ORIGINAL PAPER

Phosphonate metabolism in *Helicobacter pylori*

**Justin L. Ford · Nadeem O. Kaakoush ·
George L. Mendz**

Abstract *Helicobacter pylori* has been shown to
degrade two phosphonates, *N*-phosphonoacetyl-
L-aspartate and phosphonoacetate; however, the bac-
terium does not have any genes homologous to those
of the known phosphonate metabolism pathways
suggesting that *H. pylori* may have a novel phospho-
nate metabolism pathway. Growth of *H. pylori* on
phosphonates was studied and the catabolism of these
compounds was measured employing ^{1}H-nuclear
magnetic resonance spectroscopy. The specificity of
the catabolic enzymes was elucidated by assaying the
degradation of several phosphonates and through
substrate competition studies. *H. pylori* was able to
utilise phenylphosphonate as a sole source of phos-
phate for growth. Three strains of *H. pylori* showed
sigmoidal enzyme kinetics of phenylphosphonate
catabolism. Allosteric kinetics were removed when
lysates were fractionated into cytosolic and mem-
brane fractions. Catabolic rates increased with the
addition of DTT, Mg^{2+} and phosphate and decreased
with the addition of EDTA. The physiological
properties of *H. pylori* phosphonate metabolism were
characterised and the presence of at least two novel
phosphonate catabolism pathways that do not require
phosphate starvation growth conditions for activity
has been established.

Introduction

Phosphonates are compounds containing a carbon (C)
atom directly bonded to a phosphorus (P) atom. The
C–P bond is very stable with a similar energy to the
C–C bond, 62 and 64 kcal, respectively. Phospho-
nates are structurally similar to phosphate esters
(C–O–P), and often act as inhibitors of enzymes
primarily owing to the high stability of the C–P bond
(Nowack 2003). Phosphonates are not common in
nature, but do play vital physiological roles such as
sources of phosphorus, phosphorus storage (Kononova
and Nesmeyanova 2002), and antibiotics like the one
produced by *Streptomyces fradiae* (Ternan et al.
1998). It is thought that phosphonates may have been
significantly more prevalent in the environment
during the initial evolution of life (Kononova and
Nesmeyanova 2002). Phosphonate compounds are
found in a wide variety of organisms including
prokaryotes, eubacteria, fungi, molluscs and insects
(Nowack 2003).

J. L. Ford · N. O. Kaakoush · G. L. Mendz (✉)
School of Medicine, Sydney, The University of Notre
Dame Australia, 160 Oxford St, Darlinghurst,
NSW 2010, Australia
e-mail: GMendz@nd.edu.au

Four phosphonate metabolism pathways have been elucidated in bacteria (Kononova and Nesmeyanova 2002). The phosphonatase pathway (Kononova and Nesmeyanova 2002; Ternan et al. 1998) consists of an enzyme with two 33–37 kDa subunits and requires Mg^{2+} for activity (Kononova and Nesmeyanova 2002). The phosphonoacetate hydrolase pathway is phosphate-starvation independent, substrate inducible (Ternan et al. 1998), and consists of an enzyme with two identical subunits with a combined molecular mass of 80 kDa (McGrath et al. 1995; Kulakova et al. 2001). Metabolic activity increases in the presence of Zn^{2+}, Co^{2+} and Mn^{2+} but is unaffected by other divalent cations and dithiothreitol (DTT) (McGrath et al. 1998). One exception is the phosphonoacetate hydrolase pathway of *Penicillium oxalicum* where divalent cations and EDTA had no effect on metabolic rates (Klimek-Ochab et al. 2006). The phosphonopyruvate hydrolase pathway involves two enzymes capable of cleaving the C–P bond, and is used by organisms as a method of storing phosphorus in the form of phosphonates (Kononova and Nesmeyanova 2002; Ternan et al. 1998). The usual role of the first enzyme, phosphoenolpyruvate mutase is the anabolism of phosphonates; however, under certain conditions it can reverse this reaction cleaving the C–P bond (Ternan et al. 1998). The second enzyme, phophonopyruvate hydrolase, was purified from *Burkholderia cepacia* (Ternan et al. 2000) and from *Variovorax* spp. (Kulakova et al. 2003) with molecular masses of 232 and 63 kDa, respectively. In both cases metabolic activity increased with the addition of Co^{2+}, Ni^{2+}, and Mg^{2+} whilst activity decreased with EDTA (Ternan et al. 2000; Kulakova et al. 2003). The fourth known pathway for phosphonate metabolism is the C–P lyase pathway. This pathway has been found in *Arthrobacteriaceae*, *Bacillaceae*, *Rhodobacteriaceae*, *Alcaligenaceae*, *Pseudomonadaceae*, *Enterobacteriaceae* and *Rhizobiaceae* (Kononova and Nesmeyanova 2002), but most species of these families are not known to have phosphonate metabolising capabilities (Schowanek and Verstraete 1990a). C–P lyase pathways are capable of catabolising a variety of phosphonates, at variance with the other pathways which are acting on more specific substrates. Genetic analyses in *Escherichia coli* have determined fourteen genes (*phnC* to *phnP*) involved in the C–P lyase pathway and studies show that they are induced under phosphate starvation conditions (Metcalf and Wanner 1993). The enzymes involved in this pathway have not been purified nor the mechanism successfully elucidated but several theoretical models have been proposed (Kononova and Nesmeyanova 2002; Ternan et al. 1998).

