Polyphosphate kinase: a new colonization factor of

*Helicobacter pylori*

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Abstract

In order to elucidate the role of polyphosphate kinase (PPK) during the course of an infection by *Helicobacter pylori*, PPK deficient mutants were constructed using two genetic backgrounds: Hp141v and X47-2AL. The efficiencies of the parental strains and the derivative mutants at colonizing the gastric mucosa of mice were compared. When animals received the Hp141v and the X47-2AL parental strains, 100% of the mice remained colonized for the duration of the 45 days experiment. In contrast, none of the mice that were given the PPK deficient X47-2AL derivative strain had a detectable bacterial load in their gastric mucosa, while the deficient Hp141v derivative strain was detected in 100%, 20% and 40% of the mice at days 3, 15 and 45 post-inoculation (p.i.), respectively. The absence of PPK expression did not impair the in vitro growth of the *ppk* mutants. However, the reduced ability of the *ppk* defective mutants to colonize mice was associated with a significant decrease in both motility and in an accumulation of polyP in the bacterial cells. These results are consistent with an essential role of PPK during the initial steps of colonisation of the mouse gastric mucosa and confirm that PPK may act on the virulence of *H. pylori* partly through an energy dependent mechanism.

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1. Introduction

*Helicobacter pylori* is a spiral-shaped, microaerophilic gram-negative bacterium that colonizes the gastric mucosa of humans [1]. It is the etiologic agent of chronic gastritis and peptic ulcers and is a risk factor for the development of gastric cancer [2]. *H. pylori* is one of the bacteria that shows the highest level of genetic polymorphism [3].

In a previous study we investigated the genetic adaptation of *H. pylori* during the course of an experimental mouse infection by a fresh clinical isolate: Hp141. This strain was genetically modified in vivo. The variant, Hp141v, exhibited a deletion within the *ppk* gene that encodes polyphosphate kinase (PPK). Actually, this deletion allowed Hp141v to recover a native form of the *ppk* gene and led to a better capacity of the variant to colonize mice [4].

PPK is an enzyme that reversibly catalyzes the transfer of the gamma phosphate of ATP to inorganic polyphosphate (PolyP), a linear polymer of hundreds of orthophosphate residues linked by high energy
phosphoanhydride bonds [5,6]. It is well known that PPK and PolyP play an important role in the physiological adaptation of microbial cells during growth and development [5–9]. Reservoir of energy and phosphate, chelation of metal ions (Zn, Fe, Cu and Cd) in order to reduce their toxicity [5,6], “channelling” of DNA entry during bacterial transformation [8] and resistance to acidic conditions [9]. Furthermore, construction of ppk inhibited mutants of different pathogenic bacteria reveals that PPK and polyP contribute to wide-ranging bacterial responses to nutritional and environmental downshift by stimulating protein degradation; in addition, they also are less fitted in the stationary phase of growth and are less resistant to heat, oxidants and osmotic challenge [10–12]. Porphyromonas gingivalis ppk mutants also fail to survive in the stationary phase and are attenuated in biofilm formation suggesting that PPK may be important for the incorporation of this organism into gingival plaque [13], while Neisseria meningitides ppk mutants show a striking increase in sensitivity to killing by human serum [14]. Shigella and Salmonella spp. ppk mutants also show growth defects, defective responses to stress and starvation, loss of viability, intolerance to acid and heat and diminished invasiveness in epithelial cells [15]. Finally, Pseudomonas aeruginosa ppk mutants are aberrant in quorum sensing, unable to form a thick and differentiated biofilm, deficient in motility and less virulent in a burned-mouse model [16–18]. Taken together, these studies highlight an important role for PPK in the physiological adaptation of bacteria in response to environmental stress and suggest that this enzyme can be considered as a putative virulence factor for these organisms.

Little is known, however, about the importance of PPK for H. pylori. A massive accumulation of PolyP is detected in H. pylori during the infectious stage and it has been suggested that PPK may support the viability of the coccoid forms in vitro [16,19]. The results we obtained during experimental infection of mice also suggest an important role for PPK during colonization [4].

To further understand the role of PPK in H. pylori infection, we constructed isogenic mutants from two different H. pylori isolates by using the deletion and replacement of the ppk gene with a non-polar kanamycin cassette. We tested the ability of these mutant strains to colonize the mouse gastric mucosa and supported our findings with in vitro analysis of viability, motility, PPK expression and polyP production.

