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Peptide Transport in *Helicobacter pylori*: Roles of Dpp and Opp Systems and Evidence for Additional Peptide Transporters[▽]

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Despite research into the nutritional requirements of *Helicobacter pylori*, little is known regarding its use of complex substrates, such as peptides. Analysis of genome sequences revealed putative ABC-type transporter genes for dipeptide (*dppABCDF*) and oligopeptide (*oppABCD*) transport. Genes from each system were PCR amplified, cloned, and disrupted by cassette insertion either individually (*dppA*, *dppB*, *dppC*, *oppA*, *oppB*, and *oppC*) or to create double mutants (*dppA oppA*, *dppB oppB*, *dppB dppC*, and *oppB oppC*). Peptide-utilizing abilities of the strains were assessed by monitoring growth in a chemically defined medium where the only source of the essential amino acid isoleucine was from peptides of various lengths (two to nine amino acids long). The dipeptide system mutants lacked the ability to use certain dipeptides, hexapeptides, and nonapeptides. However, these mutants retained some ability to grow with other dipeptides, tripeptides, and tetrapeptides. Of the oligopeptide mutants, only the *oppB* strain differed significantly from the wild type. This strain showed a wild-type phenotype for growth with longer peptides (hexa- and nonapeptides) while having a decreased ability to utilize di-, tri-, and tetrapeptides. The *dppA oppA* and *dppB oppB* mutants showed similar phenotypes to those of the *dppA* and *dppB* mutants, respectively. Peptide digestion by metalloproteases was ruled out as the cause for residual peptide transport by growing mutant strains in the presence of either EDTA or EGTA. Degradation products associated with a fluorescein isothiocyanate-labeled hexapeptide (plus cells) were minimal. An as yet unidentified peptide transport system(s) in *H. pylori* is proposed to be responsible for the residual transport.

Helicobacter pylori is a gram-negative bacterium that is the causative agent of a number of stomach-related diseases, including peptic ulcer disease and certain gastric cancers (3). *H. pylori* has traditionally been grown on complex media, usually supplemented with either 5 to 10% blood or serum. Over the last 15 years, however, steps have been taken to develop defined media in order to study the nutritional and metabolic requirements of *H. pylori* (1, 7, 29, 32, 38, 43, 45). Most recently, the absolute amino acid requirements for the growth of *H. pylori*, as well as the requirements of other nutrients (such as trace metals) for *H. pylori* and other *Helicobacter* species, were determined (42). Although it has been shown to grow on glucose (26–28), *H. pylori* does not have the enzymatic machinery to break down more complex carbohydrates (11) and is thus limited in its ability to utilize carbohydrates as carbon sources. The bacterium can incorporate some simple amino acids as a carbon source, but the same studies showed that *H. pylori* requires a number of amino acids, including methionine, alanine, histidine, isoleucine, leucine, and valine, for protein synthesis (1, 38). These findings are in agreement with the genomic information available (11). On the other hand, there have not been studies concerning *H. pylori*'s ability to metabolize complex peptides as a carbon source. This is surprising considering the abundance of peptides in the stomach (11).

An analysis of sequenced *H. pylori* genomes revealed annotated systems for both dipeptide (*dppABCDF*) and oligopeptide (*oppABCD*) transport (Fig. 1A and B). Both of these systems are extremely conserved in *H. pylori* strains, indicating their essential nature (39). Based on homology, both systems belong to the ABC-type superfamily of transporters (9, 16, 17), comprised of a substrate binding domain (encoded by *dppA* and *oppA*) and two transmembrane permease domains (encoded by *dppBC* and *oppBC*). ABC-type transport systems also commonly contain one (*oppD*) or two (*dppDF*) ATP-binding domains that function as the energy source for substrate transport (17). Such transporter systems have been well studied in *Escherichia coli* and *Salmonella enterica* serovar Typhimurium (2, 12, 13, 18, 19, 21, 33) and in gram-positive organisms (4, 10, 22, 24, 36, 37, 40, 41, 44); due to the high peptide content of *H. pylori*'s unique niche and its limited ability to utilize carbohydrates as a carbon source, we wished to assess the roles of these systems in *H. pylori*.

