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) weak growth on Ham's F-12 agar, a nutritionally limiting medium (8 of 11 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. 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 Hp141v, a mutant 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, or serum, isovitalex, and antibiotics, as described previously (2, 45), or in brucella H. pylori and were chosen to be broadly representative of this species's genetic diversity. were from our laboratory collection, have been described previously (23, 25, 45),

<table>
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<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>
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<td>Decreased</td>
<td>&lt;10°/6</td>
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^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 The Δppk1 strain did not form single colonies.
^e rdxA111 and frxA-aphA are null deletion alleles (see reference 21).
^f NA, not assayed, because strain was derived from parent strain 88-3887.
^g NT, strain was obtained as a natural transformant. All other Δppk1 strains were obtained as electroporants.
^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 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.
^j The Δppk1 strain EOP was ~100-fold lower than that of the wild type, indicating increased sensitivity of the mutant.
^k Δppk1 colonies were smaller than the wild type on F-12 agar.
^l ND in the EOP column indicates that the assay was not done because the wild type grows poorly on F-12 agar (growth is <10% that on BHI blood agar).
^m UK, United Kingdom; US, United States; S. Africa, South Africa.

should be very useful in studies of metabolic networks and their plasticity and evolution: it is genetically diverse (1, 3, 32), is more fastidious and slower growing than other species in 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 μg/ml). Incubation was at 37°C under microaerobic conditions (5% O2, 10% CO2, 85% N2). For motility studies, either brucella or BHI broth containing 0.35% agar was used (46). For electroporation, exponentially growing H. pylori cells were harvested after overnight growth on BHI agar (~10^7 to 10^8 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 genetic 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 ΔureAB cat, rdxA111, and frxA-aphA 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) Δppk1-1, in which the entire 2-kb ppk1 gene was replaced with a nonpolar Cam resistance gene (cat) (with no transcription terminator; this deletion is flanked by pspk1 [hp1011] and pyrD [hp011] genes, as in sequenced strain 26695); and (ii) Δppk1-1, in which a central 1-kb segment of ppk1 was replaced with the same nonpolar 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...
homology requirements make replacement by the Δppk1-1 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 (resistance gene) was added to facilitate placement of the added ppk1 gene at the ppk1 locus. PCR tests indicated that Δppk1-1 replaces most of rdxA and selected for Mtz resistance. Lane 1, pool of MtzR electroporants; lane 2, pool of MtzR CamR electroporants made using Δppk1-1 DNA (note that only partial diploids were recovered); lane 3, 1:1 mix of wild-type and Δppk1-1 alleles (smaller wild-type allele is amplified preferentially). (D) PCR assays of the normal ppk1 locus and the rdxA locus from CamR 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 CamR 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 CamRΔppk1-1 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 MtzR 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 MtzR CamR 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 CamR 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 CamR 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.

Poly P assays. Poly P was extracted, purified, and quantified essentially as described previously (6, 35). Briefly, cells from liquid cultures were concentrated and lysed in 1× FUSE (formic acid, urea, SDS, EDTA) buffer by sonication on ice. Poly P was purified by adsorption to DE81 paper disks; digestion with DNase, RNase, and Apyrase; washing with TKP-50 mM KCl buffer; and elution with TKP-500 mM KCl buffer. It was then quantified in terms of Pi residues by ATP bioluminescence assay (Packard Instrument Co., Meriden, CT). All samples were assayed at least in triplicate.

