aeruginosa*. The rate of urea hydrolysis was not greatly affected at this pH by the presence of detergent for any bacteria, indicating that the integrity of the cytoplasmic membrane did not limit urea availability. For *Y. enterocolitica* and *M. morganii* the rate of urea hydrolysis was not affected by increasing the concentration of urea in the buffer (data not shown).

At pH 2.0 the rate of urea hydrolysis increased more than 780-fold for *Y. enterocolitica* and 20-fold for *M. morganii*, suggesting that these ureases are activated in whole bacteria under acidic conditions. Activation of urease was not due to differences in levels of expression, since all assays were done in parallel with bacteria from the same culture. Essentially no urea hydrolysis was measured for the other bacteria, suggesting that these ureases are inactivated by these conditions. The presence of detergent at pH 2.0 completely eliminated urea hydrolysis by *Y. enterocolitica* and *M. morganii*, indicating that urease is sensitive to pH 2.0 and requires cytoplasmic membrane integrity to remain active under this condition.

Urease activity was also measured for *E. coli* DH5α and the *Y. enterocolitica* urease mutant, DAV1, with and without plasmid pGY5 (Table 2). Without pGY5, there was no urease activity detected for either strain of bacteria. With the plasmid, both strains showed low levels of urease activity at pH 7.0 and an increase of urease activity at pH 2.0. The presence of detergent had no significant effect on urease activity at pH 7.0 but eliminated all activity at pH 2.0 for both bacteria, confirming that cytoplasmic membrane integrity is necessary to maintain urease activity at low pH. Further experiments will need to be done to determine why there is less urease activity when the enzyme is produced in *E. coli* DH5α; however, it has been observed for other ureases that decreased activity is due to nickel limitation (24, 31, 49). Nonetheless, heterologous expression of *Y. enterocolitica* urease allows *E. coli* to survive extreme acid assault.

Mechanism of urease activation. Activation of urease by low-pH conditions in *Y. enterocolitica* and *M. morganii* is not due to increased levels of *ure* expression, since there is no lag in time before increased urease activity is detected. This suggests that the ureases of these bacteria respond to changes in cytoplasmic pH directly and become activated by low pH. The simplest mechanism for sensing and regulating urease activity would be for the enzyme to have a low-pH optimum. To address this possibility, urease assays were performed with permeabilized cells in buffers ranging from pH 1.5 to 7.0 (Fig. 2). All assays were done with bacteria harvested from a single culture grown overnight in LB media and washed in water. The results show that there was a sharp increase in urease activity at pH 5.5 for both *Y. enterocolitica* (Fig. 2A) and *M. morganii* (Fig. 2B), but activity was relatively low at pH values above and below 5.5. This low pH optimum and the requirement for cell integrity for activity at pH 2.0 suggest that these ureases could be sensing a decrease in cytoplasmic pH. According to this model the pH optimum for urease activity should be less for whole cells compared to permeabilized cells since the cytoplasmic membrane would act as a barrier to limit the diffusion of protons into the cytoplasmic space. As the results show for whole cells, the activity optimum shifted down to pH 1.5 for *Y. enterocolitica* (Fig. 2A) and shifted down to pH 2.0 for *M. morganii* (Fig. 2B), indicating that the ureases of these bacteria may be responding to changes in cytoplasmic pH, perhaps by

