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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 Δ*ppk1* 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*

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TABLE 1. Phenotypes of Δ*ppk1 H. pylori* strains relative to wild-type parental strains.

Strain	Country of origin[m]	Motility of Δ*ppk1*	EOP on F-12 of Δ*ppk1*[a]	Mtz MIC (μg/ml) for:	
				WT[b]	Δ*ppk1* strain
88-3887[c]	UK	Decreased	$<10^{-8}$[d]	2	1
88-3887 Δ*rdxA* Δ*frxA*[c,e]	UK	NA[f]	NA	64	16
AM1	India	Same	0.001	2	2
AM2	India	Same	1	128	128
SS1	Australia	Same	0.1	1	1
SS1 (NT)[g]	Australia	Same	0.1	1	1
X47-2AL	US	Same	1	2	2
X47-2AL (NT)	US	Same	1	2	2[h]
26695[c]	UK	ND[i]	$<10^{-8}$[d]	2	2[j]
J99	US	ND	1[k]	2	2[h]
R64	S. Africa	ND	0.1	3	1
R66	S. Africa	ND	0.1	2	2[h]
HK152	Hong Kong	Same	0.001	2	2
PCM4	Hong Kong	Same	0.1	2	2
Chen13	India	Same	1	1	1
PeCan28	Peru	Same	ND[l]	2	2
A66	Lithuania	Same	ND	0.75	0.75[h]
YN1-16	China	Same	ND	64	64
YN1-18	China	Same	ND	128	128
CPY3401	Japan	Same	ND	3	3
HUP-B63	Spain	ND	ND	2	1

[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 Δ*ppk1* strain did not form single colonies.
[e] *rdxA*Δ111 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 Δ*ppk1* strains were obtained as electroporants.
[h] The Δ*ppk1* 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 Δ*ppk1* strain EOP was ~100-fold lower than that of the wild type, indicating increased sensitivity of the mutant.
[k] Δ*ppk1* 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% O_2, 10% CO_2, 85% N_2). For motility studies, either brucella or BHI broth containing 0.35% agar was used (45). For nutrient-limited growth, Ham's F-12 agar with cholesterol (100 μg/ml), bovine serum albumin (2 mg/ml), and vancomycin (6 μg/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 (~10^8 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 Δ*ureAB-cat*, *rdxA*Δ111, 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) Δ*ppk1-2*, 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 the *xerD* recombinase [*hp1009*] and *pyrD* [*hp1011*] genes, as in sequenced strain 26695); and (ii) Δ*ppk1-1*, 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 Δ*ppk1-1* for this allele to replace the intact *ppk1* gene, whereas

