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The Entner-Doudoroff Pathway Has Little Effect on *Helicobacter pylori* Colonization of Mice

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Helicobacter pylori **mutants deficient in 6-phosphogluconate dehydratase (6PGD) were constructed. Colonization densities were lower and minimum infectious doses were higher for mutant strains than for wild-type strains. In spite of better colonization, however, wild-type strains did not displace the mutant in cocolonization experiments. Loss of 6PGD diminishes the fitness of *H. pylori* in vivo, but the pathway is nonessential for colonization.**

Insight into the physiology of *Helicobacter pylori* is critical to fully comprehend how this organism colonizes and causes disease, and an understanding of its basic metabolism during an infection is central to gaining this knowledge. This information not only is of fundamental interest but also could be of value in the development of novel therapies for *H. pylori* infections.

Analysis of the complete genome sequence of *H. pylori* 26695 (2, 15) confirms that the organism has few complex metabolic pathways, which is consistent with its unique ecological niche and the absence of competition from other bacteria. *H. pylori* was at first thought to lack the ability to metabolize carbohydrates (8, 9), but it was later discovered that the bacterium can use glucose as a growth substrate via oxidative and fermentative pathways (13). Complete pathways for carbohydrate metabolism identified in *H. pylori* include the Entner-Doudoroff (ED) (12) and pentose phosphate (11) pathways. Although most of the Embden-Meyerhof-Parnas pathway enzymes are present, no evidence for use of this pathway was found (3).

Nuclear magnetic resonance spectroscopy has indicated that the most likely course of glucose metabolism in *H. pylori* is the ED pathway (12, 13), an alternative glycolytic pathway for the metabolism of sugars. The ED pathway involves two enzymes, 6-phosphogluconate dehydratase (6PGD; or ED dehydratase [EDD]) and 2-keto-3-deoxy-6-phosphogluconate aldolase (ED aldolase [EDA]), which are encoded by *edd* and *eda*, respectively (4). EDD catalyzes the dehydration of the substrate, 6-phosphogluconate (6PG), to form 2-keto-3-deoxy-6-phosphogluconate. EDA catalyzes an aldol cleavage of 2-keto-3-deoxy-6-phosphogluconate to form pyruvate and glyceraldehyde-3-phosphate. Conversion of glucose to 6PG, the true substrate of the ED pathway, requires the presence of a third enzyme, glucose-6-phosphate dehydrogenase, which is encoded by *zwf*. Thus, glucose is activated by a glucokinase to glucose-6-phosphate, which is then oxidized by EDD to form 6PG with the reduction of $NADP^+$ to NADPH. Metabolism of glucose to glyceraldehyde-3-phosphate thus requires all three genes, *zwf*, *edd*, and *eda*. These three enzymes are present and functional in *H. pylori*. Bacterial lysates have been shown to contain dehydratase, aldolase, and dehydrogenase activity (12). In addition, the *zwf*, *edd*, and *eda* genes are homologous to the same genes in *E. coli* (15), supporting their potential role in *H. pylori* metabolism.

Although the enzymes necessary for glucose metabolism are present in *H. pylori*, glucose metabolism is not necessary for survival. *H. pylori* can grow in a glucose-free medium by using only amino acids for substrates (10), which demonstrates that carbohydrates are not required if amino acids are supplied. Despite this, the ED pathway has been conserved in this organism, suggesting that it has a role, possibly in the metabolism of mucus-derived sugars, as has been described for *E. coli* (14). The purpose of this study was to elucidate the role of the ED pathway in *H. pylori* colonization of the mouse gastric mucosa.

Three *H. pylori* strains, 26695, M6, and SS1, were used in this study. All three strains were originally isolated from human stomachs and adapted for growth in piglets (strain 26695) or mice (strains SS1 and M6). *eda*, *edd*, and *zwf*, the three genes necessary for the ED pathway in *H. pylori*, were cloned from *H. pylori* 26695 and ligated into pUC18. A kanamycin resistance gene, *aphA-3*, was inserted into the 6PGD gene. The resulting plasmid, pAW105, thus carried a disrupted version of the *edd* gene and flanking sequences to allow for homologous recombination into the genome. pAW105 was transformed into *H. pylori* M6 (supplied by Steven Czinn, Case Western Reserve University, Cleveland, Ohio) and SS1 (provided by Adrian Lee, University of New South Wales, Sydney, Australia), generating M6 *edd*Ω*aphA-3* and SS1 *edd*Ω*aphA-3*, respectively. Allelic exchange and insertion of the disrupted gene into the genome were confirmed by PCR and by the loss of 6PGD activity. Supernatants were assayed spectrophotometrically for enzyme activity by using a two-step reaction in which pyruvate formed from 6PG was titrated with lactate dehydrogenase to determine the total enzyme activity (6, 7). Enzyme assays were conducted in triplicate. The results of the three trials were averaged, and the standard deviation was calculated. The num-