Several bacteria degrade industrial phosphonates that are resistant to degradation by the known phosphonate catabolism pathways (Ternan et al. 1998). In addition, phosphonate catabolism has been described in a number of bacteria known to metabolise phosphonates but that lack the genes for the known phosphonate metabolic pathways (Hayes et al. 2000; Mendz et al. 2005). The Gram-negative, ε-Proteobacterium *Helicobacter pylori* (Marshall and Warren 1983), classified as a Type I carcinogen by the World Health Organisation, has been shown to transport (Ford et al. 2007) and degrade two phosphonates, *N*-phosphonoacetyl-L-aspartate (PALA) and phosphonoacetate (Burns et al. 1998). *H. pylori* does not have any genes homologous to those of known phosphonate metabolism pathways, indicating that this bacterium has a novel pathway to catabolise phosphonates. In this study, phosphonate metabolism by *H. pylori* was investigated using nuclear magnetic resonance spectroscopy on whole cell lysates, cell-free extracts and cell pellets. The enzyme kinetics were characterised and the effects of potential cofactors were determined.

Materials and methods

Chemicals and reagents

Blood agar base No. 2, Brain Heart Infusion (BHI) broth, Luria broth, defibrinated horse blood and horse serum were from Oxoid (Heidelberg West, VIC, Australia). Amphotericin (Fungizone®) was from Bristol Myers Squibb (Noblepark, VIC, Australia). Synthetic Complete Supplement Mixture without uracil (SC-URA) and Yeast Nitrogen Base without phosphate (YNB) were from Bio101 (Carlsbad, CA, USA). α-Aminomethylphosphonate (Amephn), α-aminoethylphosphonate (AEP), phosphonoacetate (Phnace), bicinchoninic acid, bovine serum albumin and copper II sulfate, phenylphosphinate (Pheppn), phenylphosphonate (Phephn) and sodium dodecyl sulfate (SDS) were from Sigma–Aldrich (Castle

Hill, NSW, Australia). Deuterium oxide was from Cambridge Isotope Laboratories (Cambridge, UK). All other reagents were of analytical grade.

Bacterial cultures and preparation

The *H. pylori* strains employed in this study were J99, N6 (Institut Pasteur; Paris, France) and LC20, an isolate obtained from a patient with gastritis. *H. pylori* was grown on *Campylobacter* selective agar supplemented with 8% (v/v) defibrinated horse blood, 2.0 μg/ml amphotericin, 5.0 μg/ml vancomycin, 1,250 U/ml polymixin B, and 2.5 μg/ml trimethoprim. For growth curves, *H. pylori* was grown in phosphate-free defined liquid media (PFDM) containing SC-URA (1.9 g/l) and YNB (5.7 g/l) as previously described (Ford et al. 2007). Growth of *H. pylori* in PFDM supplemented with phosphate or phosphonate was measured at 24 and 48 h by plating out serial dilutions on *Campylobacter* selective agar and counting the number of colony forming units per ml (cfu/ml). Starting bacterial inoculums were approximately 10^5 cfu/ml for all conditions. All *H. pylori* cultures were incubated at 37°C under microaerobic conditions (5–10% CO_2, 5% O_2 and 90% N_2). The purity of the cultures was verified by positive urease and catalase tests, and by motility and morphology observed under phase-contrast microscopy (Kaakoush and Mendz 2005).