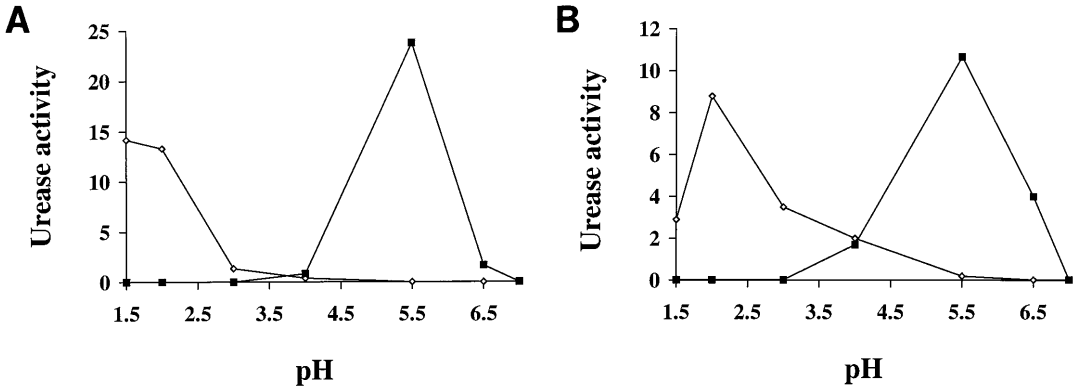

FIG. 2. The ureases of *Y. enterocolitica* and *M. morganii* have low pH optima and are highly active only under conditions that acidify the cytoplasm of the cell. Enzyme assays were done as described in Materials and Methods in buffers containing 2 mM urea. Assays were done for whole cells (diamonds) and cells permeabilized by including 0.1% CTAB in the buffer (squares). Cultures were grown overnight in LB media. Bacteria were harvested, washed twice, and resuspended in ddH_2O. Samples from the same culture were assayed in parallel at different pH values. Urease activity is expressed as micromoles of ammonia produced per minute per milligram of protein. (A) Results for *Y. enterocolitica* JB580; (B) results for *M. morganii.*

an alteration in conformation. The low pH optimum for *Y. enterocolitica* and *M. morganii* ureases contrasts sharply with other known ureases from bacteria that normally grow under neutral conditions which have activity optima of at least pH 6.8. It is also distinct from the urease of acid-tolerant *Helicobacter* bacteria which is active over a wide range of pHs and has optimum activity at pH 8.0 (11, 13, 29). Thus, the mechanism for regulating urease activity in *Y. enterocolitica* and *M. morganii* appears to be novel.

Nonactivated urease is necessary and sufficient for utilizing urea as a sole nitrogen source. Ureases provide bacteria with a pathway for obtaining nitrogen by converting urea to ammonia. For *Y. enterocolitica*, acid tolerance is associated with urease activation, but it is not clear whether activation is essential for utilization of urea as a source of nitrogen. To see if urease activity is sufficient under neutral growth conditions for urea utilization, both *Y. enterocolitica* JB580 and the urease mutant, DAV1, were examined on defined media buffered at pH 7 with urea as the sole nitrogen source. The urease mutant was unable to grow on this medium compared to the parental strain which formed colonies within 72 h (Fig. 3A). Consistent with these results, urease activity for cultures grown and assayed in defined liquid media (buffered to pH 7.0) was similar to cultures grown in LB and assayed in saline buffered to pH 7.0 (data not shown). This indicates that nonactivated urease is necessary and sufficient for *Y. enterocolitica* to utilize urea as a sole nitrogen source under neutral pH growth conditions. Further, urea utilization is probably not due to increased expression of urease, since the amount of urease activity was not affected by changes in culture media.

Introduction of plasmid pGY5 into the urease mutant restored the ability to grow on defined media with urea as the sole nitrogen source (data not shown). Growth of the mutant could also be restored on this defined medium by cross-feeding when the parental strain was streaked on the same plate (Fig. 3B). This cross-feeding is probably due to diffusion of ammonium ions generated from the hydrolysis of urea by the parental strain since growth of the mutant was best in areas closest to the parental strain. Consistent with these results, both strains grew equally well on defined media when ammonium was provided as the nitrogen source (data not shown). These results confirm that urease is necessary for *Y. enterocolitica* to utilize urea as a sole nitrogen source and that the *ure* mutation of DAV1 eliminates urease production without affecting the nitrogen assimilation pathways at steps subsequent to the conversion of urea to ammonia.

