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Roles of the Peptide Transport Systems and Aminopeptidase PepA in Peptide Assimilation by *Helicobacter pylori*

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Peptide assimilation in *Helicobacter pylori* necessitates a coordinated working of the peptide transport systems (PepTs) and aminopeptidase (PepA). We found that *H. pylori* hydrolyzes two detector peptides, L-phenylalanyl-L-3-thiaphenylalanine (PSP) and L-phenylalanyl-L-2-sulfanilylglycine (PSG), primarily before intake and excludes their antibacterial effects, whereas *Escherichia coli* readily transports them with resultant growth inhibition. PSP assimilation by *H. pylori* was inhibited by aminopeptidase inhibitor bestatin, but not by dialanine or cyanide-*m*-chlorophenylhydrazone, contrary to that of *E. coli*. RT- and qRT-PCR analyses showed that *H. pylori* may express first the PepTs (*e.g.*, DppA and DppB) and then PepA. In addition, western blot analysis of PepA suggested that the bacterium secretes PepA in response to specific inducers.

Keywords: *Helicobacter pylori*, peptide transport, PepTs, PepA , detector peptides

Helicobacter pylori is a microaerophilic gram-negative bacterium known for its unique tolerance of the acidic environment in the human stomach [4, 15]. The opportunistic residence of this bacterium is widely accepted to be the main culprit of gastritis and peptic ulcer in humans. Such pathologic transformations of the stomach mucosa can then evolve into gastric cancer if not recognized early and properly treated [20]. Thus far, a variety of antimicrobial approaches to *H. pylori* eradication have yielded unsatisfactory results [24]. Despite the increasing efforts to uncover the workings of this bacterium, the link between *H. pylori* colonization and gastric diseases is largely unknown [2, 29].

An interesting characteristic of *H. pylori* metabolism lies in its reliance on amino acids [18, 26], particularly L-alanine, L-serine, and D-amino acids [3, 23], as the main energy source. At least eight amino acids essential for the survival of *H. pylori* are acquired from the host [23]. Pyruvate is provided not by glycolysis as in other species, but by metabolism of alanine [16, 17]. Moreover, the bacterium utilizes amino acids such as glutamate or aspartate as the major intermediates for the TCA cycle [3]. Rapid uptake of amino acids, possibly along with small peptides, facilitates the cellular metabolism and growth of the bacterium, as exhibited in *Lactococcus latis* [25]. Peptide transport systems (PepTs) exist in a wide variety of living systems, microorganisms [9, 19, 21], and mammalian cells [8, 14]. *H. pylori* also possesses two sets of ABC-type peptide transporter genes (*dpp* and *opp*) [3], and the roles of the Dpp and Opp systems were studied recently with mutant strains by Weinberg and Maier [28]. The functional data on these genes specifically in regard to the transporter specificity toward di- and oligo-peptides, however, showed some inconsistencies suggestive of residual transport mechanisms yet undiscovered.

Meanwhile, we have previously found that aminopeptidase (PepA) abounds in the *H. pylori* surface [30]. This enzyme was cloned in *Escherichia coli* and characterized [6]. We have also recently purified the native PepA enzyme from *H. pylori* (paper in preparation). An accurate assessment of the peptide uptake in *H. pylori* should thus take into account both the PepTs and PepA. We have previously mentioned some underlying problems regarding the use of labeled peptides for peptide transport study [5]. The

present study thus adopted a novel approach of utilizing peptide prodrugs L-phenylalanyl-L-3-thiaphenylalanine (PSP) and L-phenylalanyl-L-2-sulfanilylglycine (PSG) that contain thiophenol and sulfanilic acid, respectively, attached to the C-terminal α-carbon [12]; synthesis and assay were carried out as described previously [5]. We first determined their assimilation by intact *H. pylori* cells spectrophotometrically, with *E. coli* as the control. Next, we explored the time-course expression of PepTs and PepA in *H. pylori*, employing RT-PCR and qRT-PCR techniques, and then bacterial PepA secretion by western blot analysis.