While the *oppABCD* system has not been studied in *H. pylori*, a *dppA* gene disruption mutant was shown to influence urease activity (8). However, this mutant was not studied with regard to peptide transport. Here we investigate the roles of the putative peptide transport systems in *H. pylori* via a mutational approach. First, the substrate binding domain associated with each system (*dppA* or *oppA*) was insertionally inactivated in order to investigate its individual role. Second, a double mutant was generated that had both of these genes (*dppA* and *oppA*) insertionally inactivated; this was done to assess the possibility of these systems having overlapping substrate binding specificities. The same inactivation procedure was used to disrupt the permease domain function from each system (*dppB*, *dppC*, *oppB*, or *oppC*). Double mutants were also generated in which both permease domains were inactivated (*dppB dppC* and *oppB oppC*) to address the possibility that the

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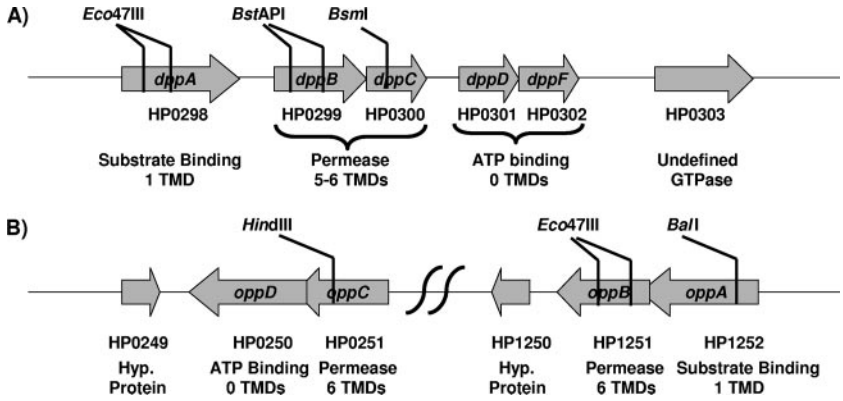


FIG. 1. Genome organization of peptide transport systems of *H. pylori*. (A) *dppABCDF* dipeptide transport system. (B) *oppABCD* oligopeptide transport system. HP numbers correspond to the sequenced 26695 strain. Gene annotations are according to the TIGR website (http://www.tigr.org). The number of transmembrane domains (TMDs) is indicated, showing either an integral membrane protein (five or six TMDs), a membrane-associated protein (one TMD), or a cytoplasmic protein (zero TMDs), as determined by TMHMM software at http://www.cbs.dtu.dk/services/TMHMM-2.0. Restriction enzymes used for insertion inactivation and their approximate cutting sites are indicated.

presence of a single permease allows for residual transport activity of these systems. In addition, inactivation of both permeases would be desirable to completely compromise the membrane-spanning pore. The oligopeptide transport system in *H. pylori* has an uncommon genome configuration for ABC-type transporters, as it is separated into two clusters on the genome (*oppAB* and *oppCD*) (Fig. 1B). Therefore, the construction of the *oppC* mutant was important to determine if the two gene clusters do in fact act in concert to yield a single functional oligopeptide transport system. Finally, a double mutant was generated in which a permease domain from each system was inactivated (*dppB oppB*). All mutants were characterized for peptide utilization ability, using a chemically defined growth medium in which the only source of the essential amino acid isoleucine (Ile) was provided as peptides of various lengths (from two to nine amino acids).

MATERIALS AND METHODS

Bacterial strains, media, and plasmids used in this study. *Helicobacter pylori* strain 43504 was used as the parent strain in all transformations. All plate cultures were grown at 37°C under conditions of 5% CO_2–10% H_2–5% O_2 on blood agar plates containing brucella agar (Becton Dickinson Co., Sparks, MD) supplemented with 10% defibrinated sheep's blood (Atlanta Biologicals, Norcross, GA). Liquid cultures were grown at 37°C under conditions of 5% CO_2–10% H_2–6% O_2 on a chemically defined medium (CDM [discussed further below]). Where necessary, plates were supplemented with kanamycin (KAN; 40 μg/ml) or chloramphenicol (Cm; 34 μg/ml).