Escherichia coli strain DH5α was grown on Luria Broth Agar plates for metabolism studies and in PFDM for growth studies. All *E. coli* cultures were incubated at 37°C under aerobic conditions. Growth of *E. coli* on PFDM supplemented with phosphate or phosphonate was determined by measuring the change in optical density at 595 nm after 5 h.

For metabolic studies cells were harvested in 150 mM NaCl and centrifuged at 16,000*g* for 10 min. The pellet was collected and the supernatant discarded. The pellet was resuspended in 150 mM NaCl, washed three times (Kaakoush and Mendz 2005) then lysed by freezing in liquid N_2 and thawing to obtain whole-cell lysates. Cell-free extracts and cell-wall fractions were separated by centrifuging whole-cell lysates at 13,000*g* for 15 min at 4°C and collecting the cytosolic fraction or resuspending the cell pellet in 150 mM NaCl, respectively (Smith et al. 1999). Protein concentrations were estimated by the bicinchoninic acid method (Kaakoush and Mendz 2005).

Genome searches

Sequence similarity searches on the *H. pylori* genomes of strains J99 and 26695 were performed using BLAST searches available at the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov/).

Nuclear magnetic resonance spectroscopy

Catabolism of phosphonates was measured employing proton nuclear magnetic resonance (^{1}H-NMR) spectroscopy. Aliquots of 150 μl of bacterial cells, whole-cell lysates, cell-free extracts or resuspended cell pellets were mixed with 50 μl ^{2}H$_2$O and 50 μl 150 mM KCl. At time zero Phephn was added from a 600 mM stock solution to obtain the required final concentration and 150 mM NaCl was added to the suspension to a final volume of 600 μl. The pH of all samples was between 7.08 and 7.12. Suspensions of bacterial lysates, cell-free extracts or cell pellets were placed in 5 mm tubes and measurements of enzyme activities were performed at 37°C. ^{1}H-NMR free-induction decays were collected using a Bruker DMX-600 spectrometer, operating in the pulsed Fourier-transform mode with quadrature detection. The instrumental parameters for the DMX-600 spectrometer were: operating frequency 600.13 MHz, spectral width 6009.61 Hz, memory size 16 K, acquisition time 1.36 s, number of transients 64, pulse angle 50° (3 μs) and relaxation delay with solvent presaturation 1.7 s. Spectral resolution was enhanced by Gaussian multiplication with line-broadening of 0.5–1.0 Hz and a Gaussian broadening factor of 0.19. Proton spectra were acquired with presaturation of the water resonance. The time-evolution of substrates and products were followed by acquiring sequential spectra of the reactions. Progress curves were constructed by measuring the integrals of resonances arising from phosphonate compounds and averaging the values corresponding to successive time points. Calibrations of the peaks to phosphonate substrate concentrations were performed by extrapolating the resonance intensity data to zero time and assigning to this intensity the initial concentration value (Kaakoush and Mendz 2005).

Calculation of kinetic parameters

Michaelis constants (K_m) and maximal velocities (V_{max}) were calculated by non-linear regression using the Sigma Plot 2002 (version 8.02) program (SPSS Inc; Chicago, IL, USA). The errors in these calculations are determined by the program as ±standard deviations.

Results

In silico analyses of phosphonate genes

The nucleotide sequences encoded by genes known to be involved in phosphonate metabolism in *E. coli*, *Salmonella typhimurium*, *Pseudomonas fluorescens* and *Burkholderia cenocepacia* were used to search the entire *H. pylori* J99 and 26695 (Tomb et al. 1997) genomes for homologues. The analysis indicated that no genes orthologous (similarity >50%) to those involved in phosphonate metabolism were found in the *H. pylori* genomes.

