J. Microbiol. Biotechnol. (2005), **15**(4), 899–902

JOURNAL OF MICROBIOLOGY AND BIOTECHNOLOGY

Characteristics of Peptide Assimilation by *Helicobacter pylori*: Evidence for Involvement of Cell Surface Peptidase

YUN, SOON-KYU, KYUNG-MIN CHOI, CHANG-SUB UHM[1], JEONG-KYU PARK[2], AND SE-YOUNG HWANG*

Graduate School of Biotechnology & Department of Bioinformatics and Biotechnology, Korea University, Seoul 136-701, Korea
[1]Department of Anatomy, College of Medicine, Korea University, Seoul 136-701, Korea
[2]Department of Microbiology, College of Medicine, Chungnam National University, Taejon 301-131, Korea

Received: December 15, 2004
Accepted: March 16, 2005

Abstract Peptide assimilation by *Helicobacter pylori* was investigated using L-phenylalanyl-3-thia-phenylalanine (PSP) as a detector peptide; the release of thiophenol upon enzymatic hydrolysis of PSP was spectrophotometrically detected with the aid of 5,5'-dithiobis[2-nitrobenzoic acid] (DTNB). By adding PSP to whole-cell suspension, thiophenol was produced progressively, resembling that found in *Esherichia coli* or *Staphylococcus aureus*. Interestingly, the rate of thiophenol production by *H. pylori* in particular was markedly reduced when cells were pretreated with trypsin, indicating surface exhibition of peptidase. According to the competitive spectrophotometry using alanyl-peptides, *H. pylori* did not appear to assimilate PSP through the peptide transport system. No discernible PSP assimilation could be ascertained in *H. pylori* cells, unless provided with some additives necessary for peptidase activity, such as Ni^{2+} or Mg^{2+} and an appropriate concentration of potassium or ammonium salts. These observations strongly suggest that, regardless of a presumptive peptide transport system, peptide assimilation of *H. plori* appears to be highly dependent upon milieu conditions, due to unique peptidase exhibition on the cell surface.

Key words: *Helicobacter pylori*, peptide transport, detector peptide, peptidase

Microorganisms can directly assimilate small peptides via di- or oligopeptide permeases [6]. Since their specificities of the peptide side-chain composition are relatively low, transport of normally impermeant compounds may greatly be facilitated if they are incorporated into peptides [7, 15]. In fact, many examples of both natural and synthetic peptide prodrugs elaborating this indication have been reported [5, 14, 22]. However, chemotherapeutic effectiveness of peptide prodrugs is thought to depend on the extent of their competition with nutrient peptides [24]. Elucidating the properties of peptide assimilation by individual pathogens is, therefore, necessary before developing peptide prodrugs.

H. pylori persistently colonizes human gastric mucosa and is considered to be a major risk factor causing various gastric diseases such as gastritis, peptic ulcer, and stomach cancer [3, 16, 18]. This bacterium is unique, in that most of the energy required for cellular metabolism are not provided from glycolysis, but rather exclusively from the unusual Kreb's cycle using amino acids as the major fuel [17, 23]. Nonetheless, many of the amino acids can not be provided by *H. pylori* itself [21], indicating an absolute dependence of nitrogen source from the host. Considering the probable impact of endothelial homeostasis of amino acids for tissue health, the rate of amino acid deprivation by *H. pylori* may be critical for destining the life-span of the host cells. More importantly, the resultant net production of ammonia [2] would be contributory to maintain ammonium clouds surrounding this bacterium, it has been considered as a unique bacterial property in relation to pathogenesis [1, 11, 13]. Meanwhile, the recent analysis of the complete genomic sequence strongly suggests that *H. pylori* may possess a peptide transport system [4]. Unexpectedly, however, *H. pylori* has been shown to be insensitive to peptide prodrugs (our observation). We, therefore, were interested in how this bacterium assimilates small peptides.

