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Motility of Urease-Deficient Derivatives of *Helicobacter pylori*

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Early studies of a *ureB* mutant derivative of *Helicobacter pylori* had suggested that urease is needed for motility and that urease action helps energize flagellar rotation. Here we report experiments showing that motility is unaffected by deletion of *ureA* and *ureB* (urease genes) or by inactivation of *ureB* alone, especially if *H. pylori* strains used as recipients for transformation with mutant alleles are preselected for motility. This result was obtained with the strain used in the early studies (CPY3401) and also with 15 other strains, 3 of which can colonize mice. We conclude that urease is not needed for *H. pylori* motility.

Helicobacter pylori is a gram-negative pathogen that is implicated in peptic ulcer disease and gastric cancer (for reviews, see references 6, 9, and 23). Mutational analyses have shown that urease, an enzyme that *H. pylori* makes in abundance, is needed for mammalian infection but is not essential in laboratory culture (7, 22, 33, 35). Urease might have several *in vivo* roles, including (i) protection from gastric acidity, via the ammonia that it generates from the urea in host tissues (22, 26); (ii) nutrition, by ammonia-elicited tissue damage and a consequent release of host metabolites that promote *H. pylori* growth (4, 11, 28); and/or (iii) generation of proton motive force through urea hydrolysis (26), which in turn helps drive flagellar rotation and bacterial motility (20, 25, 36). The third role was postulated, based in large part, on the finding that a *ureB* insertion mutant derivative of *H. pylori* strain CPY3401 was nonmotile in standard soft agar medium (25, 36). Because motility itself is needed for *H. pylori* colonization (e.g., see references 3, 8, 15, and 35), a dependence of motility on urease action would complicate efforts to examine urease's other possible roles.

H. pylori's motility can be quite unstable, in part because of a repetitive, frameshift mutation-prone sequence in *fljP*, a flagellar biosynthetic gene (14, 32). In addition, flagellar synthesis and motility probably impose a physiologic cost, estimated at some 2% of total energy expenditure for enteric species (19). As a result, nonmotile strains would tend to outgrow isogenic motile strains when motility is not needed, as in standard laboratory culture. *H. pylori* is also an extremely diverse species genetically (1, 2, 10), and recent experiments have shown that background genotype can influence whether inactivation of certain metabolic genes will affect particular phenotypes. For example, depending on the *H. pylori* strain used, inactivation of the *fdxA* ferredoxin gene can be lethal or be tolerated; *fdxA* can help downregulate the *frxA* nitroreductase gene, but only in certain strains; and in a few cases, the lethality of *fdxA* inactivation can be reversed by prior *frxA*

inactivation (24). We therefore wondered if urease-dependent motility might also be a strain-specific property.

We were intrigued by Nakazawa's proposed connection between urease, motility, and intracellular metabolic flux (25, 36). The present mutational studies were begun in the hope of confirming her interpretation, testing its generality, and characterizing its basis. Transformable, motile *H. pylori* strains from our collection (Table 1) were cultured at 37°C in a microaerobic incubator with 5% O₂ and 10% CO₂, using brain heart infusion (BHI) agar with 7% horse blood (standard conditions) (13). Motility was assayed by inoculating cultures into 0.35% agar medium containing brucella broth with serum (motility agar) and incubating the inoculated plates as described previously (25). In testing single (transformant) colonies for motility, reproducibility was improved by first streaking colonies onto ~1- to 2-cm² patches on BHI agar, growing these patches for 24 h, and then using cells from them to inoculate motility test agar. To generate urease-deficient derivatives of motile and transformable strains, we generated an allele in which *ureA* and most of *ureB* was replaced by a *cat* (chloramphenicol resistance [Cam^r]) cassette by a PCR method that does not involve recombinant DNA plasmid cloning (illustrated in Fig. 1 and described in reference 5). The PCR product containing this $\Delta ureAB$ allele was used to transform motile *H. pylori* strains, with selection for Cam^r (15 μ g/ml) (13). PCR tests of representative Cam^r transformants demonstrated the expected replacement of *ureA* and *ureB* by *cat* in each case. Motility tests were carried out on at least four single colonies and also on pools containing 20 or more colonies from each transformation. In control experiments with parental strains, motile halos of growth were evident around points of inoculation on the 3rd or 4th day of incubation with 13 of 16 strains and on the 5th day with the other 3 strains; these halos continued increasing in size for at least 3 more days. Accordingly, the motility of $\Delta ureAB$ transformants was also scored on each of several days, beginning on the 4th day after inoculation into motility agar.