Measurement of phosphonate catabolism

Measurement of phosphonate degradation in *H. pylori* employing ^{1}H-NMR spectroscopy was performed by observing the decrease in the levels of either Amephn, Phnace, Phephn, or Pheppn in the presence of *H. pylori* lysates. Bacterial cells for these assays were grown with abundant orthophosphate in the media. In the absence of bacterial lysates no reduction in the concentration of each of the four compounds was detected. A comparison of phosphonate degradation for different bacterial strains showed a difference in the specific rates measured suggesting that the catabolism of the phosphonates and phosphinate was enzymatic. In addition to lysates, catabolism of the three phosphonates was observed in metabolically active bacterial cells but lysates were chosen for further studies to avoid effects arising from the rates of substrate influx into live *H. pylori* cells (Ford et al. 2007).

Specificity of C–P bond cleavage

The ability of *H. pylori* to cleave C–P bonds of compounds of different alkyl chain length was measured for phosphono-alkyl-carboxylates and α-amino-alkyl-phosphonates with chains of 1–4 carbon atoms. The rates of degradation for the former followed the sequence acetate (100%) > formate (31%) > proprionate (10%) and no activity was observed with phosphonobutyrate (Table 1). For the α-amino-alkyl phosphonates the rates followed the sequence methyl (100%) > ethyl (78.5%) > propyl (34.5%) > butyl (16.5%) (Table 1). The degradation of Phephn by *H. pylori* lysates was completely abolished by the presence of a nitro group substituent in the para position (*p*-amino-phenylphosphonate). This result was confirmed by spectrophotometry.

Phosphonate substrate competition studies

The enzyme activities involved in C–P bond cleavage were investigated through competition experiments by measuring the rates of either 50 mM Phnace, Amephn or Phephn catabolism in whole-cell lysates in the presence of 50 mM of a second phosphonate substrate. Neither Phnace nor Phephn affected the rate of catabolism of the other but Amephn reduced the rates of Phnace and Phephn catabolism by approximately 35 and 50%, respectively. Conversely, the rate of Amephn catabolism was reduced 85% by Phnace and 45% by Phephn. The rates measured for each of the phosphonates Phephn Amephn and Phnace in competition experiments with the other two phosphonates are summarised in Fig. 1.

Table 1 Specificity of C–P bond cleavage by *H. pylori*

Substrate	V_{max} (nmol/min/mg)
Phosphonoformate	7.7 ± 0.9
Phosphonoacetate	25.1 ± 2.2
Phosphonopropionate	2.5 ± 0.3
Phosphonobutyrate	0
α-Aminomethyl phosphonate	200 ± 19
α-Aminoethyl phosphonate	157 ± 15
α-Aminopropyl phosphonate	69 ± 7
α-Aminobutyl phosphonate	33 ± 4

Rates (nmol min^{-1} mg^{-1}) of C–P bond hydrolysis by *H. pylori* whole-cell lysates for phosphono alkyl carboxylates and α-amino-alkyl phosphonates with chains with 1–4 carbon atoms were determined at initial substrate concentrations of 50 mM. Measurements ($n = 3$) were carried at 37°C, in NaCl/KCl suspensions employing ^{1}H-NMR spectroscopy. Errors were calculated as averages of the three measurements
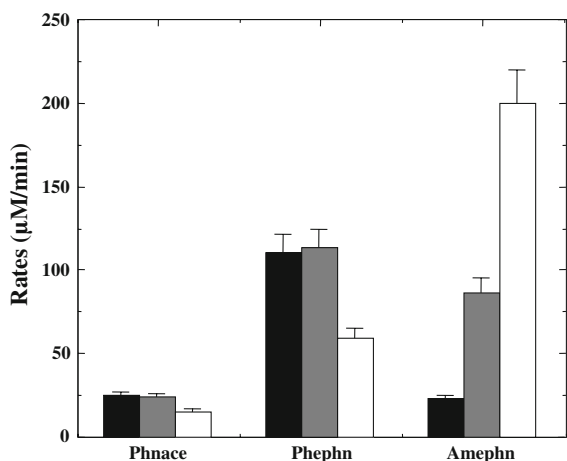
Fig. 1 Rates of C–P bond catabolism of *H. pylori* whole-cell lysates for PhnAce, AmePhn and PhePhn in the presence of a second phosphonate substrate. The phosphonate catabolised is indicated on the *horizontal axis* and the rate of catabolism is indicated in the *vertical axis*. The phosphonate added as an inhibitor is indicated as follows: Phnace in *black* (*filled square*); Phephn in *grey* (*shaded square*) and; Amephn in *white* (*open square*). The concentration of substrates and inhibitors was 50 mM. As references, 50 mM NaCl was used in place of the inhibitor for each one of the three substrates. Measurements (*n* = 3) were at 37°C, in NaCl/KCl suspensions. Errors determined as averages of the three measurements were estimated at ±15% of the substrate degradation rates