***Y. enterocolitica* and *M. morganii* ureases are homologous and distinct from other known ureases.** The primary and tertiary structure of bacterial ureases is highly conserved, suggesting that all of these enzymes hydrolyze urea by similar mech-

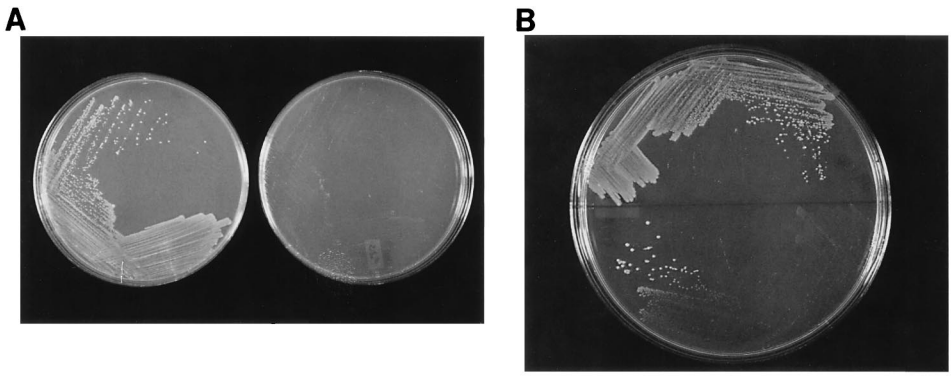

FIG. 3. Low urease activity at pH 7.0 is sufficient for utilization of urea as a sole nitrogen source. Growth of *Y. enterocolitica* strains after 72 h on defined media containing 10 mM urea and buffered to pH 7.0. (A) Strains JB580 (wild type) (left) and DAV1 (*ure*) (right) streaked for isolation on different plates; (B) strains JB580 (top) and DAV1 (bottom) streaked for isolation on the same plate. Note that colonies of DAV1 grew only in areas near the heaviest growth of strain JB580.

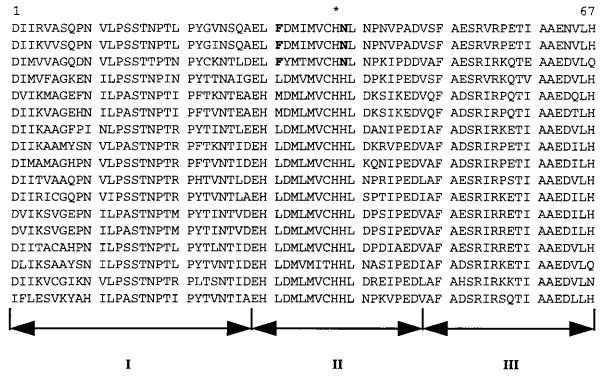

FIG. 4. Ureases with low pH optima contain conserved residues that do not occur in other ureases. Amino acid alignments were generated by the PILEUP program (University of Wisconsin, GCG). Alignment of a 67-amino-acid region of ureases is shown. *, essential H residue that serves as the catalytic base for urea hydrolysis. Residues that occur only among the ureases that exhibit a low pH optimum are boldfaced. Ye, *Y. enterocolitica* (9); Mm, *M. morganii*; Lf, *L. fermentum* (45); Ss, *Streptococcus salivarius* (3); Hf, *Helicobacter felis* (14); Hp, *Helicobacter pylori* (23); Btb, *Bacillus* species strain TB90 (24); Hi, *Haemophilus influenzae* (15); Bp, *Bacillus pasteurii* (34); Mt, *Mycobacterium tuberculosis* (4); Rm, *Rhizobium meliloti* (27); Pm, *Proteus mirabilis* (20); Pv, *Proteus vulgaris* (33); Ka, *Klebsiella aerogenes* (35); Sx, *Staphylococcus xylosus* (21); Ce, *Canavalia ensiformis* (47); Uu, *Ureaplasma urealyticum* (1).