H. pylori type strain ATCC 49503 was inoculated onto a brain heart infusion medium of pH 6.8, containing 5% horse serum plus antibiotic mixture (vancomycin, colistin, and nystatin), followed by incubation for 2 days at 37°C in a 10% CO_2 incubator; for preparation of intact cells, refer to the reference [11]. Cultivation of *E. coli* was carried out using a nutrient medium containing 1% polypeptone and 0.5% yeast extract (pH 7.0) in a reciprocal shaking chamber (150 rpm at 37°C). To study the mRNA levels of DppA, DppB, and PepA, total RNAs were isolated from the cell pellet using the Eazy-Blue/Total RNA Extraction Kit (Intron, Korea) and Trizol reagent (Gibco BRL). Primers used for (q)RT-PCR were from Cosmo Genentech Co., Korea (Table 1). cDNA preparation and RT-PCR were carried out using a ReverTra Ace (q)PCR-RT kit (Toyobo Co., Japan) and AccuPower PCR PreMix (Bioneer Co., Korea); after 26 cycles of PCR, the products were separated by electrophoresis on a 1% agarose gel. Analysis of PepA secretion by western blot analysis was carried out as follows; the cell-free supernatant (1 μg) or lysate (5.23 μg) was size fractionated by SDS-PAGE and then transferred onto a nitrocellulose membrane (Millipore). Membranes were blocked with 5% skim milk and then incubated with rabbit anti-PepA polyclonal antibody (LapFrontier, Seoul, Korea), and horseradish peroxidase-conjugated secondary antibodies (Cell Signaling Technology, Danvers, MA, USA) were used.

Table 1. Primers used in this study.

Primer	Sequences (5′ to 3′)	Length (nt)	T_m (°C)
pepAF	GCCCCACTCGCTCCAAA	17	51.9
pepAR	TGCAGGCCCAGCTTATGTG	19	53.2
dppAF	GGGATTAGGCGAGCATGAAA	20	51.8
dppAR	TCCGGATCCGCAATGTCT	18	50.3
dppBF	AGGAGATCCGGCGTTAGTGA	20	53.8
dppBR	GCGTCAATAGCGGCTTGATT	20	51.8
icdF	ACGGTGGGCATGTTTGAAG	19	51.1
icdR	ATCCAGCCCGGCGTATTTA	19	51.1
16srRNAF	GCTAAGAGATCAGCCTATGTCC	22	54.8
16srRNAR	TGGCAATCAGCGTCAGGTAATG	22	54.8

Upon PSP addition to the whole-cell suspensions of *H. pylori* and *E. coli*, thiophenol began to rise after a few minutes of time-lag in both systems (Fig. 1). Note that the slope yielded by the *E. coli* system is about four times steeper than that of *H. pylori*, similar to that of the growth rates (in the upper panel). These data, however, still do not reveal the amount of intact peptides transported into the cell. This is due to the fact that thiophenol production may result from PSP breakdown either inside or outside the cell. The solution to this problem is to measure the amount of intracellularly released molecule that correlates with the amount of peptides taken up by the cells. A dipeptide PSG with a sulfanilyl moiety is suitable for this purpose [9, 10], and thus it was used in determining the cellular location of the PSG hydrolysis in the same experiment (see Fig. 1). In *H. pylori* cells, sulfanilate progressively but quite slowly accumulated with time outside of the cells, without any detectable intracellular appearance, within the experimental error range, even after sufficient incubation. On the contrary, sulfanilate in *E. coli* was not detectable in the supernatants or the extracellular space. It accumulated

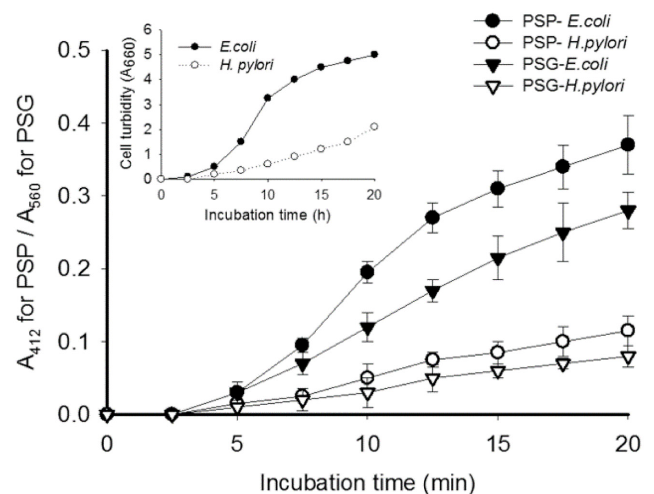

Fig. 1. Spectrophotometric determination of *H. pylori* assimilation of PSP and PSG.

An *E. coli* strain [10] with the normal PepTs was used as the control. The upper left-hand inset presents the bacterial growth curves, and the right-hand margin shows symbols for *H. pylori* (open) and *E. coli* (closed). Note the similar sigmoid plots of the rates of the bacterial peptide assimilation and growth versus incubation time. Error bars indicate standard deviations from three replicate experiments.

with time inside the cells. The slower rate of sulfanilate production than thiophenol production in the *E. coli* system accounts for the Dpp's different kinetic behavior for those substrates [10, 22]. These data evidently demonstrate that *H. pylori* hydrolyzes PSG primarily outside of the cell, in contrast to *E. coli* that carries out intracellular hydrolysis following transport into the cell.