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TABLE 1. 6PGD activity of various *H. pylori* strains and *E. coli* 1485

Strain	Presence or absence of 6PG	Enzyme activity[a]	*P* value
H. pylori 26695	−	0 ± 0	<0.05
	+	5.3 ± 0.63	<0.05
H. pylori M6	−	0.4 ± 0.70	<0.05
	+	4.4 ± 0.41	<0.05
H. pylori SS1	−	0 ± 0	<0.05
	+	3.6 ± 0.51	<0.05
H. pylori edd mutant	−	0 ± 0	NA[b]
	+	0 ± 0	NA
E. coli 1485	−	0 ± 0	<0.05
	+	16.5 ± 0.99	<0.05

[a] Expressed in nanomoles per minute per milligram of total cell protein.
[b] NA, not applicable.

bers obtained were then analyzed with the Mann-Whitney and one-way analysis of variance tests by using the Instat statistical analysis package (GraphPAD Software). C57BL/6J mice from the Jackson Laboratory, germfree C57BL/6J mice from our own colony, or germfree, recombinase-activating gene knockout (RAG-KO) 129 mice from our own colony were orally inoculated with 10^9 CFU of live *H. pylori* and sacrificed at 2 or 10 weeks after inoculation. The mouse stomachs were removed and homogenized, and serial dilutions of the homogenates were plated on blood agar plates with or without kanamycin. The number of bacteria per gram of gastric mucosa was determined by colony count. The results were averaged for each group, and the standard deviations were calculated. If the colony count was below the level of detection (10^3 CFU/g), the number utilized for statistical analysis was 1,000 (i.e., at the level of detection). Mean values were compared by using the Mann-Whitney nonparametric test from the GraphPAD package. Sections stained with hematoxylin and eosin were scored for the extent of gastritis as previously described (5). All procedures involving mice were approved by the Ohio State University International Laboratory Animal Care and Use Committee.

All three wild-type strains of *H. pylori* tested exhibited low but detectable levels of 6PGD activity (Table 1). The average activity among all strains tested, 4.43 ± 0.86 nmol/min/mg of total cell protein, was significantly lower than the activity in *E. coli*, which averaged 16.07 ± 1.61 nmol/min/mg of total cell protein ($P = 0.0001$). 6PGD activity in *H. pylori* was not significantly induced by glucose ($P = 0.35$ for strain 26695; $P = 0.2$ for strain M6), and thus the activity was constitutive. Interruption of *edd* by *aphA-3* in *H. pylori* strain M6 *edd*Ω*aphA-3* eliminated all detectable 6PG activity ($P = 0.05$) (Table 1).

TABLE 3. Effect of inoculum dose on colonization of *H. pylori*[a]

H. pylori strain	No. of mice colonized/no. of mice inoculated			
	2×10^2 CFU[b]	2×10^3 CFU	2×10^4 CFU	2×10^5 CFU
SS1	5/5	5/5	5/5	5/5
SS1 *edd* mutant	0/5	0/5	0/5	1/4
M6	0/5	0/5	2/5	4/9[c]
M6 *edd* mutant	0/5	0/5	0/5	2/9[d]

[a] All mice are strain C57BL/6J except where noted.
[b] Inoculum dose.
[c] Numbers include four RAG-KO 129 mice; two of the four were colonized.
[d] Numbers include four RAG-KO 129 mice; one of the four was colonized.

Thirty-one C57BL/6J mice and eight RAG-KO 129 mice were inoculated with M6; of the C57BL/6J mice, 15 were inoculated with the parental wild-type strain and 16 were inoculated with the mutant, M6 *edd*Ω*aphA*-3, and 4 each of the RAG-KO 129 mice received inoculations with the same strains. In addition, 10 C57BL/6J mice were inoculated, half with the SS1 wild type and half with the SS1 *edd*Ω*aphA*-3 strain. Mice were sacrificed 2 weeks after inoculation, and the numbers of bacteria (CFU per gram of gastric mucosa) were determined (Table 2). In C57BL/6J mice, both the wild-type and mutant M6 *H. pylori* strains were able to colonize, although the mutant strain had a lower colonization density ($P < 0.05$). The SS1 strains showed an even greater difference in colonization ability. The wild-type strain colonized at a high density (up to 10^7 CFU/g of gastric mucosa), while the mutant colonized at an average of 10^2 CFU/g ($P < 0.0001$). In RAG-KO 129 mice, the M6 wild-type strain colonized to a higher density than the mutant, but in this case, differences in colonization densities were not significant ($P > 0.05$).