Cloning was performed in *E. coli* strain Top10 (Invitrogen, Carlsbad, CA), which was grown in Luria-Bertani (LB) medium supplemented with ampicillin (100 μg/ml), KAN (40 μg/ml), or Cm (34 μg/ml), as needed. Cloning was performed using the cloning vector pGEM-T (Promega, Madison, WI). Primers were synthesized at Integrated DNA Technologies (Coralville, IA) (Table 1). All nonlabeled peptides were purchased from Bachem (King of Prussia, PA) (Table 2). A peptide containing an N-terminal fluorescein isothiocyanate (FITC) label was purchased from Genscript (Piscataway, NJ) and had the same amino acid sequence as the hexapeptide in Table 2.

Generation of peptide transport mutant strains of *H. pylori*. *dppA*, *dppB*, *dppC*, *oppA*, *oppB*, and *oppC* were all PCR amplified from *H. pylori* 43504 chromosomal DNA, using primers specific for each gene (Table 1). Each resulting PCR product was cloned into the cloning vector pGEM-T and transformed into *E. coli* Top10. Transformants were screened for correct inserts by restriction digestion. The *dppA* and *oppB* genes were partially deleted by restriction digestion with Eco47III, resulting in 0.3- and 0.5-kb regions, respectively, being excised from the genes. Each gene was then insertionally inactivated by ligating a Cm resistance cassette (for Cm acetyltransferase [CAT]) (47) into the excised region. *dppC* was insertionally inactivated by ligating the CAT cassette into a unique BsmI restriction site of wild-type *dppC*. *dppB* was partially deleted by restriction digestion with BstAPI, resulting in a 0.5-kb region being excised from the gene. *dppB* was then insertionally inactivated by inserting the *aphA3* gene from *Campylobacter coli*, a KAN resistance cassette (47), into the excised region. *oppA* and *oppC* were insertionally inactivated by inserting the KAN cassette into unique BalI and HindIII restriction sites of wild-type *oppA* and *oppC*, respectively. Each resulting plasmid was used to naturally transform *H. pylori* strain 43504 and selected with the appropriate antibiotic (KAN for *oppA*, *oppC*, and *dppB* and Cm for *dppA*, *dppC*, and *oppB*). This is a common method for isolating stable *H. pylori* mutants via gene replacement mediated by homologous recombination (46). The *dppA oppA* double mutant was generated by naturally transforming the *oppA* mutant strain with the pGEM-T plasmid carrying CAT-inactivated *dppA*. The *dppB oppB*, *dppB dppC*, and *oppB oppC* double mutants were

TABLE 1. Primers used in this study

Primer designation	Sequence (5′→3′)	Length (nt)	T_m (°C)
dppAF	ATGAATAATGTTTTTGTTAA	20	41
dppAR	TTATTTTTCTAAATACACCT	20	42
dppBF	ATGCTGAGTTTTATCATTAAGC	22	49
dppBR	TTATGACAACCTTATTCTAGGATC	24	50
dppCF	ATGGAGTCTTTTAGAGAGTTTATCCAA	27	54
dppCR	TTAAGAGGTGCGTTTAGGATCTAAAGC	27	57
oppAF	GTGGTCTTTAAAATTTTAGG	20	44
oppAR	TCATTGAAGATCCTTTTCTT	20	47
oppBF	ATGATTGCTTACATTCTCAAAC	22	49
oppBR	TCAACGCTTTTCAAAATC	18	46
oppCF	GTGAGCAGTTTGTTTAAAATGCGC	24	56
oppCR	TTATTTGAGCATGTTAGCGTTGAA	24	53