In an initial survey of 12 strains (Table 1, first 12 strains listed), nonmotile single transformant colonies were obtained from strains Chen13, R64, and GS5, as illustrated in Fig. 2A for strain Chen13. The lack of motility of the two single transformant colonies (labeled "sc") matches that reported earlier

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TABLE 1. List of *H. pylori* strains used in this study

Strain name	Country of origin	Source (reference)
A28-1	Lithuania	Limas Kupcinskis, Kaunas
A66-1	Lithuania	Limas Kupcinskis
PeCan28	Peru	Robert H. Gilman (16)
Chen13	India	Usha Anand Rao, Chennai
F28	Japan	Takeshi Azuma, Fukui
GS5	Japan	Teruko Nakazawa, Ube
HK192	Hong Kong	Benjamin Wong
PCM4	Hong Kong	Benjamin Wong
R64	South Africa	Issy Segal, Johannesburg
R66	South Africa	Issy Segal
R76	South Africa	Issy Segal
R82	South Africa	Issy Segal
CPY3401	Japan	Junko Akada and Teruko Nakazawa (33)
SS1	Australia	Adrian Lee (18)
X47	United States	Harry Kleanthous (17)
88-3887	United Kingdom	Kate Eaton (12, 14)

with a *ureB* derivative of strain CPY3401 (25). However, pools of Cam^r ($\Delta ureAB$) transformants (labeled “pl”) seemed fully motile in each of these three cases. In addition, all tested transformants of the nine other strains remained motile. With four of these strains, however, single transformant colonies produced subtly smaller halos than did their wild-type parents, whereas with a fifth strain (R76), $\Delta ureAB$ transformants produced slightly larger halos (Fig. 2B). Such subtle differences were sometimes difficult to reproduce or quantify reliably: they might be explained by changes in properties distinct from the strength or rate of flagellar rotation (e.g., effects on cell shape, clumping, or surface texture), and they have not been studied further to date.