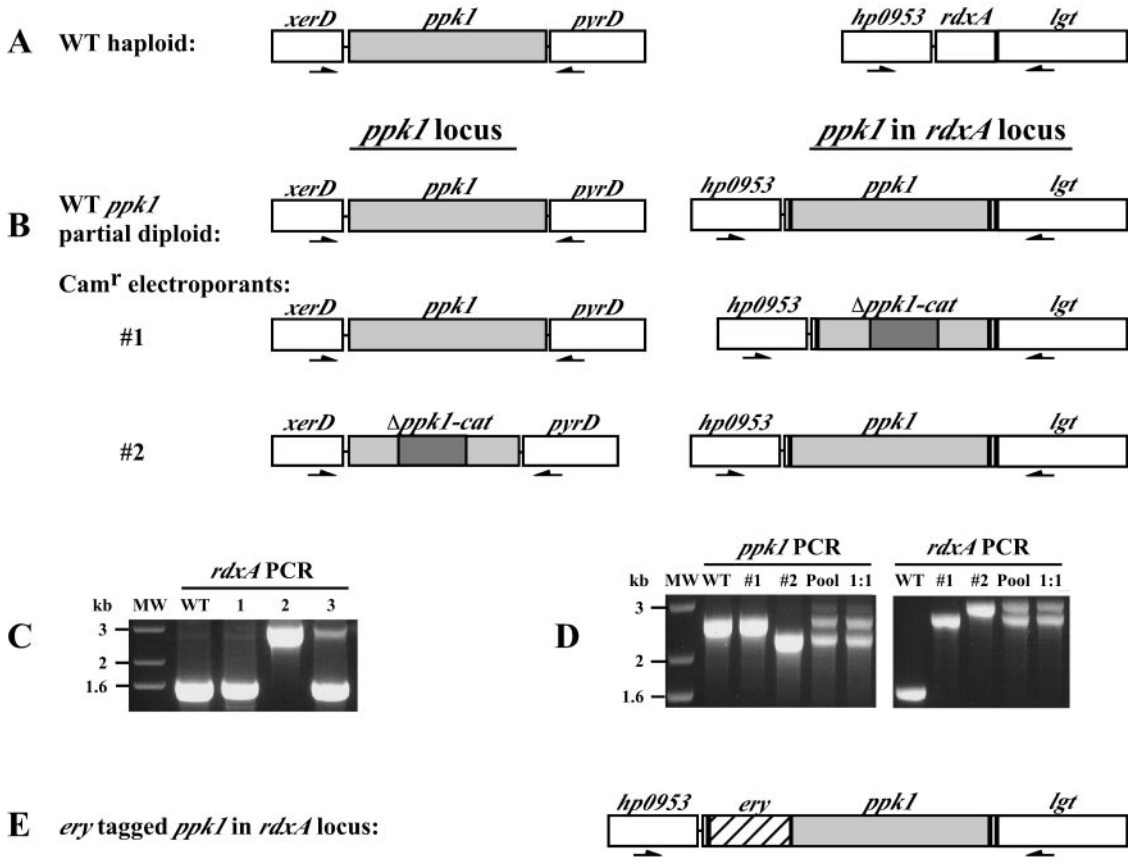

FIG. 1. *ppk1* and *rdxA* loci, structures of *ppk1* duplication strains, and PCR verifications. Approximate positions of PCR primers flanking *ppk1* and *rdxA* that were used in these analyses are indicated by half arrows (primer sequences available on request). (A) Structures of gene loci in wild-type (WT) haploid strains. (B) Gene arrangements in the *ppk1* partial-diploid parent strain and the two possible Cam^r Δ*ppk1-1* electroporants. #1, intact *ppk1* in normal *ppk1* locus (left), Δ*ppk1* in *rdxA-ppk1* locus (right); #2, Δ*ppk1* 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^r 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^r Cam^r electroporants made using Δ*ppk1-1* DNA (note that only partial diploids were recovered); lane 3, 1:1 mix of wild-type *rdxA* and Δ*rdxA*:*ppk1* alleles (smaller wild-type allele is amplified preferentially). (D) PCR assays of the normal *ppk1* locus and the *rdxA* locus from Cam^r transformants. These complementary tests show a Δ*ppk1-1* allele either in the *rdxA*:*ppk1* locus (#1) or the native *ppk1* locus (#2). Results from a pool of Cam^r electroporants (Pool) and from a reconstruction with a 1:1 mixture of Δ*ppk1-1* 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.

homology requirements make replacement by the Δ*ppk1-2* (complete deletion) allele unfeasible. For this reason, most experiments presented here used the Δ*ppk1-1* 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 *frxA-aphA* null insertion allele (*frxA* expression also makes *H. pylori* Mtz sensitive [21]), with selection for Mtz resistance (8 μg/ml), and thereby *rdxA* gene inactivation. PCR tests indicated that ~5 to 10% of Mtz^r colonies contained *ppk1* in *rdxA*, whereas the others did not, but probably contained *rdxA* point mutations (Mtz is mutagenic for *H. pylori* [42]) (Fig. 1C; also see Results). These mixed populations of haploid and partial diploid Mtz^r cells were saved as pools for further analysis (see below). A PCR product, in which the *ppk1* gene at the *rdxA* locus was tagged by insertion of an erythromycin resistance gene (*ery*) just downstream of the 3′ end of *ppk1*, was then made (Fig. 1E). Genomic DNA containing this construct could be used in transformation to move the second copy of *ppk1* to any strain of interest.

