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Diverse Phenotypes Resulting from Polyphosphate Kinase Gene (ppk1) Inactivation in Different Strains of *Helicobacter pylori*†

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Connections among biochemical pathways should help buffer organisms against environmental stress and affect the pace and trajectory of genome evolution. To explore these ideas, we studied consequences of inactivating the gene for polyphosphate kinase 1 (ppk1) in strains of *Helicobacter pylori*, a genetically diverse gastric pathogen. The PPK1 enzyme catalyzes synthesis of inorganic polyphosphate (poly P), a reservoir of high-energy phosphate bonds with multiple roles. Prior analyses in less-fastidious microbes had implicated poly P in stress resistance, motility, and virulence. In our studies, ppk1 inactivation caused the expected near-complete absence of poly P (>250-fold decrease) but had phenotypic effects that differed markedly among unrelated strains: (i) poor initial growth on standard brain heart infusion agar (five of six strains tested); (ii) weakened colonization of mice (4 of 5 strains); (iii) reduced growth on Ham’s F-12 agar, a nutritionally limiting medium (8 of 11 strains); (iv) heightened susceptibility to metronidazole (6 of 17 strains); and (v) decreased motility in soft agar (1 of 13 strains). Complementation tests confirmed that the lack of growth of one Δppk1 strain on F-12 agar and the inability to colonize mice of another were each due to ppk1 inactivation. Thus, the importance of ppk1 to *H. pylori* differed among strains and the phenotypes monitored. We suggest that quantitative interactions, as seen here, are common among genes that affect metabolic pathways and that *H. pylori*’s high genetic diversity makes it well suited for studies of such interactions, their underlying mechanisms, and their evolutionary consequences.

Many biochemical pathways are connected, in that a given metabolite can be generated and/or consumed by any of several enzymes, and the flux along one pathway can be influenced by genetic, culture-related, or environmental factors that affect traffic along complementary or competing pathways (30, 38). The resulting complex networks of interaction constitute a major focus of the new discipline of systems biology (see, e.g., reference 24); may underlie many of the epistasis (gene-gene interaction), penetrance, and quantitative-trait phenomena that are of major importance in medical, agricultural, and evolutionary genetics (13, 31); and are likely to affect the specificity and vigor of infection and virulence of pathogens. Metabolic networks exhibit intriguing formal similarities to phenomena such as food webs in natural ecosystems, patterns of human interaction, and the routing of electricity in power grids (43).

It is with this perspective that we have been studying how inactivation of the ppk1 gene, which encodes polyphosphate kinase, affects *Helicobacter pylori* (S. Tan, M. Zhang, C. D. Fraley, A. Kornberg, and D. E. Berg, Abstr. 103rd Gen. Meet.

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Am. Soc. Microbiol., abstr. 1316, 2003), a genetically diverse gastric pathogen (for reviews, see references 12 and 15). The PPK1 enzyme mediates synthesis of inorganic polyphosphate (poly P), a long-chain polymer typically containing hundreds of orthophosphate residues linked by phosphoanhydride bonds, as in ATP (for reviews, see references 10, 27, and 28). Poly P is thought to be present in all species and to constitute a reservoir of high-energy phosphate bonds. Biochemical experiments and studies of phenotypes of ppk1 mutants in fast-growing species, such as *Escherichia coli* and *Pseudomonas aeruginosa* had indicated additional roles for poly P, including inhibition of RNA degradation (9); activation of Lon protease (29); participation in membrane channel formation (50); and contribution to stress resistance, motility, quorum sensing, and virulence (10, 26, 41). Many bacterial species contain another enzyme, PPK2, that also synthesizes poly P (18, 51), but no ppk2 gene homolog was found in *H. pylori* genomes (51). In studies complementary to ours, others had reported that the ppk1 gene of *H. pylori* strain selected for an ability to maintain chronic infection in mice for 1 year, contained a 102-bp deletion, and that ppk1 inactivation reduced this strain’s vigor in mice (7, 8). They also suggested that inactivation of ppk1 in the unrelated strain X47-2AL made the strain unable to colonize mice. It is important, in terms of our experiments (see below), that their X47-2AL ppk1 null mutant seemed to be nonmotile in soft agar (7).

Most mutational analyses of ppk1’s roles have used only one strain of a bacterial species and thus have not addressed possible effects of background genotype or modifier loci that could help reveal connections among biochemical pathways. *H. pylori*
under microaerobic conditions (5% O2, 10% CO2, 85% N2). For motility studies, broth with 7% horse serum and vancomycin (6 mg/ml) was usually grown on brain heart infusion (BHI) agar with horse blood (hereafter referred to as F-12 agar). This is more fastidious and slower growing than other species in H. pylori, which poly P's role has been studied, contains a very small genome (one-third the size of E. coli's), and has relatively few regulatory genes (4, 48). These features encourage the idea that poly P might have special regulatory roles in H. pylori. Systems biology computational modeling of H. pylori metabolic networks has just begun but is focused on only one strain (26695) and has not included assessment of poly P's role(s) in analyses to date (39, 47). There is general recognition that more molecular genetic and biochemical studies are also much needed (47). Here we report that ppk1 inactivation generally decreases H. pylori fitness in culture and in vivo but with an intensity that varies markedly among strains according to the trait scored. Our results illustrate the importance of background genotype and epistatic interactions in shaping complex phenotypes in a simple prokaryote.