Transmission electron microscopy. Cells were fixed in 2% paraformaldehyde/2.5% glutaraldehyde (Polysciences Inc., Warrington, PA) in 100 mM phosphate buffer, pH 7.2, for 1 h; washed three times in phosphate buffer; postfixed in 1% osmium tetroxide (Polysciences Inc., Warrington, PA) for 1 h; rinsed extensively in distilled water (dH2O) prior to staining en bloc with 1% aqueous uranyl acetate (Ted Pella, Inc., Redding, CA) for 1 h; rinsed in dH2O; dehydrated in a graded ethanol series; and embedded in Eponate 12 resin (Ted Pella, Inc.) (all at room temperature). Sections (70 to 80 nm thick) were cut and stained with uranyl acetate and lead citrate. For negative staining, samples were fixed in 1% glutaraldehyde in phosphate-buffered saline for 10 min, then allowed to adsorb onto Formvar/carbon-coated grids for 1 min. Grids were washed in dH2O and stained with 1% aqueous uranyl acetate for 1 min. Excess liquid was gently wicked off, and grids were air dried. All samples were views on a 1200 EX transmission electron microscope (JEOL USA, Inc., Peabody, MA).
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 ppk1 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 from each 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 each from SS1 and X47-2AL 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 ΔureAB-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 ΔureAB-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 Δppk1 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 Δppk1-1 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 Δppk1 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 Δppk1 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 88-3887 was motility strongly reduced by ppk1 inactivation (Fig. 4C), whereas no reduction in motility was detected in control experiments with Cam′ ΔureAB-cat transformants of this same strain. Electron microscopy revealed clusters of flagella on Δppk1 cells that seemed normal in appearance and number per cell pole (Fig. 3B). Curiously, however, one-third of these Δppk1 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 Δppk1 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

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**FIG. 3.** Representative electron micrographs of wild-type 88-3887 (WT) and its Δppk1 derivative (Δppk1). (A) Transmission electron microscopy sections show a large distinct granule (poly P) in the wild type but not in the Δppk1 derivative. P, putative poly P granule; n, putative nucleoid (assignments based on references 16 and 40); (B) the wild-type cell shown here has flagella at one pole, and one of two Δppk1 cells has flagella at both poles.

**FIG. 4.** Progression of bacterial growth and motility in soft agar. Each wild-type (WT) or Δppk1 derivative (Δppk1) strain was stabbed in duplicate to monitor reproducibility and scored for halo formation (motility and chemotaxis) periodically, beginning day 3 or 4 after inoculation. The lack of a significant effect of ppk1 inactivation on motility shown here for strains A66 and X47-2AL (panels A and B) is typical of 12 of the 13 strains tested. Panel B shows strain X47-2AL Δppk1-2 derivatives, both of which retain motility; nc (noncolonizer) indicates a pool of X47-2AL Δppk1 natural transformants that were almost completely defective in mouse colonization; gc (good colonizer) indicates the rare X47-2AL Δppk1 electroporant that colonized mice well (Table 2). Only with 88-3887 (panel C) was motility strongly affected by ppk1 inactivation.
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 \(ppk1\) deficiency tends to delay the final separation of daughters during the cell cycle.

**Effect of \(ppk1\) 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 \(ppk1\) 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 \(ppk1\) 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 EOP on BHI agar. A variety of effects was observed: no defined culture medium, modified Ham’s F-12 agar, was used to investigate nutrient stress. Many strains exhibited diminished growth on F-12 agar. Consequently, restreaking residual growth of 26695 and 88-3887 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 \(ppk1\) 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 \(ppk1\) 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 \(ppk1\) inactivation on antimicrobial susceptibility.** We tested for effects of \(ppk1\) 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 culture plate (21) showed that \(ppk1\) inactivation increased susceptibility to Mtz in 6 of 17 strains tested (Table 1). Mtz resistance can be increased by inactivating \(rdxA\) and \(fxcA\), whose encoded nitroreductases help activate Mtz (21). Introduction of a \(\Delta ppk1\) allele into 88-3887 \(\Delta rdxA\) \(fxcA\) 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 \(\mu g/ml\)).

### TABLE 2. Effects of \(ppk1\) inactivation on mouse colonization

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

<sup>a</sup>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.

<sup>b</sup>Typical values as obtained in previous experiments with at least 10 mice per strain.

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

<sup>d</sup>The \(\Delta ppk1\) strain carries the \(\Delta ppk1\)-1 allele.

<sup>e</sup>The \(\Delta ppk1\) strain carries a natural transformant. All other \(\Delta ppk1\) strains were obtained as electroporants.

<sup>f</sup>The \(\Delta ppk1\) strain carries the \(\Delta ppk1\)-2 allele.

<sup>g</sup>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.