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 P_i residues by first using purified *E. coli* PPK1 in a reverse reaction with ADP to convert each P_i residue in poly P to ATP. ATP production was then assayed in a luciferase reaction (ATP bioluminescence assay kit CLS II; Roche Molecular Biochemicals, Indianapolis, IN), with detection in a TopCount NXT microplate luminescence counter (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 (dH_2O) prior to staining en bloc with 1% aqueous uranyl acetate (Ted Pella Inc., Redding, CA) for 1 h; rinsed in dH_2O; 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 dH_2O and stained with 1% aqueous uranyl acetate for 1 min. Excess liquid was gently wicked off, and grids were air dried. All samples were viewed on a 1200 EX transmission electron microscope (JEOL USA, Inc., Peabody, MA).

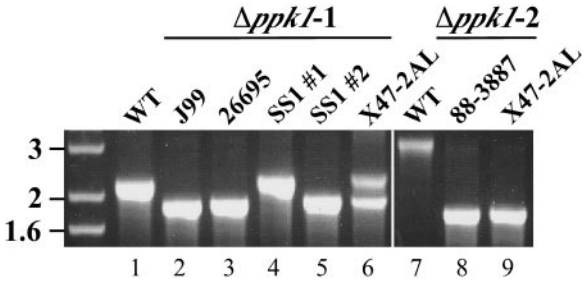

FIG. 2. PCR characterization of Cam^r (Δ*ppk1*) electroporants in representative haploid or *ppk1* partial-diploid strains, using PCR locus-specific primers (sequences available on request). Profiles shown are from representative Δ*ppk1-1* (1-kb deletion) electroporants (lanes 2, 3, and 5); representative Δ*ppk1*-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 Δ*ppk1* electroporants obtained from SS1 and X47-2AL (lanes 5 and 9, respectively). Lanes 1 and 7 show the PCR products from wild-type (WT) SS1 and X47 with intact *ppk1* alleles, obtained using primers that had detected the Δ*ppk1-1* and Δ*ppk1-2* 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 Δ*ppk1* 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 nonpolar Δ*ppk1* deletion alleles (marked with *cat*, a Cam^r determinant) in numerous *H. pylori* strains. Dozens to hundreds of normal-appearing Cam^r 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^r electroporants from each lineage showed the expected replacement of intact *ppk1* by Δ*ppk1-1* or Δ*ppk1-2* in each case (Fig. 2). In contrast, only rarely were Cam^r colonies obtained by electroporation of SS1 or X47-2AL in several different attempts and with each of the two Δ*ppk1* alleles (<1% expected frequency). Control electroporations with a Δ*ureAB-cat* allele (45) or a *cat*-marked *rpoBC* allele (37) indicated that electroporation and selection for a Cam^r 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^r electroporants of SS1 and X47-2AL identified three types: (i) one electroporant each from SS1 and X47-2AL contained intact *ppk1* and no Δ*ppk1* allele, suggesting illegitimate recombination of *cat*-containing DNA into an ectopic site; (ii) one electroporant from X47-2AL contained both intact *ppk1* and a Δ*ppk1-1* 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 Δ*ppk1* alleles in place of intact *ppk1* (Δ*ppk1-1* and Δ*ppk1-2*, respectively) (Fig. 2). The rarity of Cam^r 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 Δ*ppk1-1* DNA yielded hundreds of Cam^r electroporants, instead of the few obtained with purely haploid recipients. PCR tests of single and pooled Cam^r colonies indicated that all new transformants were of the minority, partial-diploid type (Fig. 1C); and that the Δ*ppk1* 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 Δ*ppk1-1* or Δ*ppk1-2* strains resulted in hundreds of Cam^r 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 Δ*ppk1* allele (for SS1, Δ*ppk1-1*; for X47-2AL, Δ*ppk1-2*) in every case (as in Fig. 2). New colonies formed by cells from the initial Δ*ppk1* 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 Δ*ppk1* 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^r transformants obtained using Δ*ureAB-cat* DNAs formed colonies that were normal and of uniform size. With three of these strains (J99, CPY3401, and PCM4), Cam^r (Δ*ppk1-1*) transformant colonies were mostly small (but larger than those of SS1 and X47-2AL Δ*ppk1* transformants) or heterogeneous in size, whereas those of the fourth strain (HUP-B63) were similar in size to those made with Δ*ureAB-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