Dealing with intact *H. pylori* cells *in vitro* is not easy, because of the high vulnerability of the cell envelope systems [10]. Accordingly, a chromogenic mimicry of diphenylalanine [8] was employed, which made the assay possible using intact *H. pylori* cells without disruption [19]. In practice, peptide assimilation is determined by measuring the amount of thiophenol in the medium; at neutral pH conditions, thiophenol cannot be transported, but is readily

*Corresponding author
Phone: 82-2-3290-3420/41-860-1412; Fax: 82-2-290-3420/41-865-3339;
E-mail: shwang@korea.ac.kr

extruded from bacterial cells [9]. For comparison, *E. coli* and *S. aureus* were also examined.

H. pylori ATCC 49503 was inoculated onto a brain heart infusion medium containing 5% virus-free horse serum (pH 6.8) and incubated for up to 2 days, as described previously [12]. To avoid any microbial contamination, an antibiotic mixture (1.5 mg vancomycin, 3.5 mg colistin, and 6 mg nystatin per liter) was supplemented to the medium. *E. coli* and *S. aureus* strains were aerobically grown overnight at 37°C using the Luria medium (1% bacto peptone, 0.5% yeast extract, and 1% NaCl; pH 7.0) [25].

As mentioned previously, bacterial peptide transport systems lack side chain specificity. Accordingly, we anticipated that PSP [8] would exert anti-*H. pylori* effect if *H. pylori* possesses a relevant transport system. Our preliminary data, however, have shown that this bacterium would be resistant to PSP. This finding posed the question of whether peptide transport system is indeed present in this organism. To elucidate this, we determined directly the PSP uptake by intact bacterial cells. By incubating whole cells of *H. pylori* with PSP in the presence of DTNB, it was found that thiophenol was produced progressively, having a short time-lag period at the beginning. Such phenomena were also observed in *E. coli* or *S. aureus* (Fig. 1A). The initial low rate of thiophenol production was likely due to the fact that the suspended system needs a certain period of time to achieve saturation kinetics. As presented in Fig. 1B, peptidase pools between the intact cells and washed-out fractions were compared: Substantial amounts of peptidases were present at cell surfaces of all strains, indicating that peptides may be assimilated in part as hydrolyzates during bacterial growth. To elucidate whether PSP is taken up by a carrier-mediated transport system, the peptidase-removed bacterial cells were treated with 1% chloroform; by this treatment, polar molecules in general are freely diffused across the cell membrane. As predicted, chloroform treatment of bacterial cells did not affect their ability of PSP assimilation, implicative of PSP's mediated transport. Trypsin treatment also did not affect PSP assimilation by *E. coli* or *S. aureus* cells, but a marked reduction of the PSP-assimilating capacity was seen in *H. pylori* cells (Fig. 2). This phenomenon infers that, unlike the above control strains, a large trypsin-sensitive pool may uniquely reside on *H. pylori*'s cell surface, perhaps being responsible for peptide hydrolysis. Dipeptides have been shown to selectively inhibit PSP transport through