anisms (19, 32). This includes the urease of *Y. enterocolitica* which contains identical residues at positions known to be necessary for function in other ureases because they are involved in coordination of urea, nickel, and water. There is also conservation of the essential histidine believed to serve as the catalytic base. However, the *Y. enterocolitica* and *M. morganii* ureases appear to be functionally different from most other ureases because these isozymes have an especially low pH optimum. It is likely that this low pH optimum is due to amino acid residues near the enzyme active site which are most likely to affect enzyme activity rather than residues in other parts of the holoenzyme that affect enzyme structure. To begin examining this possibility, the primary structure of the urease α subunit (UreC) of *Y. enterocolitica* and *M. morganii* was compared to other deduced urease structures. For this analysis, the deduced amino acid sequence of *Y. enterocolitica* O8 urease was already available (9). However, although the *ure* locus had been cloned from *M. morganii*, the DNA sequence was not available (18). Thus, DNA sequence of the cloned *M. morganii* locus was initiated by using a DNA primer that anneals to *Y. enterocolitica ureA*. Subsequently, additional primers were derived from *M. morganii* sequences. In all, several discontinuous regions of DNA sequence were determined spanning approximately 2 kb of the *ure* locus. Comparison of sequences from the *M. morganii ure* locus with other bacterial *ure* gene clusters showed that it contains predicted open reading frames corresponding to *ureABC*. More striking was the identity between *M. morganii* and *Y. enterocolitica* sequences, which was greater than 80% (data not shown).

The *M. morganii* DNA sequence was used to identify a region of the urease α subunit (UreC) containing the histidine thought to serve as the catalytic base (19, 32). This region was compared to *Y. enterocolitica* and comparable regions of other ureases for which a structure has been deduced (Fig. 4). In general, this region is highly conserved among the ureases, but there are some interesting differences. In particular, there are two residues located near the catalytic histidine that occur only in *Y. enterocolitica*, *M. morganii*, and *Lactobacillus fermentum*. The *L. fermentum* urease has also been shown to have an activity optimum at pH 4.0 (44). The conserved residues are Phe-31 (numbering as shown in Fig. 4), which occurs 7 residues toward the amino terminus, and Asn-39, which occurs 1 residue toward the carboxyl terminus of the protein relative to the catalytic histidine (His-38). It is not clear how these residues affect urease activity, but, given their proximity to His-38, they may affect the pH optimum of activity by affecting the pK_a of that residue.

DISCUSSION

Urease has been characterized biochemically from sources ranging from the plant jack bean to bacteria and is a cytoplasmically located multisubunit metalloenzyme that requires nickel for activity (32). This enzyme catalyzes the hydrolysis of urea to ammonia and carbonic acid. Genetic characterization of bacterial ureases has shown that its production requires several genes that encode the functional subunits and accessory proteins necessary for assembly and nickel incorporation. For most bacteria, including *Y. enterocolitica*, urease is probably necessary for utilization of urea as a nitrogen source. Fortuitously, the capacity to generate significant amounts of ammonia increases the pathogenicity of some bacteria that infect the urogenital tract (32). In these cases, production of ammonia increases the pH of the environment, resulting in the formation of struvite stones which interfere with the flow of urine that normally flushes the system of bacteria. Urease has also been shown to play a role in the establishment of infection by *Helicobacter* species which cause gastroduodenal diseases in many different animals (12, 46, 48). In this case, urease appears to be required for the production of copious amounts of ammonia that effectively neutralize acid in the environment surrounding the bacteria. Recently, survival of *Y. enterocolitica* O9 was shown to be enhanced by urease under acidic conditions, which appears to increase the likelihood of orally acquired infection because *ure*$^+$ bacteria survive passage through the stomach better than Ure$^-$ bacteria (10).

In this study, we examined the role of urease in *Y. enterocolitica* O8 survival and focused on the mechanism by which this enzyme contributes to acid tolerance. Our results indicated that *Y. enterocolitica* O8, like *Y. enterocolitica* O9 (10), had the capacity to survive in extremely acidic environments where urea is available. One such environment that *Y. enterocolitica* encounters in a host is the stomach, where the pH can be as low as 2.0. To further characterize this urea-dependent mechanism of acid tolerance, a urease mutant of *Y. enterocolitica* O8 was constructed. The mutant strain grew normally and was indistinguishable from the wild-type strain, except that it had no capacity to survive extreme acid assault, had no capacity to hydrolyze urea to ammonia, and was unable to utilize urea as a nitrogen source. Furthermore, all of these phenotypes could be restored by reintroducing the genes necessary for urease production on a plasmid.