2. Materials and methods

2.1. Bacterial strains and growth conditions

This study was conducted with two H. pylori isolates: Hp141v, the variant of the Hp141 strain in which ppk gene was rearranged during the course of an experimental mouse infection, and the mouse-adapted strain X47-2AL [4,20]. These strains were cultivated on a Skirrow medium under a microaerobic atmosphere. Kanamycin (20 μg ml⁻¹) was added to the Skirrow medium to grow of their respective ppk mutants. For microscopic observations, viability, motility assays and RNA isolation, the strains were grown in a liquid medium, consisting of Brucella broth supplemented with 5% fetal-calf serum and 20 μg ml⁻¹ kanamycin for mutants.

Escherichia coli strain MC1061 [21] was used for construction of ppk mutants. This strain was grown in Luria Bertani (LB) broth. Selection of transformants was realized on LB plates containing 100 μg ml⁻¹ spectinomycin and 20 μg ml⁻¹ kanamycin.

2.2. Construction of isogenic mutants

In order to construct ppk mutants, we used the procedure described by Contreras et al. [22]. Briefly, chromosomal DNA from strain 26695 was used as a template for gene amplification of ppk (ORF hp1010 according to Tomb et al.) [23]. The entire 2.025 kb-sequence of the ppk gene of strain 26695 was cloned into the 3.6-kb pILL570-Δ (Sp') cloning vector, then a 4.2-kb fragment from this plasmid was reverse amplified using primers complementary to sequences located 300 bp downstream, and upstream of the 5’- and 3’-end of the ppk gene, respectively. These primers were tagged at their 5’-end with KpnI and BamHI sequences. The sequences of these primers called Hp1010-1 and Hp1010-2 were respectively: 5’CGGGGTACCCATTATAGGGGTGATACAAAGCC3’ and 5’CGCGGAATTCGCAATCATTGAATGGGCTCTACGGCC3’ (sequences in italic correspond to KpnI and BamHI sequences, respectively). Following reverse amplification, PCR products were digested in KpnI and BamHI. The digested amplions were then ligated to a promoterless non polar KpnI–BamHI kanamycin cassette (0.9 kb). After selection of E. coli transformants, recombinant plasmids were purified and introduced by natural transformation into H. pylori cells. Independent kanamycin resistant H. pylori colonies (selected on Skirrow medium supplemented with 20 μg ml⁻¹ of kanamycin) were tested by PCR and were shown to have the ppk gene replaced by the 0.9 kb fragment corresponding to the non-polar kanamycin cassette. Five independent transformations were conducted, and five independent H. pylori kanamycin resistant transformants were then pooled for further comparison of mutant and parental strains properties. They were designated as Hp141vppkΩKm and X47-2ALppkΩKm.

2.3. ppk expression determined by RT-PCR

Total RNA was isolated from 10⁸ bacterial cells of parental strains and of their respective ppk mutant after
16 h of culture in a liquid medium. This was realized using RNeasy® Mini Kit (Qiagen, Courtaboeuf, France) according to the instructions supplied by the manufacturer. After Dnase treatment, first-strand cDNA was synthesized using M-MLV Reverse Transcriptase® (Invitrogen, Cergy-Pontoise, France) according to the instructions supplied by the manufacturer. The PCR amplification of a 471 bp DNA fragment of the ppk gene in the middle region was performed using the primer ppk3 (5’CTTCGACACCTTCCGCCTT3’) and ppk4 (5’CCCTAATGAGCATGATCGGC 3’) in a volume of 50 µl with a reaction mixture containing 10 mM Tris–HCl pH 8.3, 50 mM KCl, 3 mM MgCl2, 200 µM of each dNTP, 50 pM for each primer, 1.25 units of Taq polymerase (Amersham Biosciences, Orsay, France) and 1 µl of cDNA. The cycling conditions consisted in an initial denaturation at 94 ºC for 5 min followed by 35 cycles of [94 ºC, 1 min; 52 ºC, 1 min; 72 ºC, 1 min] and then by a final elongation step at 72 ºC for 7 min. The expression of the ppk gene was revealed by the presence of a 471 bp fragment after electrophoresis in 1.5% agarose gel.

2.4. Transmission electron microscopy

Observation by transmission electron microscopy of the parental and their derivative mutant strains was realized on a 36 h culture in a liquid medium. Cells were fixed in 2.5% glutaraldehyde in PBS for 90 min, and then washed three times in the same buffer. Fragments were postfixed (30 min) in osmium tetroxide. The material was then dehydrated (acetone) and embedded in an epoxy medium. Thin sections (50 nm) were cut with a diamond knife on a Reichert ultramicrotome, Ultracut S. The grids were contrasted with 2% uranyl acetate and lead citrate and examined on a JEOL 100 Cx electron microscope.