The effect of inoculation dose was determined by using C57BL/6J mice. Forty mice were inoculated with 10-fold dilutions of SS1 or SS1 *edd*Ω*aphA*-3 (20 mice per strain, 5 mice per inoculation group) (Table 3). Likewise, 48 mice were inoculated with M6 or M6 *edd*Ω*aphA*-3. Forty of these mice were C57BL/6J mice, while the remaining eight were RAG-KO 129 mice. Prior to the sacrifice date, one mouse from the wild-type SS1 group died. Mice were sacrificed 2 weeks postinoculation, and the number of CFU per gram of gastric mucosa was determined. In contrast, SS1 *edd*Ω*aphA*-3 did not colonize mice given less than 2×10^5 bacterial cells per inoculum. Wild-type SS1, as shown in Table 3, colonized mice even at the lowest inoculation dose of 200 bacteria. The lowest infectious dose for wild-type strain M6 was higher than that of SS1 (2×10^4 bacteria), and not all mice inoculated at this dose were infected. Of the strains tested, the mutant strain M6 *edd*Ω*aphA*-3

TABLE 2. Colonization of two mouse strains by *H. pylori* strain M6 or SS1 or the *edd* mutant[a]

Mouse strain	Parental bacterial strain	CFU/g of gastric mucosa (10^6) (mean ± SD)		*P* value	No. of mice infected/no. of mice colonized	
		Wild type[b]	Mutant[b]		Wild type[b]	Mutant[b]
C57BL/6J	SS1	69 ± 9.23	0.0001 ± 0	<0.0001	5/5	5/5
	M6	1.5 ± 2.06	0.21 ± 0.23	<0.05	15/15	13/16
RAG-KO 129	M6	1.0 ± 1.28	0.0011 ± 0.00214	>0.05	3/4	2/4

[a] Each mouse received one strain of bacteria (either wild type or mutant) at a dose of 10^9 CFU.
[b] Inoculum strain type.

TABLE 4. Cocolonization of two mouse strains by wild-type *H. pylori* and *edd* mutants[a]

Mouse strain	Parental bacterial strain	CFU/g of gastric mucosa (10^6) (mean ± SD)		*P* value	No. of mice infected/no. of mice colonized	
		Wild type[b]	Mutant[b]		Wild type[b]	Mutant[b]
C57BL/6J	SS1	97 ± 54.6	0.2 ± 0.34	<0.05	5/5	5/5
	M6	1.9 ± 2.31	0.05 ± 0.11	<0.05	10/10	2/10
RAG-KO 129	M6	0.023 ± 0.0356	0 ± 0	>0.05	3/4	0/4

[a] Mice were inoculated with a mixture of wild-type and mutant bacteria.
[b] Inoculum strain type.

required the highest dose to be infectious. This strain was unable to colonize well even when the mice were given 2×10^5 bacteria. Colonization results for the germfree RAG-KO mice paralleled the results for the C57BL/6J mice. Of four mice inoculated with 2×10^5 strain M6 bacteria, two became colonized while the mutant M6 *eddΩaphA*-3 colonized only one out of four mice.

Finally, the effect of a mixed inoculation dose containing high levels (10^9 CFU) of both wild-type and mutant strains was determined (Table 4). Five C57BL/6J mice were inoculated with a mixture of SS1 and SS1 *eddΩaphA*-3, 10 C57BL/6J mice were inoculated with a mixture of M6 and M6 *eddΩaphA*-3, and four germfree RAG-KO 129 mice were inoculated with a mixture of M6 and M6 *eddΩaphA*-3. In C57BL/6J mice, both SS1 and SS1 *eddΩaphA*-3 colonized all of the mice that had been inoculated, but the wild-type strain colonized at a two-fold-higher density ($P < 0.05$). In C57BL/6J mice, M6 colonized all of the mice that had been inoculated, while M6 *eddΩaphA*-3 colonized only 2 of 10 mice ($P < 0.05$). In RAG-KO 129 mice, the *H. pylori* M6 wild type colonized, although to a very low density, and M6 *eddΩaphA*-3 did not colonize ($P > 0.05$).