TABLE 2. Peptides used in this study

Peptide moniker	Sequence (N→C)
AK	Ala-Lys
AI	Ala-Ile
KI	Lys-Ile
VI	Val-Ile
RI	Arg-Ile
FI	Phe-Ile
IS	Ile-Ser
Tripeptide	Val-Trp-Ile
Tetrapeptide	Ile-Trp-Val-Asn
Hexapeptide	Val-Tyr-Ile-His-Pro-Phe
Nonapeptide	Arg-Val-Tyr-Ile-His-Pro-Phe-His-Leu

generated by similar methods. Each mutant strain was subsequently screened by PCR to identify correct incorporation of the appropriate insertionally inactivated gene into the chromosome as well as to ensure that double-crossover events had occurred. The sizes of the PCR products from the mutant strains were compared to those from the parent (43504).

Growth of *H. pylori* strains in CDM. A modified version of the Reynolds and Penn CDM (38) was used as previously reported (5, 7). Briefly, bovine serum albumin was replaced in the medium by β-cyclodextrin (1% [wt/vol]), as previously described (32). Urea (5 mmol/liter) was also added, and the medium pH was brought to 7.4 with 2 M NAOH. Unless otherwise stated, glucose was omitted from the medium. The medium was filter sterilized using a 0.2-μm vacuum-driven disposable filtration system (Millipore, Billerica, MA). After filter sterilization, ferrous sulfate was added to a final concentration of 2 μg/ml from a 1,000× stock solution (38), and a trace metal solution ($ZnSO_4 \cdot 7H_2O$, 100 mg/liter; H_3BO_3, 300 mg/liter; $CoCl_2 \cdot 6H_2O$, 200 mg/liter; $CuCl_2 \cdot 2H_2O$, 10 mg/liter; $NiCl_2 \cdot 2H_2O$, 20 mg/liter; $Na_2MoO_4 \cdot H_2O$, 30 mg/liter) (29) was added at 80 μl per 100 ml of medium. The last two solutions were γ-irradiated to ensure their sterility. The Ile-peptide source (Table 2) was added to a final concentration of 0.1 mg/ml. Cultures used for growth curve experiments contained 15 ml of medium, and those used for the Ile-dependent growth assays contained 5 ml of medium. In each case, cultures were grown in 70-ml sealed glass serum bottles aseptically flushed with an anaerobic gas mixture (5% CO_2–10% H_2–85% N_2) by use of sterile inflow and outflow needles. O_2 was added aseptically after the flush to a final headspace concentration of 6%. Cells for inoculation of liquid culture were grown for 24 h on blood agar plates with the appropriate antibiotic before harvest and resuspension into phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 4.3 mM $Na_2HPO_4 \cdot 7H_2O$, 1.4 mM KH_2PO_4). Liquid cultures were inoculated to an initial optical density at 600 nm (OD_{600}) of ~0.05 (~8.5×10^7 viable cells/ml) and shaken at 37°C for 24 h. Growth yield was measured by determining the OD_{600} in a Beckman DU-640B spectrophotometer. Results shown are the averages for at least three independent assays, and a two-tailed Student *t* test (31) was performed to determine which mutants differed significantly from the wild type. One-milliliter samples were taken every 4 h to check the OD_{600} during growth curve experiments. Initial experiments also included plate counts to ensure that an increase in OD_{600} corresponded to an increase in cell number within the growth range tested. OD_{600} values corresponded to cell counts, with an R^2 value of 0.96 (data not shown).

Extracellular peptidases of *H. pylori*. The presence of extracellular peptidases of *H. pylori* was assayed both indirectly and directly. For the indirect assay, cells were grown as described above in CDM supplemented with either 10 μM EDTA or 500 μM EGTA, and either the Lys-Ile dipeptide or the hexapeptide was provided as the Ile source.