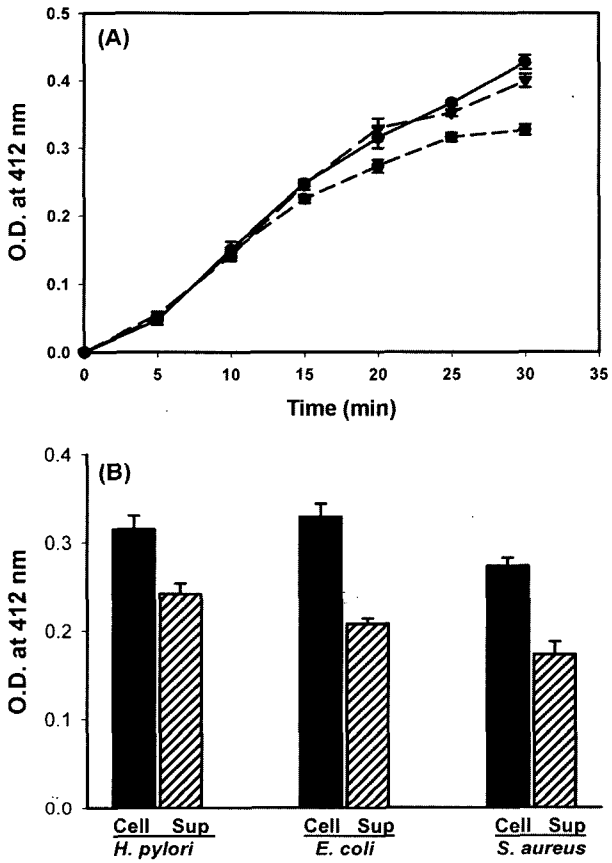

Fig. 1. Determination of thiophenol production from PSP.
(A) PSP-assimilating capacity of bacterial intact cells. Fresh cells were washed twice with saline solution, and then suspended to a turbidity of 0.2 O.D. at 660 nm in 50 mM HEPES-KOH or PBS, pH 7.4, for *H. pylori* and control strains, respectively. To the whole-cell-suspension (0.98 ml) placed in a 1-cm light-path cuvette was added 10 µl of 10 mM DTNB. Reaction was initiated by the addition of 10 µl of 5 mM PSP, and monitored spectrophotometrically using UV2101PC (Shimazu, Japan). Symbols used: ●, *H. pylori*; ▼, *E. coli*; ■, *S. aureus*. (B). Thiophenol production from PSP by intact cells and extracellular peptidase. Total peptidases washed out from cells were reacted with PSP for 20 min and compared with the data of (A). Abbreviation; HEPES, N-2-hydroxyethylpiperazine-N'-2-ethanesulfonate.

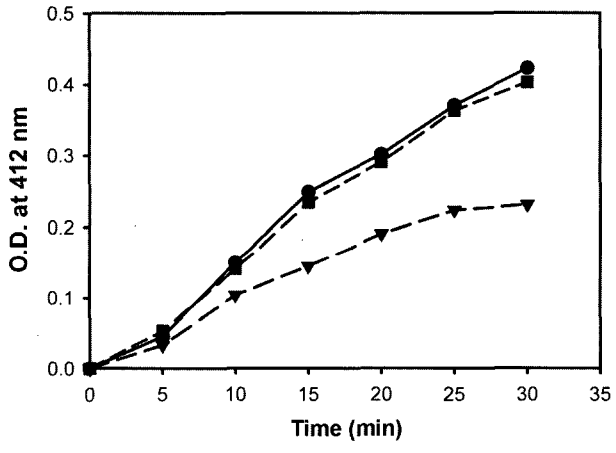

Fig. 2. Effect of organic and enzymatic pretreatment of *H. pylori* cells on PSP assimilation.
H. pylori cell-suspensions (A_{660} = 0.2; 50 mM HEPES-KOH, pH 7.4) containing trypsin (1 mg/ml) or chloroform (1.5% in final conc.) were incubated for 30 min at 37°C, and then centrifuged (9,000 ×*g* for 10 min). The resulting cell pellets were carefully washed using a hand homogenizer before assay. Symbols used: ●, *H. pylori* control; ▼, *H. pylori* treated with trypsin; ■, *H. pylori* treated with chloroform.

Table 1. Inhibitory effect of alanyl-peptides on bacterial PSP assimilation.

	Percent inhibition for PSP assimilation			
	Ala	$(Ala)_2$	$(Ala)_3$	$(Ala)_4$
H. pylori	17	18	47	20
E. coli	NI	69	18	ND
S. aureus	NI	63	48	ND

Bacterial cell-suspensions containing 0.05 mM PSP, 0.1 mM DTNB, and 50 mM of optimum buffers (pH 7.4) for individual strains (detailed buffer compositions are listed in Table 2 and Fig. 3) were incubated with or without 0.1 mM each of competitor for 10 min at 37°C; in *H. Pylori* assay system, 0.1 mM $NiCl_2$ was added. NI, not inhibitory; ND, not determined.