It is remarkable that sulfanilate is practically impermeable to the cell membrane. However, it is shown here to be transported as part of a dipeptide in *E. coli* [7]. Of note, sulfanilate binds with much higher affinity than sulfanilamide to dihydropteroate synthase [9], theoretically exerting a more potent antibacterial effect. Despite such favorable applicability as an antimicrobial agent, however, *H. pylori* did not allow peptide-assisted entry of the anti-metabolite; namely, *H. pylori* should basically be resistant to peptide prodrugs. Experimental data to support this are presented in Table 2; as peptide side-chains, the antibacterial efficacies of thiophenol and sulfanilate increased considerably against *E. coli*, but not in *H. pylori*, indicating most of them are released extracellularly.

The Dpp transports only dipeptides [1, 12, 19] so that the transportation of PSP should specifically be inhibited by dipeptides. Indeed, the rate of thiophenol production from PSP by intact *E. coli* cells was substantially inhibited only by dialanine, and the absence of such a specific inhibition in the cell extract further supported the hypothesis that dialanine competes with PSP for the *E. coli* Dpp (Table 3). Thiophenol production from PSP was not inhibited specifically by dialanine in either the *H. pylori* cell suspension or cell extract, suggestive of its enzymatic hydrolysis largely before transport. We also employed a protonophore, cyanide-*m*-chlorophenylhydrazone (CCCP), that interrupts di- and oligo-peptide transport [19, 22]. As predicted, this compound almost completely blocked PSP assimilation by *E. coli* but not by *H. pylori*. In addition, when PSP was added to the *H. pylori* cell suspension containing bestatin, a potent aminopeptidase inhibitor [27], thiophenol production was markedly reduced, but it had little effect on PSP assimilation in *E. coli*. Taken altogether, the immediate site for PSP hydrolysis may largely be restricted to the cell surface and the cytoplasm of *H. pylori* and *E. coli*, respectively. In our future study, the utilization of a PepA-defective condition, such as with a PepA mutant, will be useful in quantifying peptide transport in *H. pylori*.

We next determined the relative expression ratio between the PepTs (here, DppA and DppB) and PepA, by means of qRT-PCR, with isocitrate dehydrogenase (ICD) as the control. As a result, 1-day cultured cells contained about 2-fold more PepA mRNA than DppA and DppB, followed by further widening the gap with incubation time (Fig. 2A). A more clear-cut difference could be observed when the ratio of individual yields relative to that of the control ICD were computed; as can be seen in Fig. 2B, their expression levels in fact appeared to diverge with time, with a decrease in the Dpps level accompanied by an increase in the PepA level. To see what would happen at an earlier stage, carefully washed and concentrated *H. pylori* cells were co-cultured with a human gastric epithelial cell line, AGS (#CRL-1739 ATCC, USA), and observed for their RT-PCR products by agarose gel electrophoresis, with 16S rRNA as the control (Fig. 2C). Strikingly, the mRNA level of DppB appeared to stay constant up to 24 h, whereas that

Table 2. Antibacterial effects of thiophenol and sulfanilate as side chains of dipeptide.

Compound	Conc. (μmol)	Disc zone diameter (mm)	
		H. pylori	*E. coli*
Thiophenol	1	7 ± 1	7 ± 1
	10	15 ± 1	14 ± 1
Sulfanilate	1	0	0
	10	(10 ± 1)	(11 ± 1)
PSP	1	7 ± 1	15 ± 1
	10	14 ± 1	26 ± 1
PSG	1	(7 ± 1)	(17 ± 1)
	10	(11 ± 1)	(28 ± 1)

Solutions of the compounds were added to filter paper discs (6 mm diameter) and the discs were transferred to agar plates with a defined medium [20], seeded with fresh cells (approx. 10^7 seeds/ml). After incubation at 37°C overnight, the diameters of the zones of inhibition were measured; parentheses indicate static zones. Data values are the means ± standard errors in a triplicate assay.

Table 3. Differential effects of additives on thiophenol production from PSP by intact cells and cell-free extracts.

Additives	Conc. (mM)	% Reduction in rate[a]			
		H. pylori		*E. coli*	
		Cells	Extract	Cells	Extract
(Ala)2	1	10 ± 4	13 ± 1	90 ± 5	16 ± 2
(Ala)3	1	22 ± 7	26 ± 3	12 ± 2	14 ± 2
(Ala)4	1	17 ± 6	20 ± 2	11 ± 3	15 ± 2
Bestatin	0.01	80 ± 8	91 ± 7	5 ± 1	90 ± 6
CCCP	0.01	ND	ND	100	100

[a]Rates of thiophenol release from 0.1 mM PSP by intact cell suspensions (turbidity; A_{600} ≈. 1.0) or cell extracts were measured in the presence and absence of additives. Data represent the percentage decrease in amount of thiophenol produced from PSP by additives; ND, not determinable.
Values are the means ± standard errors in a triplicate assay.