An FITC-labeled hexapeptide was utilized for the direct assay for extracellular peptidases. *H. pylori* cells (wild type and *oppA*, *dppA*, and *oppA dppA* mutant) were grown in complete CDM (containing all amino acids) containing 50 μg/ml of the FITC-labeled peptide, but they were harvested at 20 h instead of 24 h to limit cell lysis. The spent medium was centrifuged at 10,000 × *g* for 10 min at 4°C, and the resulting supernatant was passed through a 0.22-μm syringe filter into a sterile 15-ml conical tube. The presence of the labeled hexapeptide was determined in this sterile, spent medium by high-performance liquid chromatography (HPLC) analysis with excitation at 485 nm and emission at 518 nm. As controls, uninoculated cultures, with or without the labeled hexapeptide and treated identically to the above cultures, were also subjected to HPLC analysis. The HPLC analysis was performed at the University of Georgia proteomics facility (Athens, GA).

Bioinformatics-based homology search. Amino acid sequence homology searches for all domains of each peptide transport system were performed using the BLASTP database on the NCBI website (http://www.ncbi.nlm.nih.gov). Amino acid sequences were based on nucleotide sequences from the sequenced strain 26695 genome.

RESULTS

Construction of *H. pylori* mutant strains. Inactivation of the substrate binding domains (*dppA* or *oppA*) or the permease domains (*dppB*, *dppC*, *oppB*, or *oppC*) and construction of the double mutants (*dppA oppA*, *dppB oppB*, *dppB dppC*, and *oppB oppC*) were done by insertional inactivation as described in Materials and Methods. PCR confirmation of the resulting mutant strains showed bands corresponding to either a 1.4-kb

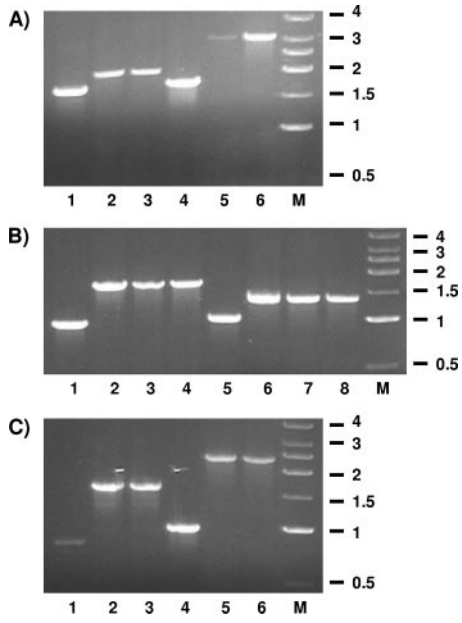


FIG. 2. Confirmation of insertional inactivation mutants by PCR. (A) PCR products using primers specific for either *dppA* (lanes 1 to 3) or *oppA* (lanes 4 to 6). The source of genomic DNA for each PCR was as follows: lane 1, WT strain 43504; lane 2, *dppA* mutant; lane 3, *dppA oppA* mutant; lane 4, WT strain 43504; lane 5, *oppA* mutant; and lane 6, *dppA oppA* mutant. (B) PCR products using primers specific for either *dppB* (lanes 1 to 4) or *oppB* (lanes 5 to 8). Genomic DNAs were isolated from the following: lane 1, WT strain 43504; lane 2, *dppB* mutant; lane 3, *dppB dppC* mutant; lane 4, *dppB oppB* mutant; lane 5, WT strain 43504; lane 6, *oppB* mutant; lane 7, *oppB oppC* mutant; and lane 8, *dppB oppB* mutant. (C) PCR products using primers specific for *dppC* (lanes 1 to 3) or *oppC* (lanes 4 to 6). Genomic DNAs were isolated from the following: lane 1, WT strain 43504; lane 2, *dppC* mutant; lane 3, *dppB dppC* mutant; lane 4, WT strain 43504; lane 5, *oppC* mutant; and lane 6, *oppB oppC* mutant. The gels were 1% agarose gels. The KAN gene is 1.4 kb long, and the CAT gene is 0.8 kb long. Sizes (in kb) are indicated to the right of each gel.

increase over the wild-type size for KAN insertion (*dppB*, *oppA*, and *oppC* mutants) or a 0.8-kb increase over the wild-type size for CAT insertion (*dppA* and *oppB* mutants) (Fig. 2A to C), minus the size of any deletions described previously (Fig. 1). All PCRs yielded single products, indicating a successful double-crossover event for each disruption. A downstream polar effect has already been shown for a *dppA* mutation, affecting the expression of other proteins of the dipeptide transport system (8). There are no genes immediately downstream of the *dppABCDF* operon, however, making any further polar effects unlikely. Possible polar effects with regards to the oligopeptide transport mutants are discussed later.