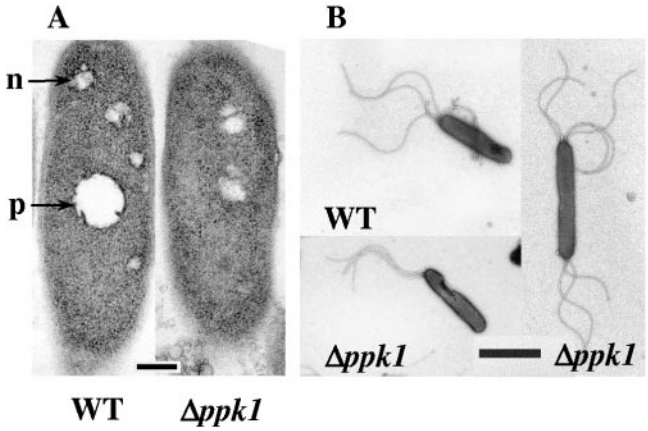

FIG. 3. Representative electron micrographs of wild-type 88-3887 (WT) and its Δ*ppk1* derivative (Δ*ppk1*). (A) Transmission electron microscopy sections show a large distinct granule (poly P) in the wild type but not in the Δ*ppk1* derivative. P, putative poly P granule; n, putative nucleoid (assignments based on references 16 and 40); (B) the wild-type cell shown here has flagella at one pole, and one of two Δ*ppk1* cells has flagella at both poles.

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

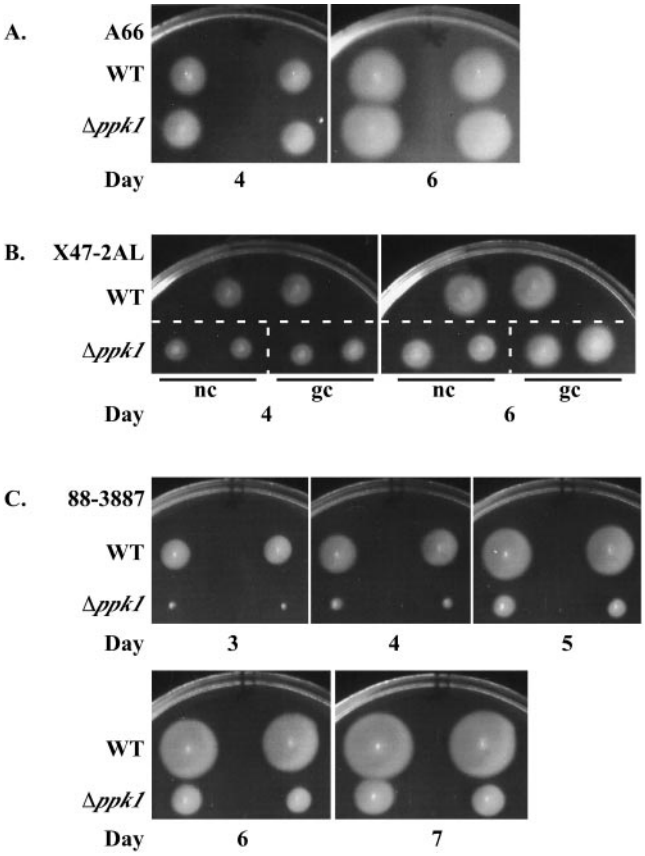

FIG. 4. Progression of bacterial growth and motility in soft agar. Each wild-type (WT) or Δ*ppk1* derivative (Δ*ppk1*) strain was stabbed in duplicate to monitor reproducibility and scored for halo formation (motility and chemotaxis) periodically, beginning day 3 or 4 after inoculation. The lack of a 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 Δ*ppk1-2* derivatives, both of which retain motility; nc (noncolonizer) indicates a pool of X47-2AL Δ*ppk1* natural transformants that were almost completely defective in mouse colonization; gc (good colonizer) indicates the rare X47-2AL Δ*ppk1* electroporant that colonized mice well (Table 2). Only with 88-3887 (panel C) was motility strongly affected by *ppk1* inactivation.