To evaluate the role of EDD in gastritis, 10 C57BL/6J mice were inoculated with *H. pylori* M6, 5 with the wild-type and 5 with the mutant strain, and were sacrificed 10 weeks following inoculation. At sacrifice, the bacterial densities were $(2.40 \times 10^5) \pm (1.77 \times 10^4)$ and $(9.5 \times 10^4) \pm (1.1 \times 10^4)$ CFU/g of gastric mucosa for the wild-type and mutant strains, respectively. The difference in densities between the two strains was not significant ($P > 0.05$). Histologic examinations of these mice were conducted, and gastritis was mild to moderate in mice killed at 10 weeks postinoculation (data not shown). There was no difference in the extent of gastritis in mice infected with the M6 wild-type strain and that in mice infected with M6 *eddΩaphA*-3.

H. pylori is unusual in its relatively small genome and its lack of many of the metabolic pathways that are crucial for the survival of other gram-negative enteric bacteria. However, our results demonstrated that low but detectable EDD activity was present in three strains of *H. pylori*, indicating that the *edd* gene and the ED pathway are conserved within the species and may serve some essential function.

We showed that while the ED pathway is not essential for colonization, it does confer an added advantage by increasing fitness in vivo. Both of the mouse-colonizing strains examined colonized significantly less densely than the wild type did, and the difference in fitness was reflected in differences in infectious dose. Interestingly, cocolonization studies suggested that in spite of decreased fitness for initial colonization, there is no evidence of competition for colonization sites in vivo between wild-type and EDD-negative *H. pylori*. In mice cochallenged with a large dose of SS1 and SS1 *eddΩaphA*-3, both strains colonized all mice. The colonization density of SS1 *eddΩaphA*-3 was lower than that of SS1, but the difference was similar to colonization densities in mice challenged with each strain separately, indicating that cocolonization did not alter colonization density. The explanation for this lack of competition is not clear, but it may reflect the multifocal distribution of *H. pylori* in vivo. The mutant and wild-type bacteria may colonize different anatomic regions of the stomach, as has been shown previously (1). Finally, our results suggest that loss of 6PGD does not diminish the persistence of *H. pylori* in the stomach. The colonization densities of M6 *eddΩaphA*-3 were not statistically different between mice killed 10 weeks after inoculation and mice killed 2 weeks after inoculation.

The role of the ED pathway in *H. pylori* is not known. Our studies show that, in contrast to *E. coli*, the pathway is not inducible in *H. pylori*. This may be due to the ability of *H. pylori* to utilize only glucose instead of a range of sugars, thus necessitating the capability to quickly metabolize the sugar whenever it is present. The pathway may allow the organism to utilize aldonic acids such as gluconate (12). Gluconate is present in mouse cecal mucus (14) and thus may be present in the stomach and could represent an energy source for *H. pylori* in the absence of competition from other bacteria. However, paralogs for gluconate transporters have not been found on the *H. pylori* genome (data not shown), and the use of this pathway for gluconate metabolism is as yet unproven.

In addition to providing information about the role of 6PGD in colonization by *H. pylori*, our findings are applicable to future studies using genetically altered *H. pylori* in mice. We found significant differences in colonization patterns that were dependent on both the parental *H. pylori* strain and the mouse strain. In C57BL/6J mice, strain SS1 was clearly the better and more consistent colonizer. Thus, differences between mutant and wild-type *H. pylori* strains were clearly defined and statistically significant. In contrast, differences between the colonizing abilities of M6 and M6 *eddΩaphA*-3 were smaller and did not reach significance in some experiments. The mouse strain also affected the colonization rate. The same wild-type strain, M6, colonized C57BL/6J mice better than it colonized RAG-KO 129 mice, thus suggesting that C57BL/6J mice are preferable for evaluation of mutant strain effects. These results suggest that both the parental bacterial strain and the mouse strain should be chosen with care when colonization mutants of *H. pylori* are being evaluated in animal models.

The precise role of the ED pathway in *H. pylori* metabolism remains unclear. It may serve as a reserve system for scavenging carbon or producing energy when few other sources are available. For example, when amino acids are not prevalent, the organism may be able to utilize glucose, a commonly available substrate. Further work in this direction may help elucidate details on the importance of this pathway in the colonization and survival of *H. pylori*.

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