MATERIALS AND METHODS

H. pylori strains and general methods. The H. pylori strains used (Table 1) were from our laboratory collection, have been described previously (23, 25, 45), and were chosen to be broadly representative of this species's genetic diversity. H. pylori was usually grown on brain heart infusion (BHI) agar with horse blood or serum, isovitalex, and antibiotics, as described previously (2, 45), or in brucella broth with 7% horse serum and vancomycin (6 mg/ml). Incubation was at 37°C under microaerobic conditions (5% O2, 10% CO2, 85% N2). For motility studies, each brucella or BHI broth containing 0.35% agar was used (45). For nutrient-limited growth, Ham’s F-12 agar with cholesterol (100 mg/ml), bovine serum albumin (2 mg/ml), and vancomycin (6 mg/ml) (hereafter referred to as F-12 agar) was used (46).

For electroporation, exponentially growing H. pylori cells were harvested after overnight growth on BHI agar (10^7 to 10^9 cells), washed twice in 10% glycerol, and suspended in 100 μl of 10% glycerol at 4°C, and then 6 μl of purified PCR fragment or 3 μl of genomic DNA (100 to 300 ng) was added. The suspension was subjected to single-pulse electroporation (initial voltage of 2.5 kV; Bio-Rad Gene Pulser) in a prechilled 0.2 cm-gap cuvette; spread on BHI agar; incubated for ~20 h; and then transferred to BHI agar with chloramphenicol (Cam) (15 μg/ml), metronidazole (Mtz) (8 μg/ml), or erythromycin (Ery) (10 μg/ml) as appropriate and incubated for 3 to 7 days to select electroporants.

For natural transformation, cells grown overnight in brucella broth with shaking (early stationary phase) were diluted in fresh medium to an optical density at 600 nm of 0.1, 2 to 5 μg of genomic DNA was added as previously recommended (19), incubation was continued for 4 h, and cells concentrated by centrifugation from 1.5 ml of culture were spread on BHI Cam agar.

H. pylori genomic DNAs were isolated using QIAamp DNA mini kits (QIAGEN, Inc., Valencia, CA). Specific PCR for construction and scoring of mutant alleles was carried out using appropriate primers (sequences available on request). The Δppk1∆111, and frxA-ppk1 alleles used here have been described previously (21, 45). Two new ppk1 alleles were constructed directly by PCR without recombinant DNA cloning, as described previously (11, 45): (i) Δppk1-3, in which the entire 2-kb ppk1 gene was replaced with a nonpolared cat resistance gene (cat) (with no transcription terminator; this deletion is flanked by the xerA recombinase [hp1009] and pryD [hp1011] genes, as in sequenced strain 26695); and (ii) Δppk1-1, in which a central 1-kb segment of ppk1 was replaced with the same nonpolared cat cassette. Many H. pylori strains contain genes other than xerD just upstream of ppk1. In these strains there is still sufficient homology in the 0.5 kb of ppk1 sequences upstream and downstream of the 1-kb deletion/cat insertion of Δppk1-1 for this allele to replace the intact ppk1 gene, whereas

### TABLE 1. Phenotypes of Δppk1 H. pylori strains relative to wild-type parental strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Country of origin</th>
<th>Motility of Δppk1</th>
<th>EOP on F-12 of Δppk1</th>
<th>Mtz MIC (μg/ml) for:</th>
<th>WT</th>
<th>Δppk1 strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>88-3887</td>
<td>UK</td>
<td>Decreased</td>
<td>&lt;10^-6d</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>89-3887 ΔrdxA ΔfrxA-e</td>
<td>UK</td>
<td>NA</td>
<td>NA</td>
<td>64</td>
<td>16</td>
<td></td>
</tr>
<tr>
<td>AM1</td>
<td>India</td>
<td>Same</td>
<td>0.001</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>AM2</td>
<td>India</td>
<td>Same</td>
<td>1</td>
<td>128</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>SS1</td>
<td>Australia</td>
<td>Same</td>
<td>0.1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>SS1 (NT)</td>
<td>Australia</td>
<td>Same</td>
<td>0.1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>X47-2AL</td>
<td>US</td>
<td>Same</td>
<td>1</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>X47-2AL (NT)</td>
<td>US</td>
<td>Same</td>
<td>2</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>26695</td>
<td>UK</td>
<td>ND</td>
<td>&lt;10^-6d</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>J99</td>
<td>US</td>
<td>ND</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>R64</td>
<td>S. Africa</td>
<td>ND</td>
<td>0.1</td>
<td>3</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>R66</td>
<td>S. Africa</td>
<td>ND</td>
<td>0.1</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>HK152</td>
<td>Hong Kong</td>
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<td>0.001</td>
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<td>2</td>
<td></td>
</tr>
<tr>
<td>PCM4</td>
<td>Hong Kong</td>
<td>Same</td>
<td>0.1</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>Chen13</td>
<td>India</td>
<td>Same</td>
<td>1</td>
<td>1</td>
<td>1</td>
<td></td>
</tr>
<tr>
<td>PCan28</td>
<td>Peru</td>
<td>Same</td>
<td>ND</td>
<td>2</td>
<td>2</td>
<td></td>
</tr>
<tr>
<td>A66</td>
<td>Lithuania</td>
<td>Same</td>
<td>ND</td>
<td>0.75</td>
<td>0.75h</td>
<td></td>
</tr>
<tr>
<td>YN1-16</td>
<td>China</td>
<td>Same</td>
<td>ND</td>
<td>64</td>
<td>64</td>
<td></td>
</tr>
<tr>
<td>YN1-18</td>
<td>China</td>
<td>Same</td>
<td>ND</td>
<td>128</td>
<td>128</td>
<td></td>
</tr>
<tr>
<td>CPY3401</td>
<td>Japan</td>
<td>Same</td>
<td>ND</td>
<td>3</td>
<td>3</td>
<td></td>
</tr>
<tr>
<td>HUP-B63</td>
<td>Spain</td>
<td>ND</td>
<td>ND</td>
<td>2</td>
<td>1</td>
<td></td>
</tr>
</tbody>
</table>