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^r Δ*ureAB-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. 3B). 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 *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 size but not 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 $< 10^{-8}$). However, restreaking residual growth of 26695 Δ*ppk1* and 88-3887 Δ*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 Δ*ppk1*'s inability to grow on F-12 agar was due to Δ*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 Δ*ppk1* was transformed to Ery^r with genomic DNA from a strain carrying this construct. The presence of both intact *ppk1* in *rdxA* and Δ*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 Δ*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 Δ*ppk1* cultures were estimated on different halves of the same plates (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 *frxA*, whose encoded nitroreductases help activate Mtz (21). Introduction of a Δ*ppk1* allele into 88-3887 Δ*rdxA* Δ*frxA* also diminished Mtz resistance (the MIC was reduced from 64 to 16 μg/ml [Table 1]). In complementary tests using other antimicrobials, no effect of a Δ*ppk1* allele on susceptibility of strain 88-3887 to clarithromycin or amoxicillin was detected (MICs of 0.5 and 2 μg/ml, respectively), whereas the Δ*ppk1* allele made X47-2AL slightly more sensitive to amoxicillin (MIC of 1 μg/ml versus 2 μg/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 μg/ml).

TABLE 2. Effects of *ppk1* inactivation on mouse colonization

H. pylori strain	C57BL/6J mouse strain[c]	Mean no. of CFU/stomach[a]		No. of Δ*ppk1* inoculated mice
		Wild type[b]	Δ*ppk1*	
88-3887[d]	IL-12β KO	1×10^5	2×10^4	3
AM1[d]	IL-12β KO	5×10^4	4×10^3	3
AM2[d]	IL-12β KO	5×10^4	5×10^3	5
SSI[d]	WT	1×10^6	7×10^5	5
SSI[d](NT)[e]	WT	1×10^6	1×10^6	2
X47-2AL[f]	IL-12β KO	1×10^6	6×10^5	5
X47-2AL[f]	WT	1×10^6	1×10^6	5
X47-2AL[f](NT)[e]	WT	1×10^6	<10[g]	11
X47-2AL[f] PD[i]	WT		1×10^6	10[h]

[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 Δ*ppk1* strain carries the Δ*ppk1-1* allele.
[e] NT, Δ*ppk1* strain obtained as a natural transformant. All other Δ*ppk1* strains were obtained as electroporants.
[f] The Δ*ppk1* strain carries the Δ*ppk1-2* allele.
[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*$^+$/Δ*ppk1*) partial diploid derived from X47-2AL Δ*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 Δ*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), Δ*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 Δ*ppk1*, the most vigorous of these three strains. Mice were inoculated with 1:1 mixtures of the Δ*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^s) (significantly different from the 50% expected if Δ*ppk1* did not affect fitness; $P = 0.002$; 1-sample sign test) (Fig. 5). An equivalent result was obtained using derivatives of 88-3887 Δ*ppk1* that had been passaged once in mice to select for any possibly better-adapted derivatives. Thus, Δ*ppk1*, while allowing these strains to establish low-grade mouse infections, decreased their vigor in vivo.