Other gram-negative pathogens that produce urease were examined to determine if they could also survive an extreme acid assault when urea was available. Results from these experiments distinguished *M. morganii* as another bacterial species with the capacity to survive acidic environments where urea is available. This suggested that there may be functional similarities between the *Y. enterocolitica* and *M. morganii* ureases that distinguish them from other ureases. As results from this study show, *Y. enterocolitica* and *M. morganii* hydrolyze urea at significantly higher rates under acidic conditions compared to the other bacteria which hydrolyze little or no urea under acidic conditions. In addition, increased urea hydrolysis was due to increased enzyme activity of preexisting urease at

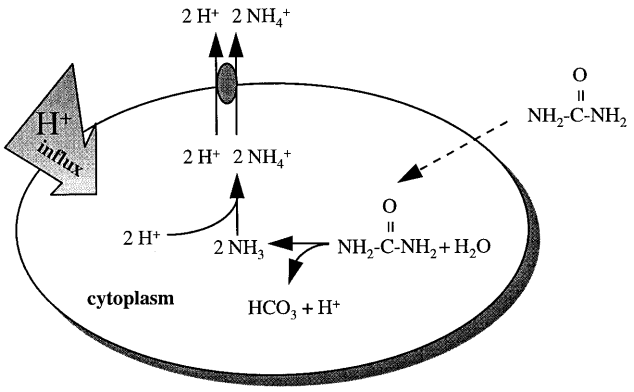

FIG. 5. A hypothetical model describing the mechanism of urease-dependent acid tolerance in *Y. enterocolitica*. The large oval represents the cytoplasmic membrane. The small shaded oval represents a hypothetical ammonium/proton transporter. The periplasm and outer membrane have been omitted for clarity. Stochiometric equivalents of reaction participants are depicted. Under low-pH conditions there is an influx of protons to the cytoplasm which results in an increase of urease activity. Urease hydrolyzes urea, which freely diffuses into the cytoplasm, producing ammonia and carbonic acid. The ammonia spontaneously protonates to ammonium, effectively neutralizing 1 mol of protons per mol of urea hydrolyzed. Ammonium that exits the cytoplasm via a symport results in proton efflux. This ammonium-driven proton transport increases the cell's capacity to counter proton influx.

low pH. However, when the cytoplasmic membrane is compromised by including detergent in the assay buffer, activation of urease at pH 2.0 does not occur and activity is abolished. Thus, the cytoplasmic membrane plays an essential role in activation of urease under acidic conditions for both *Y. enterocolitica* and *M. morganii*. One way that the cytoplasmic membrane could influence urease activity is to act as a diffusion barrier against protons to limit the rate at which the pH of the cytoplasm would decrease. Consistent with this hypothesis, the pH optimum for urease activity was 1.5 for *Y. enterocolitica* and 2.0 for *M. morganii* for whole cells compared to pH 5.5 for both ureases when measured with permeabilized cells. It is likely that activation of these ureases in whole cells occurs as the pH of the cytoplasm approaches 5.5, since this is the pH where activation occurs for urease in permeabilized samples. Another hypothetical role that the cytoplasmic membrane could play in acid tolerance is to maintain a membrane-located ammonium transport system (Fig. 5). Such a system could couple the efflux of ammonium ions and protons from the cytoplasmic space. The energy to drive the system would come from the ammonium ion gradient generated as copious amounts of urease-produced ammonia spontaneously convert to ammonium. In this way the hydrolysis of 1 mol of urea could effectively neutralize 3 mol of protons within the cytoplasmic space. This is three times greater than the effect that protection by the production of ammonia alone would afford. This system would be beneficial to the bacteria because under normal conditions it could reverse the transport process to import ammonium which is used as a nitrogen source.