Phosphonate catabolism by cell lysates and fractions

Phnace hydrolysis was detected in whole-cell lysates and in the cell-wall fractions of *H. pylori*; no degradation was detected in the cytosolic fractions. Phephn was catabolised at similar rates by whole-cell lysates and cell-wall fractions and at rates 65% lower in cytosolic fractions. In contrast, separation of whole lysates into fractions decreased significantly the rates of Amephn catabolism; activity in the cell-wall fractions were about 30% of those measured for whole-cell lysates and for cytosolic fractions.

Growth on phosphonates

The effects of Phnace, Amephn and Phephn on *H. pylori* growth were studied by supplementing BHI broth with each of the phosphonates at concentrations between 0.5 and 10 mM. The presence of up to 10 mM concentrations of either of the three phosphonates did not affect cell growth indicating that the compounds were not cytotoxic at these concentrations.

The phosphonates used contained trace amounts of phosphate (<0.7%) (Mendz et al. 2005). To determine if this amount would support bacterial growth, phosphate growth curves were constructed. *H. pylori* grew only at phosphate concentrations higher than 100 μM. In contrast, *E. coli* DH5α was able to grow at phosphate concentrations as low as 100 nM.

To determine whether Phnace, Amephn or Phephn could be employed as the sole phosphorus source to support bacterial growth in vitro, *H. pylori* was grown in defined media containing 10 mM of phosphate or 10 mM of one of the three phosphonates. Media without phosphonate or phosphate was used as a control. Growth curves showed that Phephn was able to support bacterial growth as a sole phosphorus source, but Phnace or Amephn did not.

Phephn catabolism

Phephn catabolism was observed in bacterial cells, whole-cell lysates, cell pellets and cell-free extracts of *H. pylori* strains J99, LC20 and N6, employing ^{1}H-NMR spectroscopy. This substrate is catabolised by C–P lyase pathways. The ^{1}H-NMR spectrum of Phephn gave two clear resonance peaks proportional to its concentration, and the rates of degradation were determined from the decrease with time of this signal. The enzymatic origin of the reactions was established by determining that no activity was present in suspensions of whole-cell lysates which had been denatured by either heating at 80°C for 48 h or by treating with 1% SDS. All catabolism experiments were conducted at pH 7.0 and no change in the pH occurred over the duration of the reaction.

The rates of Phephn degradation by whole-cell lysates in three *H. pylori* strains showed sigmoid kinetics (Fig. 2); the K_m and V_{max} for the three strains are given in Table 2. The kinetic parameters for the three strains indicated that there were significant differences between strains. Interestingly, for cell-free extracts and cell-wall fractions the kinetics were of the Michaelis–Menten type. The K_m for the cell-wall fractions and cell-free extracts of strains J99 and N6 (Table 2) were greater than the maximum Phephn concentrations achievable in the samples under the experimental conditions owing to precipitation of Phephn, thus, the values quoted may overestimate the real ones. The K_m and V_{max} for the fractions were significantly higher than for whole-cell lysates. The

K_m and V_{max} for LC20 cell-wall and cytosolic fractions could not be reasonably calculated at the achievable concentrations of Phephn.

To elucidate putative effects of oxidation on Phephn catabolism, whole-cell lysates were treated with 25 mM DTT. Phephn catabolism in the lysates increased fivefold in the presence of DTT (data not shown). DTT had no effect on Phephn in the absence of cell lysates. The addition of 100 or 200 μM phosphate increased Phephn catabolism by 200 and 400%, respectively. MgCl$_2$ (100 mM) increased activity by 40% whilst EDTA (100 mM) reduced activity by 20% (Fig. 3). To determine the effects of monovalent cations in the catabolism of Phephn LiCl, NaCl, KCl, RbCl and CsCl were added to lysates and degradation rates were measured. None of the cations significantly affected the rate of Phephn catabolism (Fig. 3).