a Colony counts as determined by EOP on Ham's F-12 medium (see Materials and Methods).

b WT, wild type.

c 88-3887 and 26695 are closely related strains and are considered here as one lineage.

d ΔrdxAΔfrxA and rdxA-ppk1 are null deletion alleles (see reference 21).

e NA, not assayed, because strain was derived from parent strain 88-3887.

f NT, strain was obtained as a natural transformant. All other Δppk1 strains were obtained as electroporants.

g The Δppk1 strain did not form single colonies.

h The Δppk1 strain EOP at the MIC was ~10-fold lower than that of the wild type, indicating increased sensitivity of the mutant.

i The Δppk1 strain colonies were smaller than the wild type on F-12 agar.

j ND in the Motility column indicates that the assay was not done. This is because the wild type was not sufficiently motile. Same, same as that of the wild type.

k The Δppk1 strain was normally grown on brain heart infusion (BHI) agar with horse blood.

l Colony counts as determined by EOP on Ham's F-12 medium (see Materials and Methods).

m UK, United Kingdom; US, United States; S. Africa, South Africa.
homology requirements make replacement by the Δppk1-2 (complete deletion) allele unfeasible. For this reason, most experiments presented here used the Δppk1-1 allele.

To make strains with two copies of ppk1, a PCR product containing ppk1 in place of nearly all of rdxA (nitroreductase gene) between the normally flanking genes hp0953 and lgt was made without DNA cloning, as described previously (11, 45). This PCR product was used to transform derivatives of strains SS1 and X47-2AL that already carried an aphA-frxA-erdxA (nitroreductase gene) between the normally flanking hp0953 and lgt genes (11, 45). This PCR product was used to transform derivatives of strains SS1 and X47-2AL electroporants made using a PCR product which in intact ppk1 replaces most of rdxA and selected for Mtz resistance. Lane 1, pool of Mtz' electroporants (the −3 kb band generated from the ppk1 partial diploid is nearly invisible because of its size and low abundance); lane 2, pool of Mtz' Cam' electroporants made using Δppk1-1 DNA (note that only partial diploids were recovered); lane 3, 1:1 mix of wild-type rdxA and ΔrdxA/ppk1 alleles (smaller wild-type allele is amplified preferentially). (D) PCR assays of the normal ppk1 locus and the rdxA locus from Cam' transformants. These complementary tests show a Δppk1-1 allele either in the rdxA/ppk1 locus (①) or the native ppk1 locus (②). Results from a pool of Cam' electroporants (Pool) and from a reconstruction with a 1:1 mixture of Δppk1-1 and ppk1 wild-type purified DNAs (1:1) are shown. (E) Structure of the rdxA region in which rdxA was replaced by intact ppk1, and ery (resistance gene) was added to facilitate placement of the added ppk1 locus in other strains.

FIG. 1. ppk1 and rdxA loci, structures of ppk1 duplication strains, and PCR verifications. Approximate positions of PCR primers flanking ppk1 and rdxA that were used in these analyses are indicated by half arrows (primer sequences available on request). (A) Structures of gene loci in wild-type (WT) haploid strains. (B) Gene arrangements in the ppk1 partial-diploid parent strain and the two possible Cam' Δppk1-1 electroporants. ①, intact ppk1 in normal ppk1 locus (left), Δppk1 in rdxA-ppk1 locus (right); ②, Δppk1 in normal ppk1 locus (left), intact ppk1 in rdxA-ppk1 locus (right). (C) Characterization by PCR of the rdxA locus in X47-2AL electroporants made using a PCR product in which intact ppk1 replaces most of rdxA and selected for Mtz resistance. Lane 1, pool of Mtz' electroporants (the −3 kb band generated from the ppk1 partial diploid is nearly invisible because of its size and low abundance); lane 2, pool of Mtz' Cam' electroporants made using Δppk1-1 DNA (note that only partial diploids were recovered); lane 3, 1:1 mix of wild-type rdxA and ΔrdxA/ppk1 alleles (smaller wild-type allele is amplified preferentially). (D) PCR assays of the normal ppk1 locus and the rdxA locus from Cam' transformants. These complementary tests show a Δppk1-1 allele either in the rdxA/ppk1 locus (①) or the native ppk1 locus (②). Results from a pool of Cam' electroporants (Pool) and from a reconstruction with a 1:1 mixture of Δppk1-1 and ppk1 wild-type purified DNAs (1:1) are shown. (E) Structure of the rdxA region in which rdxA was replaced by intact ppk1, and ery (resistance gene) was added to facilitate placement of the added ppk1 locus in other strains.
FIG. 2. PCR characterization of Cam′ (Δppk1) electroporants in representative haploid or ppk1 partial-diploid strains, using PCR locus-specific primers (sequences available on request). Profiles shown are from representative Δppk1-1 (1-kb deletion) electroporants (lanes 2, 3, and 5); representative Δppk1-2 (2-kb deletion) electroporants (lanes 8 and 9); a rare ectopic integration of the cat determinant (lane 4); a rare spontaneous ppk1 duplication in X47-2AL (lane 6); and the only bona fide Δppk1 electroporants obtained from SS1 and X47-2AL (lanes 5 and 9, respectively). Lanes 1 and 7 show the PCR products using primers that had detected the Δppk1-1 and Δppk1-2 alleles, respectively.