To test whether differences in motility of single colony trans-

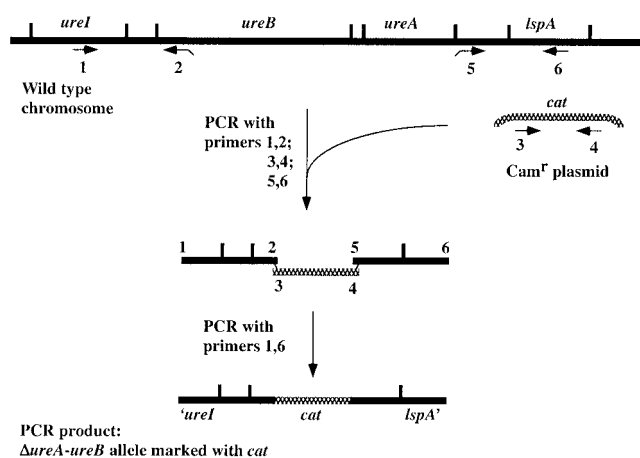


FIG. 1. Construction of $\Delta ureA-ureB$ (deletion) allele marked with a resistance determinant. This construction involved PCR without cloning, essentially as described previously (5). The following six primers were used (see the figure for positions): 1 (*ureABR2*), 5'-TCCCTAAAGGGATTTTCAAGATGT; 2 (*ureCAMF2*), 5'-CCCAGTTTGTCGCACTGATAACCATGTGTTTCGTGGATGGCAA; 3 (C2), 5'-TTATCAGTGCACAAACTGGG; 4 (C1), 5'-GATATAGATTGAAAAGTGGAT; 5 (*ureCAMR1*), 5'-ATCCACTTTTCAATCTATATCATTCTCCTATTCTTAAAGTGT; and 6 (*ureABF1*), 5'-CATGGGGCGTGGTGGATTA. Overlaps between primers 2 and 3 and between primers 4 and 5 are underlined. PCR product sizes were 797 bp (primers 1 and 2), 742 bp (primers 3 and 4), 513 bp (primers 5 and 6), and 2,010 bp (primers 1 and 6).

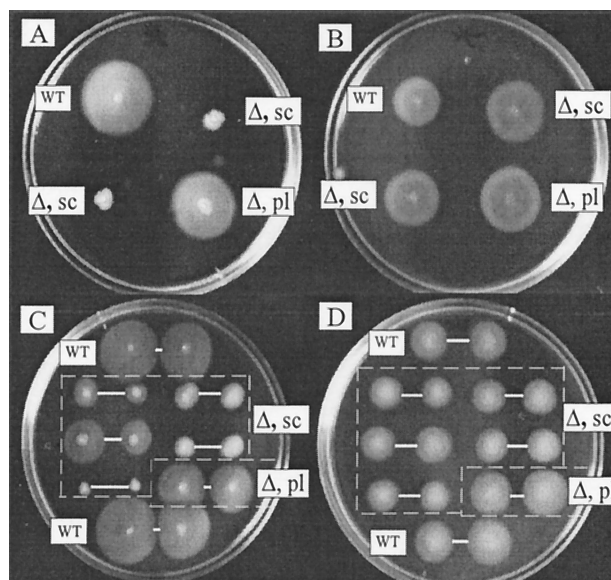


FIG. 2. Motility tests of representative *H. pylori* wild-type (WT) strains and isogenic $\Delta ureAB$ transformants (Δ, sc , single colony; Δ, pl , pool) stabbed into motility agar and incubated for 6 days. The strains shown are Chen13 (A), R76 (B), R64 (C), and SS1 (D). In panels C and D, each culture was stabbed twice to score reproducibility of the motility assay. Duplicate stabs are designated by white bands.

formants versus pools from 3 of 12 strains (as in Fig. 2A) could be ascribed to heterogeneity in the recipient population, we prepared subpopulations of strains Chen13, R64, and GS5 from pools of bacterial cells recovered from edges of halos after 4 days of growth in motility agar. These “motility-selected” cell populations were then used as recipients in transformation. Single Cam^r ($\Delta ureAB$) transformant colony derivatives of Chen13 prepared in this way were fully as motile as their parents (data not shown). In further tests, two of four colonies from the original nontransformed Chen13 stock were nonmotile. Thus, our initial finding of urease-deficient derivatives of the original Chen13 stock that were nonmotile can be ascribed to heterogeneity in this stock, to variants that had accumulated before transformation. In contrast, marked diversity in halo size was again seen after transformation of motility-selected populations of strains R64 and GS5. This is illustrated in Fig. 2C for strain R64, in which each transformant colony tested was picked twice into motility agar to monitor reproducibility (duplicate stabs identified by white connecting bars). Of five transformants scored here, the halo size of one was about two-thirds that of its R64 wild-type parent, the halos of three others were more sharply reduced, and a fifth transformant seemed completely nonmotile. Similar heterogeneity was also seen in a motility-selected nontransformed R64 wild-type population. In further tests, two of six $\Delta ureAB$ transformants (single colonies) of motility-selected GS5 also seemed completely nonmotile, whereas the other four formed halos that were about 80% as large as those of their wild-type parent. We infer that metastability of motility can be characteristic of certain *H. pylori* clinical isolates, as exemplified by strains R64 and GS5, independent of urease gene status.