<sup>h</sup>Five mice each for two different pools of X47-2AL complemented partial-diploid pools.

<sup>i</sup>PD, complemented \((ppk1/\Delta ppk1)\) partial diploid derived from X47-2AL. \(\Delta ppk\) (NT) that colonized mice very poorly, if at all.

**Effect of \(ppk1\) inactivation on ability to colonize mice.** The effect of \(ppk1\) 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<sup>+</sup>) (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<sup>+</sup> (\(\Delta ppk1\)) (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),
as described above, and a pool of Ery transformants was due to
/H9004 of colonization ability of X47-2AL inoculated with several different pools of such natural trans-
onies were obtained from any of the other 9 mice that had been
strains were recovered by quantitative culture 2 weeks after
pylori used as hosts were as follows: wild-type C57BL/6J (E)
Cam susceptibility (wild type) versus resistance (Δppk1). All Δppk1 strains used were generated by electroporation, except where indicated
The Δppk1 fraction was estimated by scoring 20 colonies per mouse for
Cam susceptibility (wild type) versus resistance (Δppk1). All Δppk1 strains used were generated by electroporation, except where indicated by the symbol ○, which stands for natural transformation. The mice used as hosts were as follows: wild-type C57BL/6J (○, ◊), C57BL/6J IL-12β deficient (●), and C57BL/6J IL-10 deficient (□).

FIG. 5. Effect of ppk1 inactivation on H. pylori fitness in mice. H. pylori strains were recovered by quantitative culture 2 weeks after mixed infection (a 1:1 mixture of wild-type and isogenic Δppk1 strains). The Δppk1 fraction was estimated by scoring 20 colonies per mouse for Cam susceptibility (wild type) versus resistance (Δppk1). All Δppk1 strains used were generated by electroporation, except where indicated by the symbol ○, which stands for natural transformation. The mice used as hosts were as follows: wild-type C57BL/6J (○, ◊), C57BL/6J IL-12β deficient (●), and C57BL/6J IL-10 deficient (□).

about 10% of colonies recovered were Cam⁺ (significantly differ-
ent from the ~1% found with X47-2AL Δppk1 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 Δppk1 natural trans-
formants of X47-2AL seemed nearly incapable of mouse col-
onization, although they had near-normal motility (Fig. 4).
Only five and seven H. pylori colonies per stomach were ob-
tained from 2 of 11 inoculated mice, respectively, and no col-
onies were obtained from any of the other 9 mice that had been
inoculated with several different pools of such natural trans-
formants. 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 Δppk1 natural transform-
ments was due to Δppk1 itself. Cells of strain X47-2AL Δppk1 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 transformants was
used to inoculate C57BL/6J 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
Δppk1 natural transformant to colonize mice efficiently is likely
due to Δppk1 itself, not a suppressor possibly selected during
outgrowth.

In contrast to the results for strain X47-2AL, Δppk1 deriv-
atives of strain SS1, generated by natural transformation or by
electroporation, each seemed fully capable of mouse coloniza-
tion 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/6J mice and their cytokine IL-10- and IL-12β-deficient
derivatives (Fig. 5), which exhibit stronger and weaker inflam-
matory 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 diver-
sity in phenotypic effects came from efforts to generate Δppk1
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, Δppk1 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
Δppk1 electroporants of SS1 and X47-2AL may stem from
poor recovery from electric shock or overgrowth by nontrans-
formed wild-type siblings during the ensuing ~20 h of incuba-
tion.

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
Δppk1 (EOP ≤ 10⁻³) and J99 Δppk1 (EOP 1); Δppk1 deriv-
atives 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 extrap-
olation from E. coli (9, 29), this poor-growth phenotype might
also be ascribed to effects of poly P on RNA or protein turn-
over. Identification of suppressor mutations that restore the
ability of 26695 or 88-3887 Δppk1 to grow on F-12 agar (per-
haps 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. Al-
though 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 Δppk1

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transfectant 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 ppk1 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 ppk1 but its basis is not known. Among possible models, we are drawn to those invoking effects of ppk1 but its basis is not known. Among possible models, we are...