Experimental infections. C57BL/6j wild-type mice and their isogenic cytokine interleukin 12β (IL-12β) and IL-10 knockout derivatives (Jackson Laboratories, Bar Harbor, ME) were maintained in the Washington University Medical School Animal Quarters with water and standard mouse chow given ad libitum and used in an Animal Studies Committee-approved protocol. Mice were inoculated with exponentially growing bacteria (2), and colonization was scored 2 weeks later by quantitative culture and genetic testing of individual colonies (2, 44). The 1-sample sign test or Mann-Whitney test was used to assess statistical significance, as appropriate.

RESULTS

Isolation of Δppk1 derivatives of H. pylori. An electroporation protocol, which includes ~20 h of outgrowth for recovery from electric shock and expression of an introduced resistance gene, was used first to replace wild-type ppk1 alleles with non-polar Δppk1 deletion alleles (marked with cat, a Cam′ determinant) in numerous H. pylori strains. Dozens to hundreds of normal-appearing Cam′ electroporant colonies were obtained using strains 26695 (also 88-3887, the motile, mouse-colonizing variant of 26695 [22]) and J99, whose genomes were previously sequenced (4, 48), as recipients, and then with 13 of 15 additional strains tested. PCR carried out with several Cam′ electroporants from each lineage showed the expected replacement of intact ppk1 by Δppk1-1 or Δppk1-2 in each case (Fig. 2). In contrast, only rarely were Cam′ colonies obtained by electroporation of SS1 or X47-2AL in several different attempts and with each of the two Δppk1 alleles (<1% expected frequency). Control electroporations with a ΔureAB-cat allele (45) or a cat-marked pscBC allele (37) indicated that electroporation and selection for a Cam′ phenotype was efficient in these two strains when other chromosomal loci were involved. Thus, it seemed that ppk1 inactivation might usually be particularly deleterious or lethal in strains SS1 and X47-2AL.

PCR tests of the rare Cam′ electroporants of SS1 and X47-2AL identified three types: (i) one electroporant each from SS1 and X47-2AL contained intact ppk1 and no Δppk1 allele, suggesting illegitimate recombination of cat-containing DNA into an ectopic site; (ii) one electroporant from X47-2AL contained both intact ppk1 and a Δppk1-1 allele, which might reflect spontaneous duplication of the ppk1 gene segment (as in reference 5); and (iii) one electroporant from each strain contained the desired Δppk1 alleles in place of intact ppk1 (Δppk1-1 and Δppk1-2, respectively) (Fig. 2). The rarity of Cam′ electroporants of these two strains, and their often aberrant nature, suggested that ppk1 inactivation was often deleterious or lethal. Such bona fide but rare ppk1-deficient electroporants might carry suppressors that bypass the need for a functional ppk1 gene.

Support for this inference came from studies of SS1 and X47-2AL populations in which some 5 to 10% of cells contained two copies of ppk1—one at the normal ppk1 locus and a second in the rdxA locus (Fig. 1)—and the remaining ≈90% carried only one ppk1 gene (see Materials and Methods). Electroporation of these mixed haploid and partially diploid populations using Δppk1-1 DNA yielded hundreds of Cam′ electroporants, instead of the few obtained with purely haploid recipients. PCR tests of single and pooled Cam′ colonies indicated that all new transformants were of the minority, partial-diploid type (Fig. 1C); and that the Δppk1 allele was incorporated into the normal ppk1 locus and the added ppk1 gene with equal frequency (Fig. 1D). This recovery of only partial diploids from the mixed haploid/partial-diploid population indicates that loss of ppk1 function is deleterious or lethal in these strains.

An alternative natural-transformation protocol (19) was used to further assess the importance of ppk1 for SS1 and X47-2AL. No electric shock was used, and just a few hours of outgrowth was allowed (to express the introduced resistance marker), in contrast to the overnight growth used in a standard electroporation protocol. Natural transformation of SS1 and X47-2AL with genomic DNAs from Δppk1-1 or Δppk1-2 strains resulted in hundreds of Cam′ colonies. These colonies were heterogeneous in size, and most were minute and slow growing initially (many were detected only after 5 days, rather than the usual 3 days, of incubation). Nevertheless, PCR tests of representative single colonies and pools of colonies showed replacement of intact ppk1 by the appropriate Δppk1 allele (for SS1, Δppk1-1; for X47-2AL, Δppk1-2) in every case (as in Fig. 2). New colonies formed by cells from the initial Δppk1 colonies were uniform in size and grew nearly as well as their wild-type parents. Collectively, these results indicated that an intact ppk1 gene contributes to, or is needed for, normal growth in these strains and suggested that the slow growth of Δppk1 derivatives of strains SS1 and X47-2AL may be compensated by suppressor mutations elsewhere in the genome.