Discussion

Presence of multiple enzymes

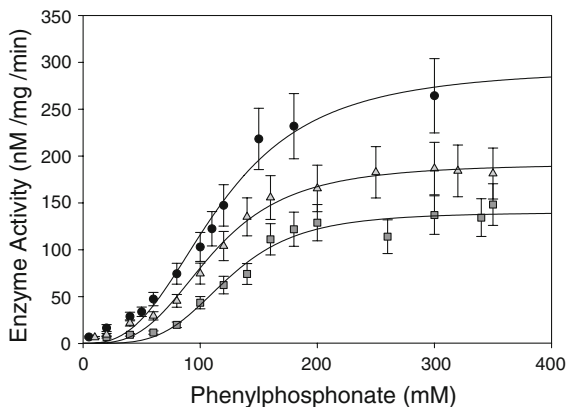Physiological studies have shown the presence of phosphonate metabolism in *Campylobacter* spp.

Fig. 2 Phephn catabolism rates by *H. pylori*. Phephn catabolism rates were measured in *H. pylori* strains J99 (*filled circle*), LC20 (*shaded square*) and N6 (*shaded triangle*) using ^{1}H-NMR spectroscopy. Bacterial cells were grown in the presence of abundant phosphate. The Phephn signal was measured every 172 s, and it decreased with time as the compound was catabolised. The rate of catabolism was calculated by measuring the fastest decrease in three or more signal measurements over the duration of the reaction. Errors were estimated at ±15%. The curve was fitted to the three parameter Hill sigmoidal function (Keller et al. 2002) by Sigma Plot 2002 (version 8.02)

(Mendz et al. 2005) which are closely related to *H. pylori*. Bioinformatic analyses on the genomes and proteomes of *H. pylori* have guided genetic and metabolic investigations of this bacterium; however, this approach is limited to proteins with homologues

Table 2 Phephn catabolism activities in *H. pylori*

Fraction	Isolate	K_m (mM)	V_{max} (nmol/min/mg)
Whole cell	J99	117 ± 9	295 ± 23
	N6	111 ± 4	191 ± 5
	LC20	124 ± 6	140 ± 6
Cytosolic	J99	299 ± 30	501 ± 28
	N6	273 ± 27	411 ± 24
	LC20	–	–
Cell wall	J99	433 ± 132	1,682
	N6	476 ± 97	648
	LC20	–	–

Phephn catabolism rates were measured in whole-cell lysates, cell-free extracts and cell-wall fractions of *H. pylori* strains J99, LC20 and N6 using ^{1}H-NMR spectroscopy. Catabolic rates were calculated by measuring the fastest decrease in three or more signal measurements over the duration of the reaction. K_m and V_{max} were calculated using the three parameter Hill sigmoidal function, and K_m was taken as the point of inflection. Errors in the kinetic parameters were calculated from non-linear regression fits to the data
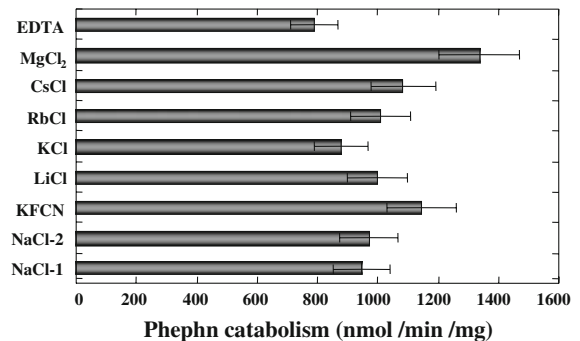

Fig. 3 The effects of different compounds and metal ions on *H. pylori* Phephn catabolism rates. Phephn catabolism rates were measured in *H. pylori* using ^{1}H-NMR spectroscopy. The Phephn signal was measured every 172 s, and it decreased with time as the compound was catabolised. The rate of catabolism was calculated by measuring the fastest decrease in three or more signal measurements over the duration of the reaction. The final concentration of the compounds and metal ions added to each assay mixture was 100 mM. Measurements ($n = 3$) were at 37°C, in NaCl/KCl suspensions. Errors determined as averages of the three measurements were estimated at ±15% the substrate rate. KFCN, potassium ferricyanide; NaCl-1, 250 mM NaCl; NaCl-2, 150 mM NaCl

characterised in other organisms. The catabolism of phosphonates by *H. pylori* described in this study demonstrated the presence of a function previously unknown in the genus.