bacterial dipeptide transport systems [20]. This, in turn, reflects that the specificities recognizing peptide length are much narrower than that of cellular peptidase [8]. To show the probable effect of surface peptidase on *H. pylori*'s peptide transport, a comparative experiment was undertaken by employing the competitive spectrophotometry [9] with alanyl-peptides. As predicted, di-alanine selectively inhibited PSP assimilation of both *E. coli* and *S. aureus*, but not that of *H. pylori* (Table 1). The discrepancy in *H. pylori* is interpreted to be due to nonspecific inhibition and signifies that *H. pylori* does not fully adopt a peptide transport system for assimilating such small peptides as PSP, but permits perpetual action of peptidase on the surface to a great extent. It was also found that *H. pylori* cells continuously released corresponding peptidase, harvestable simply by cell resuspension and centrifugation (9,000 ×*g*, 10 min). During

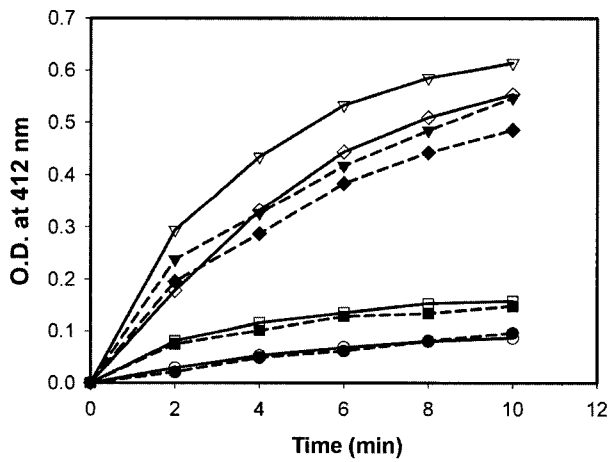

Fig. 3. Factors affecting thiophenol production from PSP by *H. pylori* cells and cell-free extract.

Detailed conditions for thiophenol production from PSP were described under the legend in Fig. 1. Cellular peptidase was isolated from whole cells by sonic treatment and ultracentrifugation (see Ref. 25 for conditions). Cells and cell-free extract were suspended or dissolved in 50 mM HEPES-Tris, pH 7.4, and then reacted with PSP in the presence or absence of 0.1 mM $NiCl_2$ and/or 50 mM salts. Symbols used: Cell-free extract - ●, none; ▼, $NiCl_2$; ■, $NiCl_2$ plus NaCl; ◆, $NiCl_2$ plus KCl. Intact cells - ○, none; ▽, $NiCl_2$; □, $NiCl_2$ plus NaCl; ◇, $NiCl_2$ plus KCl.

Table 2. Comparison of medium compositions for bacterial PSP assimilation.

Compositions	*Relative extent of PSP assimilation		
	H. pylori	*E. coli*	*S. aureus*
HEPES-KOH	0.33	ND	ND
HEPES-KOH+Ni^{2+}	1.00	ND	ND
PBS	0.23	0.98	0.94
PBS+Ni^{2+}	0.52	0.22	0.59
PBS+Mg^{2+}	0.38	1.00	1.00
HEPES-Tris	0.06	0.43	0.91
HEPES-Tris+Ni^{2+}	0.63	0.35	0.74
HEPES-Tris+Mg^{2+}	ND	0.39	ND

*Data values are presented as the ratio of the degree of thiophenol production relative to those observed at an optimum condition. Turbidity of bacterial cell-suspensions; A_{660} = 0.2. All buffers were 50 mM at pH 7.4. Ni^{2+}, 0.1 mM; Mg^{2+}, 10 mM; PBS, phosphate buffer containing 0.7% NaCl.

or after enzyme harvest, the intracellular peptidase pool was found to be almost constant (data not shown). Although our understanding of what is beneficial to this bacterium is beyond the present scope, versatile expression or externalization of this peptidase may be conceivable in this bacterium. Interestingly, both whole cells and cell-free extract appeared to be identical for their requirements for PSP hydrolysis and/or assimilation (Fig. 3). In fact, intact *H. pylori* cells did not appear to assimilate PSP, unless the peptidase was artificially activated; having the characteristic of an apoenzyme, it was virtually inactive without extra provision of heavy metals such as $NiCl_2$, along with appropriate concentrations of K^+, but not Na^+. All of these requirements for peptidase action did not show any positive effect on PSP assimilation by the other strains (Table 2). It was remarkable that ammonium ions also served the same effect as K^+, suggesting a somewhat differential environment of *H. pylori* habitats in particular. These findings suggest that the extent of peptide assimilation by *H. pylori* would strongly depend on its surrounding circumstances, due to robust peptidase across the cell wall.