The generality of these effects was tested by natural transformation of four additional H. pylori strains, chosen because preliminary experiments had shown that Cam′ transformants obtained using ΔureABC-cat DNAs formed colonies that were normal and of uniform size. With three of these strains (J99, CPY3401, and PCM4), Cam′ (Δppk1-1) transformant colonies were mostly small (but larger than those of SS1 and X47-2AL Δppk1 transformants) or heterogeneous in size, whereas those of the fourth strain (HUP-B63) were similar in size to those made with ΔureABC-cat DNA. These outcomes indicate that ppk1 inactivation can result in a continuum of growth effects, ranging from negligible to severe depending on the strain, and thus its genotype, and at least partial compensation for its
Deleterious effects can be achieved by suppressor mutations at unknown loci.

Poly P levels in *H. pylori* strains. Poly P levels in five representative wild-type *H. pylori* strains (88-3887 and J99, whose genomes have been sequenced previously, and also X47-2AL, SS1, and CPY3401) and in their isogenic Δ*ppk1* derivatives were measured in a standard assay, which entails production of ATP from poly P and ADP, and then by ATP quantitation in a luciferase reaction. The levels of poly P found in wild-type strains ranged from 15 to 116 nanomoles of phosphate per mg of total cell protein (depending on strain and/or growth phase), much as has been seen in other gram-negative bacterial species. Most important for the present studies, in each case, *ppk1* inactivation resulted in severe reduction in poly P levels (at least 250-fold) during both exponential and early stationary phases of growth (data not shown). In accordance with these quantitative data, transmission electron microscopy of wild-type strain 88-3887 revealed large bodies, generally interpreted as poly P granules (33, 40), in more than half of the cell sections, whereas putative nucleoids (16) but no such granules were detected in >100 sections of its Δ*ppk1-1* derivative (Fig. 3A).

Effect of *ppk1* inactivation on motility. With each of a half-dozen bacterial species studied previously, *ppk1* inactivation had caused marked reductions in motility in soft agar (36, 41) and was similarly reported by others (7) to cause a near-complete loss of motility in *H. pylori* strain X47-2AL. In contrast, we found that *ppk1* inactivation had little if any effect on motility in 12 of the 13 *H. pylori* strains tested, as illustrated in Fig. 4A and B. Of particular note, X47-2AL’s Δ*ppk1* derivatives—both the natural transformants that colonized mice very poorly (noncolonizers) and the single electroporant that had colonized mice well in single infection (good colonizer)—exhibited near-normal motility (Fig. 4B). This outcome differed from that reported by others (7) using a Δ*ppk1* derivative of the same strain, X47-2AL. This discrepancy may be explained by our use of recipient bacterial populations that recently had been cultured from mice and/or preselected for high motility in soft agar and by the tendency of nonmotile subclones to accumulate if there is no such preselection (45).

Of the 13 strains tested, only in 88-3887 was motility strongly reduced by *ppk1* inactivation (Fig. 4C), whereas no reduction in motility was detected in control experiments with *Cam*' *SureAB-cat* transformants of this same strain. Electron microscopy revealed clusters of flagella on Δ*ppk1* cells that seemed normal in appearance and number per cell pole (Fig. 4B). Curiously, however, one-third of these Δ*ppk1* cells from each of two independent cultures contained flagella at both poles (Fig. 3B), whereas only one-sixth of wild-type parent cells had such a bipolar arrangement (200 cells were scored in each group). The Δ*ppk1* cells with flagella at both poles seemed slightly longer than those with flagella at one pole, as expected (3.33 ± 0.54 microns versus 2.39 ± 0.49 microns; sample size, 20 cells of each type). The corresponding lengths of isogenic
wild-type cells were 2.95 ± 0.25 and 2.25 ± 0.25 microns. Among the possible explanations for these length distributions and standard deviations, we are drawn to a model in which \( pppk1 \) deficiency tends to delay the final separation of daughters during the cell cycle.

**Effect of \( pppk1 \) inactivation on growth on Ham’s F-12 agar.** A defined culture medium, modified Ham’s F-12 agar, was used to test for effects of \( pppk1 \) inactivation on growth under apparent nutrient stress. Many \( H. pylori \) strains can grow on F-12 agar (46), although, in our experience, often with reduced colony-forming efficiency (efficiency of plating [EOP]) and growth rate, relative to those on standard BHI agar. The effect of \( pppk1 \) inactivation was tested using 11 unrelated strains whose EOP on F-12 agar were at least 10% of their respective EOP on BHI agar. A variety of effects was observed: no detected growth deficiency with three strains; decreased colony size but no EOP with one strain (J99); modest decreases (10- to 1,000-fold) in EOP in six strains; and an inability to form colonies in two strains (26695 and the related 88-3887) (EOP < 10\(^{-6} \)) and, however, restrengthening residual growth of 26695 \( \Delta ppk1 \) and 88-3887 \( \Delta ppk1 \) after 7 days of incubation on fresh F-12 agar allowed the recovery of a few colonies. Their F-12-adapted phenotype was maintained after passage on BHI agar, which indicates presence of compensatory (suppressor) mutations, not epigenetic change.