2.5. Viability assay

Parental strains and their derivative mutants were cultivated for 6 days in a liquid medium. Daily, 100 µl of the culture were serially diluted and 20 µl of each dilution was plated on Skirrow medium supplemented by 20 µg ml−1 of kanamycin for the PPK deficient mutants. The number of viable H. pylori cells was determined after an incubation period of 7 days.

2.6. Motility assay

Motility was first assessed by direct microscopic observation of bacterial cells cultivated for 24 h in a liquid medium. Then, the motility of the strains was measured in 0.3% agarose brucella broth complemented with 5% fetal calf serum. For each strain, a bacterial suspension of 10^7 CFU/ml was stabbed into the thickness of the soft agar using a sterile toothpick [24]. The halo obtained after culturing for 7 days was measured.

2.7. Experimental infection

Experimental infections were conducted as previously described with 6-week-old female C57BL/6 inbred mice checked to be Helicobacter free by culturing, microscopic examination after Gram staining, and enzyme-linked immunosorbent assay on mice sera [4,25]. Strains Hp141v, X47-2AL, and their respective derivative ppk mutants were used to prepare the infecting inocula: 0.5 ml suspensions adjusted at 10^10 CFU per ml, were administered intragastrically, twice at 2 h-intervals. Fifteen animals were infected with each strain at day 0 with Hp141v, X47-2AL or the ppk mutants. Five animals were individually sacrificed at day 3, 15 and 45 post-inoculation (p.i.). As a control, 9 mice were given Brucella broth only in order to check for the absence of contamination by human strains during the experiment, and 3 of them were sacrificed at each experimental step. The stomachs were entirely removed after sacrifice and ground into 1 ml of Brucella broth. The suspensions were serially diluted and cultured during 7 days for the determination of viable counts. This experimental infection protocol received the approval of the Ethics Committee of the University of Poitiers (No. MIC/2001/07/AC).

2.8. Statistical analysis

Statistical analysis presented in this study was performed using the Mann–Whitney U non-parametric test.

3. Results

3.1. Influence of ppk mutation on colonization of mice by H. pylori

Mice were infected with the parental strains and their respective derivative ppk mutants. All the animals infected with either Hp141v and X47-2AL were colonised and remained so for the duration of the 45 days experiment. In contrast, none of the mice that were given the PPK deficient X47-2AL derivative strain had a detectable bacterial load in their gastric mucosa, while Hp141vppkKm was detected in 100%, 20% and 40% of the mice at days 3, 15 and 45 post-inoculation (p.i.), respectively.

With one exception at day 3 p.i. with Hp141vppkKm (p = 0.094), the infection rates obtained with the ppk mutants were found to be significantly lower at each point in time when compared with the parental strain (p < 0.05) (Fig. 1).
3.2. Effect of ppk mutation on the expression of PPK and PolyP production

The expression of PPK in the parental strains, Hp141v and X47-2AL, and their respective ppk mutants was studied by RT-PCR amplification of the ppk gene. Results revealed a signal corresponding to a 471 pb DNA fragment in both parental strains. This signal was absent in Hp141vppkΩKm and X47-2ALppkΩKm kanamycin-resistant mutants. This suggests the absence of ppk transcription in the ppk mutants.

Moreover, observation by transmission electron microscopy of the parental strains Hp141 and X47-2AL showed the presence of large, bright spots that represented an accumulation of long PolyP chains, commonly observed in H. pylori (Fig. 2) [26]. These structures were present in about 90% of the parental bacterial cells but were totally absent in their derivative mutants, confirming the absence of synthesis of long chain polyP in Hp141vppkΩKm and X47-2ALppkΩKm resistant kanamycin mutant strains.

3.3. Influence of ppk mutation on viability and motility

The study of in vitro growth curves did not show any difference between Hp141v, X47-2AL growth and that of their respective derivative mutants during the 6 day-follow up. Direct microscopic observation of parental strains showed that both parental strains as well as their respective ppk mutants were motile. Diffusion assay in soft agar revealed that Hp141v was more motile than X47-2AL, as mean diameters of the halos were 15 and 9 mm, respectively. We also observed that both ppk mutants were less motile than their respective parental strains with mean diameter of the halo of 4 mm for the Hp141v mutant and 2 mm for the X47-2AL mutant (p < 0.05).