In the course of this study on possible use of peptide prodrugs against *H. pylori*, we found that this pathogen was relatively insensitive to those prodrugs. In order to elucidate the mechanism of the bacterial resistance, we studied the characteristics of peptide assimilation using a chromogenic dipeptide mimicry, PSP. As a result, PSP hydrolysis was found to occur to a large extent before transport, due to substantial surface coverage by peptidase. Some portion of this enzyme pool could be removed by trypsin treatment, suggesting its distinct association with the cell wall. During a consecutive cropping of the enzyme by cell washing, both surface and intracellular peptidase were found to be constantly maintained. Such unique cellular distribution of peptidase may be responsible for the bacterium to resist peptide prodrugs in general. In addition, because of the

presence of enzyme throughout the cellular compartments, *H. pylori* should not be able to assimilate small peptides unless peptidase is activated. Its nature of an apoenzyme character should also be mentioned, raising the question of its physiological role for this pathogen. Apart from this uncertain point, it is apparent that a single ubiquitous peptidase contributes to peptide assimilation by *H. pylori*, regardless of its conceivable possession of a peptide transport system, and the activity determines the rate of peptide assimilation by this organism.

Acknowledgment

This work was supported by a grant (R05-2000-000-00108-0) from the Korea Science and Engineering Foundation.