A complementation test was used to assess whether 88-3887 \( \Delta ppk1 \)'s inability to grow on F-12 agar was due to \( \Delta ppk1 \) itself or a putative modifier mutation selected during outgrowth of transformants on F-12 agar. To accomplish this, an Ery resistance marker was placed immediately downstream of the intact \( pppk1 \) gene that had been inserted into the rdxA locus (Fig. 1E). Then, 88-3887 \( \Delta ppk1 \) was transformed to Ery\(^+ \) with genomic DNA from a strain carrying this construct. The presence of both intact \( pppk1 \) in \( rdxA \) and \( \Delta ppk1 \) at the normal \( ppk1 \) locus (between \( xerD \) and \( pyrD \)) was verified by PCR in representative transformants. Phenotype tests showed that these partial-diploid transformants formed colonies as efficiently as their wild-type ancestor on F-12 agar. Thus, the \( \Delta ppk1 \)-associated growth deficiency is due to this null allele itself, not a modifier mutation elsewhere in the genome.

**Effect of \( pppk1 \) inactivation on antimicrobial susceptibility.** We tested for effects of \( pppk1 \) inactivation on susceptibility to metronidazole (Mtz), an agent whose activation products cause extensive DNA breakage and mutagenesis (42). The results of tests in which the viability of wild-type and isogenic \( \Delta ppk1 \) cultures were estimated on different halves of the same 2 plates (21) showed that \( pppk1 \) inactivation increased susceptibility to Mtz in 6 of 17 strains tested (Table 1). Mtz resistance can be increased by inactivating \( rdxA \) and \( frxA \), whose encoded nitroreductases help activate Mtz (21). Introduction of a \( \Delta ppk1 \) allele into 88-3887 \( \Delta rdxA \) \( \Delta frxA \) also diminished Mtz resistance (the MIC was reduced from 64 to 16 \( \mu g/ml \) [Table 1]). In complementatory tests using other antimicrobials, no effect of a \( \Delta ppk1 \) allele on susceptibility of strain 88-3887 to clarithromycin or amoxicillin was detected (MICs of 0.5 and 2 \( \mu g/ml \), respectively), whereas the \( \Delta ppk1 \) allele made X47-2AL slightly more sensitive to amoxicillin (MIC of 1 \( \mu g/ml \) versus 2 \( \mu g/ml \) for the wild type) and made SS1 slightly more sensitive to clarithromycin (EOP of \( <10^{-3} \) [no distinct colonies] versus EOP of 0.01 for the wild type at a MIC of 0.01 \( \mu g/ml \)).

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### Table 2. Effects of \( pppk1 \) inactivation on mouse colonization

<table>
<thead>
<tr>
<th>( H. pylori ) strain</th>
<th>C57BL/6J mouse strain</th>
<th>Mean no. of CFU/ stomach(^a)</th>
<th>No. of ( \Delta ppk1 ) inoculated mice</th>
</tr>
</thead>
<tbody>
<tr>
<td>88-3887(^d)</td>
<td>IL-12β KO</td>
<td>1 × 10(^7)</td>
<td>2 × 10(^4)</td>
</tr>
<tr>
<td>AM1(^a)</td>
<td>IL-12β KO</td>
<td>5 × 10(^4)</td>
<td>4 × 10(^3)</td>
</tr>
<tr>
<td>AM2(^d)</td>
<td>IL-12β KO</td>
<td>5 × 10(^4)</td>
<td>5 × 10(^3)</td>
</tr>
<tr>
<td>SSF(^f)</td>
<td>WT</td>
<td>1 × 10(^6)</td>
<td>7 × 10(^5)</td>
</tr>
<tr>
<td>SSF(^f)(NT)(^f)</td>
<td>WT</td>
<td>1 × 10(^6)</td>
<td>1 × 10(^6)</td>
</tr>
<tr>
<td>X47-2AL(^g)</td>
<td>IL-12β KO</td>
<td>1 × 10(^6)</td>
<td>6 × 10(^5)</td>
</tr>
<tr>
<td>X47-2AL</td>
<td>WT</td>
<td>1 × 10(^6)</td>
<td>1 × 10(^6)</td>
</tr>
<tr>
<td>X47-2AL(^f)(NT)(^f)</td>
<td>WT</td>
<td>1 × 10(^6)</td>
<td>5 × 10(^5)</td>
</tr>
<tr>
<td>X47-2AL/ PD(^h)</td>
<td>WT</td>
<td>1 × 10(^6)</td>
<td>1 × 10(^6)</td>
</tr>
</tbody>
</table>

\(^a\) The entire stomach was removed from each mouse, homogenized without weighing to minimize aerobic killing of \( H. pylori \), and plated out for isolation of individual colony-forming units.

\(^b\) Typical values as obtained in previous experiments with at least 10 mice per strain.

\(^c\) All mice were either C57BL/6J IL-12β deficient or wild-type (WT) C57BL/6J, KO, knockout.

\(^d\) The \( \Delta ppk1 \) strain carries the \( \Delta ppk1-1 \) allele.

\(^f\) NT, \( \Delta ppk1 \) strain obtained as a natural transformant. All other \( \Delta ppk1 \) strains were obtained as electroporants.