Earlier phosphonate experiments in *H. pylori* showed phosphonatase activity in the cytosolic fraction of cell lysates which were capable of metabolising PALA and Phnace but this activity was not detected in cell-wall fractions (Burns et al. 1998). A simple interpretation of the published data and of our results on phosphonate catabolism by cell-wall and cytosolic fractions and of the effects of competing substrates on the rates of phosphonate catabolism (Fig. 1) is that there are at least two different C–P bond cleavage activities in *H. pylori*. One was exclusively associated with the cell wall of the bacterium and able to hydrolyse phosphonoalkyl carboxylates and PALA. The other activity was found in both the cell-wall and cytosolic fractions and able to cleave Phephn. In addition, both activities were capable of catabolising Amephn at different rates.

The catabolism of Phephn indicated the presence of a C–P lyase also able to cleave the C–P bond of Amephn, similar to those found in other Gram-negative bacteria such as *Agrobacterium radiobacter* (Wackett et al. 1987), *Alcaligenes eutrophus* (Schowanek and Verstraete 1990b), *E. coli* (Metcalf and Wanner 1993), *Rhodobacter capsulatus* (Schowanek and Verstraete 1990b), and various species of *Klebsiella* (Ternan et al. 1998, 2000), *Kluyvera* (Wackett et al. 1987), *Pseudomonas* (Schowanek and Verstraete 1990b; Stover et al. 2000) and *Rhizobium* (Parker et al. 1999; Stevens et al. 2000). C–P lyases acting on these substrates have been observed also in the Gram-positive bacteria *Arthrobacter atrocyanus* (Baker et al. 1998), *Arthrobacter* spp. GLP-1 (Schowanek and Verstraete 1990b), and *Bacillus megaterium* (Quinn et al. 1989). The other phosphonate cleaving activity could have been a phosphonatase or a phosphonoacetate hydrolase, since it could catabolise both substrates. Commonly, but not always, the expression of the former is regulated by phosphate while the latter is not (Baker et al. 1998; Kulakova et al. 2001; Lee et al. 1992; McGrath et al. 1995). Thus, the *H. pylori* enzyme is more likely to be a hydrolase because it was not regulated by phosphate.

Phosphate starvation dependence

In most bacteria the catabolism of phosphonate is under phosphate starvation control and the corresponding enzymes are part of the Pho regulon (Bardin et al. 1996; Kononova and Nesmeyanova 2002; Stover et al. 2000; Wanner 1994). At variance with *E. coli* and many other bacteria, the two phosphonate catabolism activities were expressed in *H. pylori* lysates even when there was abundant orthophosphate in the culture medium, suggesting that the enzymes are different from known C–P cleaving enzymes and that phosphonate degradation by *H. pylori* could occur in environments with substantial backgrounds of phosphate as seen in *B. cepacia* Pa16 (Ternan et al. 2000), *Pseudomonas paucimobilis* strain MMM101 (Schowanek and Verstraete 1990b), *Pseudomonas putida* NG2 (Ternan and Quinn 1998), and *Rhizobium huakii* PMY1 (McGrath et al. 1998). This interpretation is supported by the lack of *H. pylori* genes orthologous to those coding for any of the four known types of phosphonate catabolising enzymes. In addition, the expression of these enzyme activities in lysates from cells grown in media abundant in phosphate suggested that they may have other physiological roles.

Bacterial growth

Phosphonates are thought to have been far more common in early evolutionary history (Cooper et al. 1992), and this may have led to a number of phosphonate metabolism pathways being retained in modern bacteria. In the case of *H. pylori*, the constraints of its small genome and over 10,000 years of close evolution with its human host (Cooke et al. 2005) would make this unlikely. On the other hand, phosphonates are typically used as alternative sources of phosphate in phosphate starvation conditions.