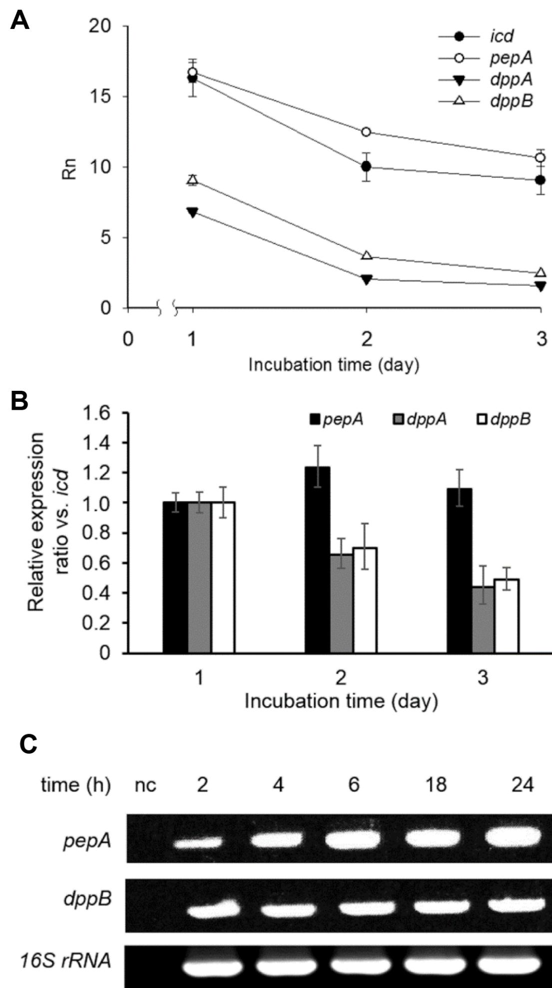

Fig. 2. Determination of the PepTs and PepA levels in *H. pylori*.
(**A**) qRT-PCR of DppA, DppB, PepA, and ICD as control. Rn is the reporter signal normalized to the fluorescence of the ROX^{TM}, passive dye, i.e. the ratio of the fluorescence of the reporter dye (SYBR® Green) divided by the fluorescence of the ROX dye. (**B**) Computed ratio of the levels relative to that of ICD in A. (**C**) RT-PCR of DppB and PepA in *H. pylori* co-cultured with AGS cell line in 1 day, with 16S rRNA as the control. Note that mRNA levels of DppB and 16S rRNA appear to be constant within the period examined, whereas that of PepA increases gradually with time. Error bars indicate standard deviations from three replicate experiments. The 'nc' means negative control.

of PepA increased progressively with time. These data show that in *H. pylori*, the PepTs may preexist and PepA is inducible. More interestingly, western blotting of PepA appeared to be positive in the *H. pylori* medium with peptone but not with amino acids or casein (Fig. 3), connoting that there may be a putative sensing mechanism [13] in this bacterium to secrete the enzyme. Although beyond the scope of this study, we hypothesize that the PepTs *per se* might be the sensory system; the association between the PepTs and PepA secretion is under investigation.

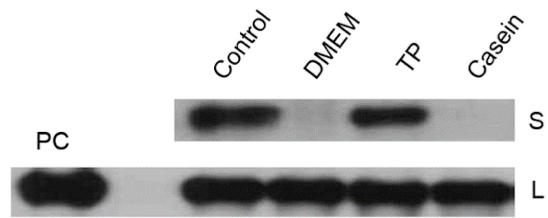

Fig. 3. Western blot for PepA: effects of medium composition on PepA secretion in *H. pylori*.
Lanes contain 1 μg or 5.23 μg proteins in supernatant (S) or cell lysate (L), respectively. Cells placed in different medium conditions for 1 h (37°C; 10% CO_2) were used; detailed procedures are in the text. Control, 20 mM HEPES-KOH, pH 7.4; DMEM, Dulbecco's Modified Eagle's Medium; TP, 1% Tryptone-Peptone, pH 7.4; Casein, 1%, pH 7.4; PC, positive control with purified PepA protein.

In conclusion, we showed for the first time that *H. pylori* expresses the PepTs but secretes PepA. Thus, peptides are mostly predigested before uptake. Although difficult to reason, *H. pylori*'s properties of taking up digested rather than intact peptides may be an evolutionary advantage for bacterial colonization. However, it is deleterious to rapidly growing human gastric mucosal cells with the PepTs. Further investigation into this topic will help unravel the pathogenic mechanisms of *H. pylori* and aid in the development of pharmacologic therapies for its eradication.

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