\(^g\) Five and seven colonies were seen in direct smears of stomach tissues from 2 of 11 mice, respectively, and none were found in the other 9 mice.

\(^h\) Five mice each for two different pools of X47-2AL complemented partial-diploid pools.

\(^i\) PD, complemented \( ppk1^{-1}/\Delta ppk1 \) partial diploid derived from X47-2AL. 

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**Effect of \( pppk1 \) inactivation on ability to colonize mice.** The effect of \( pppk1 \) inactivation in vivo was tested by inoculating C57BL/6J mice or cytokine IL-12β-deficient derivatives (recommended for many \( H. pylori \) strains [17]) with \( \Delta ppk1 \) derivatives of five distinct lineages and scoring densities of \( H. pylori \) in the gastric mucosa 2 weeks later. With strains of three lineages (88-3887, AM1, and AM2), \( \Delta ppk1 \) derivatives achieved densities some 5- to 12-fold lower than those achieved by their isogenic wild-type parents (Table 2). Competition tests were carried out with 88-3887 \( \Delta ppk1 \), the most vigorous of these three strains. Mice were inoculated with 1:1 mixtures of the \( \Delta ppk1 \) mutant and its wild-type parent and sacrificed 2 weeks later; gastric homogenates were spread on Cam-free agar, and individual colonies (20 per mouse) were tested for Cam susceptibility. These tests indicated that >99% of recovered \( H. pylori \) strains were wild type (Cam\(^+\)) (significantly different from the 50% expected if \( ppk1 \) did not affect fitness; \( P = 0.002; 1\)-sample sign test) (Fig. 5). An equivalent result was obtained using derivatives of 88-3887 \( \Delta ppk1 \) that had been passaged once in mice to select for any possibly better-adapted derivatives. Thus, \( \Delta ppk1 \), while allowing these strains to establish low-grade mouse infections, decreased their vigor in vivo.

Several distinct phenotypes were observed with \( \Delta ppk1 \) derivatives of strain X47-2AL. The single electroporant colonized IL-12β-deficient, and also wild-type, C57BL/6J mice at densities similar to those of its \( ppk1 \) wild-type parent when inoculated alone (Table 2). However, only ~1% of \( H. pylori \) strains recovered after coinoculation of the \( \Delta ppk1 \) electroporant and its wild-type parent (1:1 mixture) were Cam\(^+\) (Fig. 5). In an equivalent test, but using a pool of \( \Delta ppk1 \) derivatives that had been cultured from mice (after 2 weeks of infection),
about 10% of colonies recovered were Cam\(^t\) (significantly different from the \(<1\%\) found with X47-2AL \(\Delta ppk1\) that was not passed in mice; \(P < 0.02;\) Mann-Whitney test) (Fig. 5). This suggested emergence of a partial suppressor of deleterious effects of \(ppk1\) inactivation. Finally, the \(\Delta ppk1\) natural transformants of X47-2AL seemed nearly incapable of mouse colonization, although they had near-normal motility (Fig. 4). Only five and seven \(H. pylori\) colonies per stomach were obtained from 2 of 11 inoculated mice, respectively, and no colonies were obtained from any of the other 9 mice that had been inoculated with several different pools of such natural transformants. In contrast, thousands of colonies were routinely recovered from each mouse inoculated with wild-type X47-2AL (Table 2).

A complementation test was used to assess whether the loss of colonization ability of X47-2AL \(\Delta ppk1\) natural transformants was due to \(\Delta ppk1\) itself. Cells of strain X47-2AL \(\Delta ppk1\) that had failed to colonize mice were transformed with genomic DNA from the \(ery\)-marked partial-diploid strain (Fig. 1E), as described above, and a pool of \(Ery^t\) transformants was used to inoculate \(C57BL/6\) mice. Quantitative culture 2 weeks later indicated that each of the 10 mice tested had become infected at bacterial densities matching those achieved with wild-type X47-2AL (Table 2). Thus, the inability of X47-2AL \(\Delta ppk1\) natural transformant to colonize mice efficiently is likely due to \(\Delta ppk1\) itself, not a suppressor possibly selected during outgrowth.

In contrast to the results for strain X47-2AL, \(\Delta ppk1\) derivatives of strain SS1, generated by natural transformation or by electroporation, each seemed fully capable of mouse colonization when inoculated alone (Table 2) or in competition with the wild type (Fig. 5). The relative yields were also not much affected by mouse genotype: yields were similar for wild-type \(C57BL/6\) mice and their cytokine IL-10- and IL-12β-deficient derivatives (Fig. 5), which exhibit stronger and weaker inflammatory responses to infection, respectively (14, 17).

**DISCUSSION**

We found that \(ppk1\) inactivation, which resulted in a near absence of long-chain poly P, generally decreased \(H. pylori\)’s fitness but with an intensity that differed markedly among strains and the phenotypes scored. A first indication of diversity in phenotypic effects came from efforts to generate \(\Delta ppk1\) strains by electroporation: this was far more difficult in SS1 and X47-2AL than in any of the 15 other \(H. pylori\) strains tested. In contrast, \(\Delta ppk1\) derivatives of SS1 and X47-2AL were easily made by natural transformation. Initially the transformants grew far more slowly than did those of many other strains, but faster-growing variants accumulated as the transformants were cultured. These results indicated that \(ppk1\) inactivation can be deleterious for growth in culture and that the severity of this effect depended on the background genotype. The rarity of \(\Delta ppk1\) electroporants of SS1 and X47-2AL may stem from poor recovery from electric shock or overgrowth by nontransformed wild-type siblings during the ensuing \(<20\ h\) of incubation.