References

1. Choi, K. M., J. K. Park, and S. Y. Hwang. 2003. Pathogenetic impact of vacuolar degeneration by accelerated transport of *Helicobacter pylori* VacA. *J. Microbiol. Biotechnol.* **13:** 666–672.
2. Chung, M. I., M. H. Lim, Y. J. Lee, I. H. Kim, I. Y. Kim, J. H. Kim, K. H. Chang, and H. J. Kim. 2003. Reduction of ammonia accumulation and improvement of cell viability by expression of urea cycle enzymes in Chinese hamster ovary cells. *J. Microbiol. Biotechnol.* **13:** 217–224.
3. Cover, T. and M. Blaser. 1992. *Helicobacter pylori* and gastroduodenal disease. *Annu. Rev. Med.* **42:** 135–145.
4. Doig, P., B. L. de Jonge, R. A. Alm, E. D. Brown, M. Urica-Nickelsen, B. Noonan, S. D. Mills, P. Tummino, G. Carmel, B. C. Guild, D. T. Moir, G. F. Vovis, and T. J. Trust. 1999. *Helicobacter pylori* physiology predicted from genomic comparison of two strains. *Microbiol. Mol. Biol. Rev.* **63:** 675–707.
5. Gilvarg, C. 1981. *In* Ninet, L., P. E. Bost, D. H. Bovanchaud, and J. Florent (eds.). *The Future of Antibiotherapy and Antibiotic Research*, pp. 351–365. Academic Press.
6. Hwang, S. Y., D. A. Berges, J. J. Taggart, and C. Gilvarg. 1989. Portage transport of sulfanilamide and sulfanilic acid. *J. Med. Chem.* **32:** 694–698.
7. Hwang, S. Y., M. R. Ki, S. Y. Cho, and I. D. Yoo. 1995. Transport of sulfanilic acid via microbial dipeptide transport system. *J. Microbiol. Biotechnol.* **5:** 315–318.
8. Hwang, S. Y., W. D. Kingsbury, N. M. Hall, D. R. Jakas, G. L. Dunn, and C. Gilvarg. 1986. Determination of leucine aminopeptidase using phenylalanyl-3-thia-phenylalanine as substrate. *Anal. Biochem.* **154:** 552–558.
9. Hwang, S. Y., M. R. Ki, S. Y. Cho, W. J. Lim, and I. D. Yoo. 1996. Competitive spectrophotometry for microbial dipeptide transport systems. *J. Microbiol. Biotechnol.* **6:** 92–97.
10. Keenan, J., T. Day, S. Neal, B. Cook, G. Perez-Perez, R. Allardyce, and P. Bagshaw. 2000. A role for the bacterial outer membrane in the pathogenesis of *Helicobacter pylori* infection. *FEMS Microbiol. Lett.* **182:** 259–264.
11. Ki, M. R., S. K. Yun, K. M. Choi, and S. Y. Hwang. 2003. Potential and significance of ammonium production from *Helicobacter pylori*. *J. Microbiol. Biotechnol.* **13:** 673–679.
12. Ki, M. R., S. K. Yun, W. J. Lim, B. S. Hong, and S. Y. Hwang. 1999. Synergistic inhibition of membrane ATPase and cell growth of *Helicobacter pylori* by ATPase inhibitors. *J. Microbiol. Biotechnol.* **9:** 414–421.
13. Kim, J. M., J. E. Shin, M. J. Han, S. H. Park, and D. H. Kim. 2003. Inhibitory effect of ginseng saponins and polysaccharides on infection and vacuolation of *Helicobacter pylori*. *J. Microbiol. Biotechnol.* **13:** 706–709.
14. Kim, M. W., C. S. Shin, H. J. Yang, S. H. Kim, H. Y. Lim, C. H. Lee, M. K. Kim, and Y. H. Lim. 2004. Naltriben analogues as peptide anticancer drugs. *J. Microbiol. Biotechnol.* **14:** 881–884.
15. Kingsbury, W. D., J. C. Boehm, D. Perry, and C. Gilvarg. 1984. Portage of various compounds into bacteria by attachment to glycine residues in peptides. *Proc. Natl. Acad. Sci. USA* **81:** 4573–4576.
16. Lui, S. Y., K. L. Ling, and B. Ho. 2003. *rdxA* Gene is an unlike marker for metronidazole resistance in the asian *Helicobacter pylori* isolates. *J. Microbiol. Biotechnol.* **13:** 751–758.
17. Mendz, G. L., S. L. Hazell, and L. van Gorkom. 1994. Pyruvate metabolism in *Helicobacter pylori*. *Arch. Microbiol.* **162:** 187–192.
18. Parsonnet, J., S. Hansen, L. Rodriguez, A. B. Gelb, R. A. Warnke, E. Jellum, N. Orentreich, J. H. Vogelman, and G. D. Friedman. 1994. *Helicobacter pylori* infection and gastric lymphoma. *New Engl. J. Med.* **330:** 1267–1271.
19. Payne, J. W. and T. M. Nisbet. 1980. Limitation to the use of radioactively labeled substrates for studying transport in microorganisms. *FEBS Lett.* **119:** 73–76.
20. Perry, D. and C. Gilvarg. 1984. Spectrophotometric determination of affinities of peptides for their transport systems in *Esherichia coli*. *J. Bacteriol.* **169:** 943–948.
21. Reynolds, D. J. and C. W. Penn. 1994. Characteristics of *Helicobacter pylori* growth in a defined medium and determination of its amino acid requirements. *Microbiology* **140:** 2649–2656.
22. Scannell, J. P. and D. L. Pruess. 1974. Naturally occurring amino acid and oligopeptide antimetabolites, pp. 189–243. *In* B. Weinstein (ed.). Chemistry and Biochemistry of Amino Acids, Peptides, and Proteins: 3. Dekker, NY, U.S.A.
23. Stark, R. M., M. S. Suleiman, I. J. Hassan, J. Geenman, and M. R. Miller. 1997. Amino acid utilisation and deamination of glutamine and asparagine by *Helicobacter pylori*. *J. Med. Microbiol.* **46:** 793–800.
24. Terada, T., K. Sawada, H. Saito, Y. Hashimoto, and K. I. Inui. 1999. Functional characteristics of basolateral peptide transporter in the human intestinal cell line-Caco-2. *Am. J. Physiol.* **276:** G1435–G1441.
25. Yun, S. K., M. R. Ki, J. K. Park, W. J. Lim, and S. Y. Hwang. 2000. Cation flux-mediated activation of P-type ATPase in *Helicobacter pylori*. *J. Microbiol. Biotechnol.* **10:** 441–448.