In addition to acid tolerance, urease provides *Y. enterocolitica* with a way to utilize urea as a nitrogen source, but it was not clear that this required urease to be at activated levels. When *Y. enterocolitica* and an isogenic urease mutant were streaked onto defined media (buffered at pH 7.0) containing urea as the sole source of nitrogen, only the wild-type strain formed colonies. Also, the rate of urea hydrolysis for *Y. enterocolitica* grown and assayed in this defined medium was similar to rates measured for cultures grown in LB and assayed in saline buffered at pH 7.0, indicating that under neutral conditions low urease activity was sufficient to provide a pool of ammonia for growth. Taken together, these data indicate that *Y. enterocolitica* produces a bifunctional urease that has high activity under low-pH conditions to protect the cell from the lethal effect of cytoplasmic acidification and low, but sufficient, activity under neutral conditions to maintain a pathway for utilizing urea as a nitrogen source. This control of urease activity may enhance the overall survival of *Y. enterocolitica* by maintaining a mechanism that can be immediately activated for survival of a rapidly changing environment, as occurs when the bacteria pass through the stomach of a host. Conversely, decreased activity under neutral conditions ensures that ammonia is not produced at levels that could be toxic to the bacteria by increasing the pH to high levels in either a host or free in the environment, as observed for *H. pylori* (5).

The mechanism of acid tolerance for *Y. enterocolitica* O8 and *M. morganii* appears different than the mechanism that occurs in *H. pylori* with respect to both the location in the cell where ammonia is produced and enzyme activity. For *Y. enterocolitica* O8 and *M. morganii*, urease is located in the cytoplasm, and this location is essential for acid tolerance since it is labile below pH 5.5. Also, enzyme activity is regulated to maintain low levels of activity under conditions where high levels may be detrimental. Furthermore, the protective effect of ammonia does not extend beyond the cell where it is produced since wild-type *Y. enterocolitica* O8 failed to rescue the urease mutant when both strains were coincubated in low-pH buffer (data not shown). In contrast, *H. pylori* urease is most active at pH values above 7.0 (optimum of 8.0), but it maintains partial activity under low-pH conditions (13). This mechanism is also complicated because the enzyme is normally located in the cytoplasmic space, but, when released from dying cells, it remains active and will associate with the outer surface of viable bacteria (17, 38). For *H. pylori* it appears that both cytoplasmic and surface-located ureases contribute to acid tolerance.

To examine structural relationships between the *Y. enterocolitica* and *M. morganii* ureases that may distinguish them from other ureases, several appropriate regions of the peptide subunits that compose urease holoenzymes were compared by amino acid sequence alignments and construction of phylogenetic matrices. Initial results from this analysis comparing a 67-amino-acid region that included the histidine residue that serves as the catalytic base for hydrolysis Fig. 4) suggested that the *L. fermentum* urease was related to the ureases of *Y. enterocolitica* and *M. morganii*. This result was somewhat surprising because previous studies that compared available nucleotide sequence for genes encoding the structural subunits of the enzyme did not show any association between *L. fermentum* and *Y. enterocolitica* (9, 24). Interestingly, *L. fermentum* urease has an activity optimum of pH 4.0 (44), which is similar to the pH optimum determined for the *Y. enterocolitica* and *M. morganii* ureases. Further computer analysis narrowed the region common to these ureases to a segment of 20 amino acids (region II [Fig. 4]). This region includes a phenylalanine and an asparagine residue that flank the essential catalytic histidine. In all other characterized ureases the position where phenylalanine occurs is a highly conserved leucine or a methionine, as occurs in *Helicobacter* ureases. The asparagine residue occurs at a position that is an absolutely conserved histidine in other characterized ureases. Further experiments will need to be done to determine the significance of these residues, but it is possible that these two residues influence urease activity by affecting the pK_a of the catalytic histidine. Control experiments using other regions of the same protein or other subunits show no significant similarities between the *L. fermen-*

tum urease primary structure and the *Y. enterocolitica* or *M. morganii* urease primary structures, suggesting that the similarity within the catalytic region is an example of functional coevolution. In contrast, there is close relationship between *Y. enterocolitica* and *M. morganii* at both the nucleotide level and the amino acid sequence level throughout the *ure* locus. Taken together, these observations do not prove but further suggest that the region near the urease active site is critical for the low pH optimum for these enzymes.

ACKNOWLEDGMENTS

We thank Carleen Collins for advice and for critical review of the manuscript. We also thank B. Jones, H. Mobley, C. Collins, and J. Miller for providing plasmids used in this study.

This work was supported by National Institutes of Health grants AI-27342 and AI-01230 to V.L.M., who is a Pew Scholar in the Biomedical Sciences.

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