With these results in hand, we investigated effects of deletion of *ureA* and *ureB* on an additional four strains, using

subpopulations selected for high motility, as just described. These strains were CPY3401, early study of which (25) had suggested that motility was urease dependent; and SS1, X47, and 88-3887, each of which can colonize mice (12, 17, 18). In our hands, urease gene inactivation had no effect on halo size of the motility-selected CPY3401 recipient population, nor did it cause loss of motility in any of the other three motility-selected strains. With SS1, however, single colony transformants exhibited subtly reduced halo size (Fig. 2D). We do not yet know if the smaller halos of $\Delta ureAB$ transformants of SS1 and 5 other strains (of the 16 tested) stem from urease gene inactivation per se, versus rapid accumulation of changes in other genes that affect motility or other determinants of halo size. In this context, we note that derivatives of strain X47 selected directly for high motility form halos approximately 20% larger in diameter than do derivatives recovered from infected mice (D. Dailidienė and D. E. Berg, unpublished observations), in which motility is also required. Subtle differences in halo size could be affected by many factors, such as cell shape and texture, as well as speed and pattern of flagellar rotation.

Two further control experiments were carried out. First, the effect of specific urea supplementation on *H. pylori* motility was tested, even though *H. pylori* can also derive urea from arginine in culture media (21). No difference in motility of $\Delta ureAB$ transformants versus isogenic parents was seen in any of four lineages (CPY3401, SS1, X47, or 88-3887) on medium that was either free of additional urea or contained 1 or 5 mM urea (concentrations that have been used in other studies and that are in the range of those in the human gastric mucosa) (26, 27, 31, 34).

Second, we also studied the effect on motility of a *ureB*-null insertion allele, very similar to the one Nakazawa and collaborators had used (25). These *ureB* alleles each contained an *aphA-3* (kanamycin resistance) gene inserted without deletion at the *Bam*HI site in *ureB* (nucleotide position 1013, some 60% from this gene's 5' end). (Our allele was generated by PCR in vitro, essentially as shown in Fig. 1 [primer sequences available on request].) *ureB*-deficient derivatives of strains CPY3401, SS1, X47 and 88-3887 were then generated by DNA transformation. In each case, the motility halos of *ureB*-deficient transformants matched those of isogenic $\Delta ureAB$ strains and also those of their wild-type parents in three cases (CPY3401, X47, and 88-3887); halo sizes of *ureB* insertion derivatives of SS1 were about 80% of those of the parental wild type, as was also the case with SS1's $\Delta ureAB$ derivatives (noted above). This outcome argued against a possible physiologic imbalance model, involving the UreA urease subunit without UreB, as an explanation for the reported nonmotility of a *ureB* *H. pylori* strain (25).

We suggest that the reported (25, 33, 36) nonmotility of one *ureB* derivative of CPY3401 stems from an unanticipated heterogeneity in the bacterial population used as transformation recipients and the unfortunate choice of a *ureB* transformant of a preexisting nonmotile subclone. Nonmotility would, in this case, be due to genetic change at another locus distinct from *ureB*: perhaps a locus such as *fliP*, which is metastable due to a frameshift-prone, repetitious DNA motif (14, 32). We had also seen nonmotility in some transformants of our original stock of strain Chen13. This result was interpreted to be spurious, be-

cause such transformants were not found with a motility-selected Chen13 recipient population. We suggest that the availability of numerous motile, urease-deficient, mouse-adapted strains of *H. pylori* and also of special achlorhydric mice (29, 30) should contribute to the definition of the *H. pylori* urease's true role or roles in vivo: e.g., critical tests of whether urease is needed solely to neutralize gastric acidity or whether this abundant enzyme also has additional vital roles.

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