Several distinct phenotypes were observed with Δ*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 Δ*ppk1* electroporant and its wild-type parent (1:1 mixture) were Cam^r (Δ*ppk1*) (Fig. 5). In an equivalent test, but using a pool of Δ*ppk1* derivatives that had been cultured from mice (after 2 weeks of infection),

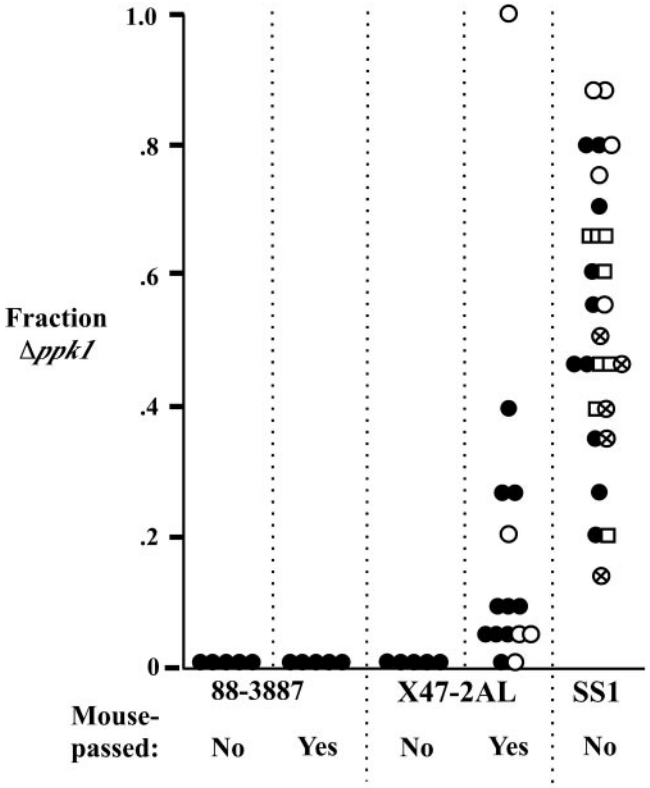

FIG. 5. Effect of *ppk1* inactivation on *H. pylori* fitness in mice. *H. pylori* strains were recovered by quantitative culture 2 weeks after mixed infection (a 1:1 mixture of wild-type and isogenic Δ*ppk1* strains). The Δ*ppk1* fraction was estimated by scoring 20 colonies per mouse for Cam susceptibility (wild type) versus resistance (Δ*ppk1*). All Δ*ppk1* 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^r (significantly different from the ~1% found with X47-2AL Δ*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 Δ*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 Δ*ppk1* natural transformants was due to Δ*ppk1* itself. Cells of strain X47-2AL Δ*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^r 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 Δ*ppk1* natural transformant to colonize mice efficiently is likely due to Δ*ppk1* itself, not a suppressor possibly selected during outgrowth.

In contrast to the results for strain X47-2AL, Δ*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/6J 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 Δ*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, Δ*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 Δ*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 Δ*ppk1* (EOP $\leq 10^{-8}$) and J99 Δ*ppk1* (EOP 1); Δ*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 Δ*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 Δ*ppk1*

transformant of a preexisting nonmotile variant subclone. This said, we found motility to be reproducibly reduced in Δ*ppk1* transformants of strain 88-3887. One explanation for this curious effect, based on the unexpected abundance of Δ*ppk1* 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* 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 Δ*ppk1* strains.

Quantitative differences among strains in the need for *ppk1* were also evident in vivo. With SS1, *ppk1* inactivation had no significant effect on fitness even in competition tests using the wild-type strain and its isogenic Δ*ppk1* derivatives. Fitness was also not much affected by use of wild-type mice or congenic animals that lacked cytokine IL-12β or IL-10, which have diminished and heightened host responses to infection, respectively (14, 17).

In contrast, with strain X47-2AL, Δ*ppk1* derivatives obtained by natural transformation were nearly incapable of mouse colonization, even when inoculated alone. The rare Δ*ppk1* 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 *ppk1* 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 *ppk1* gene restored their ability to colonize mice. It is therefore likely that X47-2AL has a strong intrinsic need for *ppk1* and poly P in vivo and that this need is partially suppressed by the mutation that allowed recovery of a Δ*ppk1* electroporant.

The Δ*ppk1* 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 Δ*ppk1* 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 *ppk1* 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 Δ*ppk1* mutations seemed to emerge easily in many Δ*ppk1 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 *ppk1* 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 *ppk1* inactivation found here may be quite typical of genes for nonessential components of metabolic webs in any genetically diverse species.

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