Effects of \(ppk1\) inactivation that differed quantitatively among strains were also evident on nutritionally limiting F-12 agar. At the extremes were sequenced strains 26695/88-3887 \(\Delta ppk1\) (EOP \(\leq\) \(10^{-8}\)) and J99 \(\Delta ppk1\) (EOP 1); \(\Delta ppk1\) derivatives of many other strains showed intermediate (\(<10\)- to 1,000-fold) reductions in EOP (Table 1). The poor growth of some strains might be explained by specific auxotrophy caused by the \(ppk1\) deficiency (although F-12 agar contains a full complement of free amino acids, vitamins, and other organics) or by death of cells before adaptation to F-12 agar. By extrapolation from \(E. coli\) (9, 29), this poor-growth phenotype might also be ascribed to effects of poly P on RNA or protein turnover. Identification of suppressor mutations that restore the ability of 26695 or 88-3887 \(\Delta ppk1\) to grow on F-12 agar (perhaps as discussed in reference 34 or 49) should help identify mechanisms involved and thereby better define poly P’s roles.

The lack of effect of a \(ppk1\) deficiency on motility in most \(H. pylori\) strains seemed remarkable, given its effects in other species (10, 36, 41) and the appeal of models invoking poly P as a regulator of flagellar motor assembly or energy source for flagellar movement. In this, our results with X47-2AL differ from those of other investigators (7), who interpreted \(ppk1\) inactivation as causing a nearly complete loss of motility. Although further study is needed, precedent (45) suggests that the lost motility they reported could be due to heterogeneity in their recipient population and their fortuitous use of a \(\Delta ppk1\)
transfomrant of a preexisting nonmotile variant subclone. This said, we found motility to be reproducibly reduced in Δppk1 transformants of strain 88-3887. One explanation for this curious effect, based on the unexpected abundance of Δppk1 cells with flagella at both ends, assumes delays in the cell cycle or disruption of normal coupling between flagellar synthesis and cell division and less effective directional swimming by such “bipolar” cells. Further study is needed to understand how motility and flagellar distributions can be linked to, or disengaged from, poly P availability and other metabolic functions.

Mtz susceptibility was also increased by Δppk1 inactivation in more than one-third of H. pylori strains tested. This effect was slight but credible because mutant and wild-type strains were assayed on different halves of the same Mtz-containing plate, but its basis is not known. Among possible models, we are drawn to those invoking effects of Δppk1 inactivation on general cell permeability or stress resistance (27, 28, 50), because slight increases in susceptibility to clarithromycin or to amoxicillin were also observed in some Δppk1 strains.

Quantitative differences among strains in the need for Δppk1 were also evident in vivo. With SS1, Δppk1 inactivation had no significant effect on fitness even in competition tests using the wild-type strain and its isogenic Δppk1 derivatives. Fitness was also not much affected by use of wild-type mice or congenic animals that lacked cytokine IL-12 or IL-10, which have diminished and heightened host responses to infection, respectively (14, 17).

In contrast, with strain X47-2AL, Δppk1 derivatives obtained by natural transformation were nearly incapable of mouse colonization, even when inoculated alone. The rare Δppk1 electroporant seemed as vigorous as the wild type during single infection, but it was less fit during mixed infection. One explanation for these findings assumes that Δppk1 is not very important in vivo for X47-2AL and that inability of natural transformants to infect mice was due to a suppressor mutation that might have been selected in culture. However, complementation with an intact Δppk1 gene restored their ability to colonize mice. It is therefore likely that X47-2AL has a strong intrinsic need for Δppk1 with poly P in vivo and that this need is partially suppressed by the mutation that allowed recovery of a Δppk1 electroporant.

The Δppk1 allele caused mild impairment of growth in vivo in three other lineages, 88-3887, AM1, and AM2 (intermediate between those seen in SS1 and those in X47 natural transformants). The decreased fitness of 88-3887 Δppk1 might be due to its weak motility (Fig. 4), but other explanations are needed for the other two strains.

Evolutionary inferences. We suggest that the variety of phenotypes caused by Δppk1 inactivation in H. pylori illustrates this pathogen’s extraordinary genetic diversity and points to connectedness, redundancy, and resilience in metabolic pathways. The patterns observed suggest a significant repertoire of potential modifiers or suppressors. Formally, this repertoire may be the microbial counterpart of the quantitative trait loci that underlie much of the phenotypic diversity within higher organism species (13, 31), most of which are still poorly understood at a mechanistic level. Mutations that modify the impact (penetrance) of Δppk1 mutations seemed to emerge easily in many Δppk1 H. pylori strains and may preexist in others. The need for such suppressors in some strains and their preexistence in others can be ascribed to (i) genetic drift or (ii) selection, albeit for other traits that only inadvertently affect consequences of Δppk1 inactivation. These findings recall Francois Jacob’s early suggestion that evolution be likened to a tinkerer who mostly modifies and adjusts already-established genes or processes, not a design engineer (20). The complexity of effects of Δppk1 inactivation found here may be quite typical of genes for non-essential components of metabolic webs in any genetically diverse species.

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