The inability of *H. pylori* to catabolise the antibiotic glyphosate (Burns et al. 1998) indicates that the phosphonate catabolising pathways are primarily directed towards obtaining phosphorus. Levels of nutrients containing phosphate are high in the known habitat of *H. pylori* in the human stomach; however, the bacterium may undergo phosphate limitation en route to a new host, a situation that could have led to the development of these pathways. Nevertheless, environmental levels of phosphonate are typically too

low to measure (Nowack 2003), and alone are unlikely to provide *H. pylori* with enough phosphate. It is possible that *H. pylori* uses phosphonates as a supplement to other obtainable sources of phosphate such as orthophosphate. The ability for *E. coli* to grow on a larger range of phosphonates may be a reflection of its adaptation to grow in a wider range of habitats not commonly experienced by *H. pylori*.

Properties of Phephn catabolism by *H. pylori*

Helicobacter pylori can utilise Phephn but not Amephn or Phnace as a sole source of phosphate. Of the four known phosphonate metabolism pathways only the C–P lyase pathway is known to metabolise Phephn.

Whole cell lysates from all three strains of *H. pylori* showed the ability to catabolise Phephn. Cells were grown in abundant phosphate showing that the enzymes responsible for catabolism do not require phosphate starvation conditions for expression. All three strains showed sigmoid enzyme kinetics of Phephn catabolism with the clinical isolate LC20 having a higher K_m and lower V_{max} than the lab-adapted strains J99 and N6. This suggested that the continual passaging of *H. pylori* onto nutrient rich media may have caused a reduction in the efficiency of its phosphonate catabolism pathways relative to the requirements in vivo. On the other hand, *H. pylori* has a high level of genetic variation between strains (Cooke et al. 2005; Salama et al. 2000), and this may cause differences in the rate at which it metabolises some substrates. Thus, the variation of Phephn catabolism rates between strains LC20, J99 and N6 may be due to the different genetic backgrounds and not as a result of adaptation to lab conditions.

Sigmoid enzyme kinetics are known to be caused by a change in binding efficiency with increasing substrate saturation. In the case of metabolic enzymes, the activity is usually subject to feedback inhibition or activation resulting in conformational changes in the enzyme. Known allosteric effectors on enzyme kinetics include pH, complex formation, metal coordination and conformational changes owing to substrate binding in both active and non-active sites (White et al. 1954). The cause of the allosteric enzyme kinetics of Phephn in whole cell lysates is unknown. Sigmoid enzymes kinetics have been shown in a variety of organisms including

H. pylori (Pitson et al. 1999), but are undocumented in phosphonate metabolism. The sigmoid enzyme kinetics may be the result of Phephn being bound to sub-cellular components in the whole-cell lysates. Binding of Phephn other than to enzymes involved in its catabolism effectively reduces the concentration of Phephn available to the catabolic enzymes. Once the other components reach saturation, their effects are negated. The putative sub-cellular components would not be present in the fractionated cell lysates, thus, removing the sigmoid effect. Fractionation of lysates into cell-wall and cytosolic fractions removed the allosteric effector yielding Michaelis–Menten kinetics in both the cell-wall and cytosolic fractions.

There is evidence that the enzymes involved in phosphonate catabolism may require phosphorylation for maximum activity. Both phosphate and DTT increased the rate of Phephn catabolism. DTT can maintain proteins in a phosphorylated state (Potter et al. 2002); nonetheless, its ability to decrease protein degradation owing to oxidative stress is likely to have an effect on the in vitro rate of Phephn catabolism. Catabolic rates were reduced in the presence of EDTA consistent with previous *H. pylori* phosphonate catabolism studies (Burns et al. 1998). Rates of Phephn degradation were unaffected by monovalent cations but increased in the presence of Mg^{2+}. The presence of Mg^{2+} increases catabolism in both the phosphonatase and the phosphonopyruvate hydrolase pathways (Kononova and Nesmeyanova 2002; Kulakova et al. 2003; Ternan et al. 2000). The effects of Mg^{2+} on the C–P lyase pathway are undocumented, and only Zn^{2+}, Co^{2+} and Mn^{2+} affect catabolism in the phosphonoacetate hydrolase pathway. Mg^{2+} does not affect the rate of PALA catabolism (Burns et al. 1998) providing further evidence for the presence of at least two novel phosphonate metabolism enzymes in *H. pylori*.

Conclusions

This study characterised phosphonate metabolism in *H. pylori*, focusing on Phephn. Several factors that influence catabolism of Phephn have been identified. Evidence for at least two novel phosphonate catabolism pathways capable of metabolising Phephn that do not require phosphate starvation conditions for activity have been elucidated.

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