4. Discussion

In order to determine the role of PPK in H. pylori during colonization, two ppk mutants were constructed. To ensure that the deletion and disruption of hp1010 did not have any polar effects on the expression of ORF downstream, we used for the construction a non-polar
cassette (aphA3) that was previously validated as having a non-polar effect within the ureI–ureE operon of H. pylori [27]. This cloned cassette carries a new ribosome-binding site (RBS) and an ATG codon that is in phase with the last hundred codons of the inactivated genes, so that translation can be initiated if transcription/translation are coupled in the operon. By using independent mutants introduced into two independent genetic backgrounds, we also expected to minimize the influence of uncontrolled secondary mutations during the construction of our model.

Taking these precautions, we can postulate that the mutation of the ppk gene did not provoke any growth impairment, although it was correlated with a significant drop in its colonization capacity. Our data are consistent with a crucial role for PPK in vivo but there is also a non-essential role of this enzyme in the in vitro growth of the two tested H. pylori strains. This is especially true for the strain X47-2AL since the colonization of the animal was undetectable with the corresponding ppk mutant even at the early stage of infection. In this genetic background, PPK appears as a crucial factor in the colonization of the gastric mucosa. In contrast, in the Hp141v strain and its derivative mutant, PPK activity does not appear to play an important role, since some animals inoculated by the derivative mutant remained infected whereas for the others, especially at the latest stages of the infection, colonization was not detectable any more (Fig. 2).

No residual PPK expression was present in Hp141vppkΩKm nor in X47-2ALppkΩKm. Although PPK is the principal enzyme for the synthesis of long polyP chains, several other possible routes of polyP production need to be considered. In E. coli, mutants lacking PPK were no longer able to produce long polyP chains (ca. 750 residues) but were still able to produce short chains (ca. 60 residues) by an undefined pathway [5]. Several plausible routes are possible for their biosynthesis: from ADP by reversal of an AMP phosphotransferase, from acetyl phosphate, from 1,3-diphosphoglycerate, from dolichyl pyrophosphate and by proton motive forces, known to fix inorganic phosphate in inorganic polyphosphate as well as in ATP [5]. Recently, in a P. aeruginosa mutant lacking the ppk gene, a previously uncharacterised PPK activity (designated PPK2) was identified. It can be distinguished from PPK1 (subject of this study) by the following characteristics: PPK2 synthesises polyP from GTP or ATP while PPK1 uses only ATP. PPK2 activity is stimulated 10-fold by the addition of polyP but no such effect was observed with PPK1. Moreover, a remarkable feature of PPK2 is that the catalytic potency in the polyP-driven synthesis of GTP from GDP is far greater than the synthesis of polyP. The ratio of polyP utilization to polyP synthesis is more than 1000-fold greater for PPK2 than PPK1 [28,29]. Sequences homologous to PPK2 are present in 32 other bacteria, suggesting that PPK2 is widely conserved. Although no deduced PPK2 homologue has been found in the 2 sequenced H. pylori genomes until today, it can be suggested that compensatory mechanisms could be more active in the Hp141v background than in the X47-2AL background [28].

We have shown here that ppk mutants were less motile than the parental strains and also that the Hp141v mutant is more motile than the X47-2AL mutant. The increased efficiency of Hp141vppkΩKm at colonizing a mouse’s stomach could also be explained by an increase in motility. The impairment in motility observed in the ppk mutants was not the consequence of a morphologic alteration of flagella since these structures appear intact when observed by negative stain transmission electronic microscopy (data not shown) as has been reported for other pathogens, for example E. coli, P. aeruginosa, K. pneumoniae, V. cholerae and Salmonella spp. [16]. The mechanisms involving PPK1 and polyP in motility are not clear. It has been proposed that polyP might interfere in the cellular Ca2+ level and act directly on the flagellar motor [16]. Moreover, polyP granules have been found at the base of the flagella in H. pylori [30]. This suggests that PPK1 affect motility at a functional level.

In conclusion, this study clearly demonstrates that the decrease in the colonizing capacity of defective ppk mutants is accompanied by a decrease in motility and a loss of polyP granules in cells. The basic metabolism of H. pylori is not entirely elucidated. PolyP granules represent a reservoir for stored energy and phosphorus. They may be a reservoir for an alternative energy source when ATP is no longer available. A link between the presence of PPK1 and virulence has been reported for several pathogens. We show here that the PPK1 of H. pylori also plays a crucial role during the initial steps of colonisation of the mouse gastric mucosa and confirm that PPK may act on the virulence of H. pylori partly through an energy dependent mechanism.

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