Once constructed, each mutant strain was grown in complete CDM (containing all amino acids) for comparison to growth of the wild type. Mutant strain growth compared favorably to that of the wild type in this medium, with all strains displaying a similar lag phase (~8 h) before beginning exponential growth and entering stationary phase at approximately the same time (~24 h) (representative examples are shown in Fig. 3A). All strains (including the wild type) grew to approximately the same OD_{600} (~0.4 to 0.5) over the growth period of 24 h (Fig. 3A). Since late log/early stationary phase was reached within

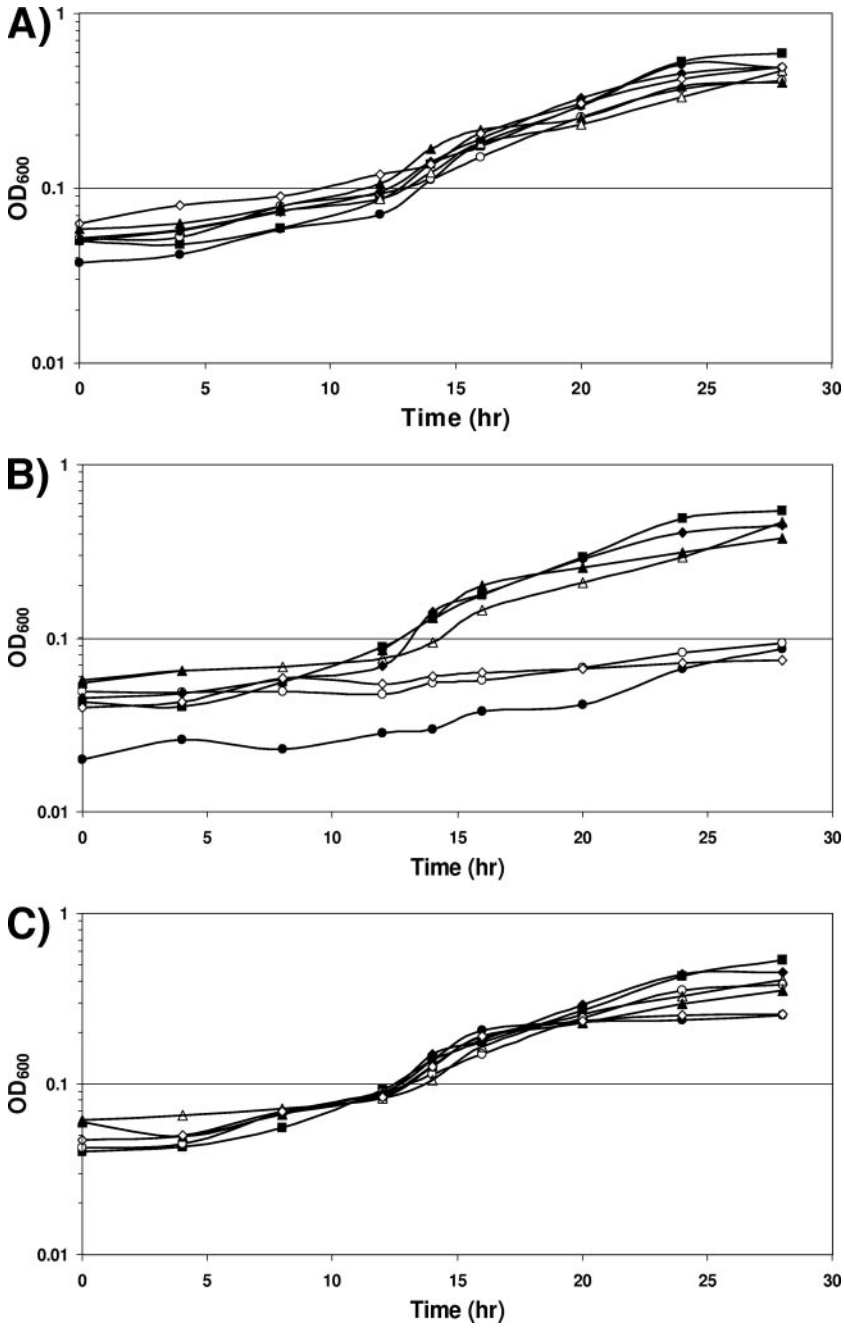
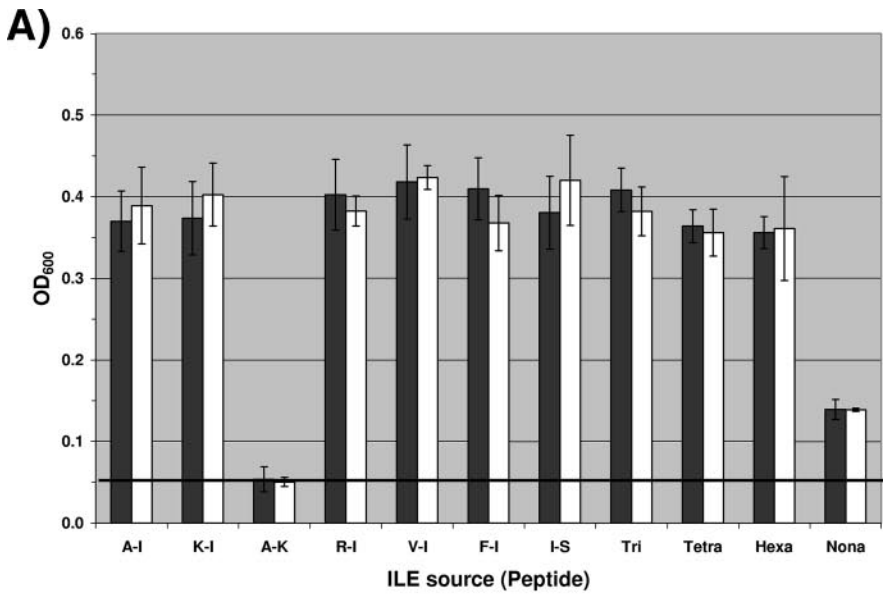
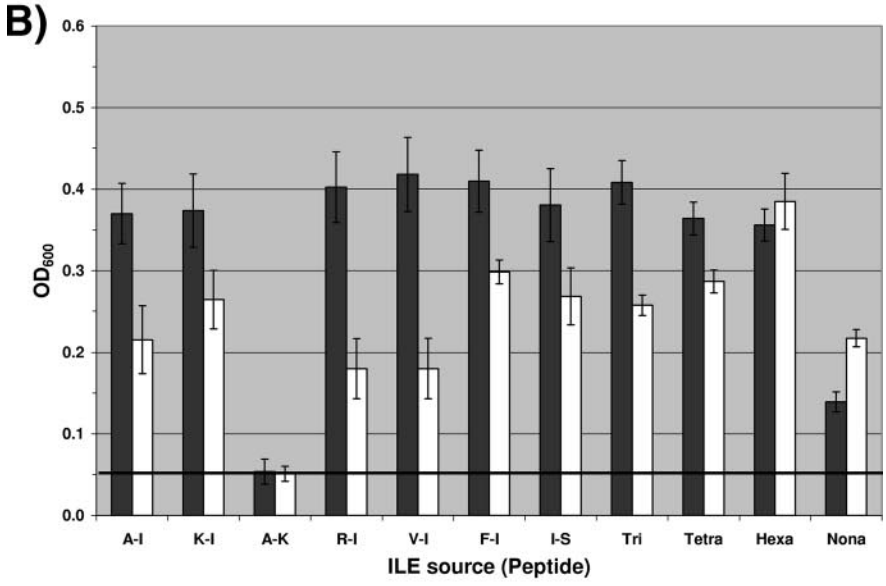


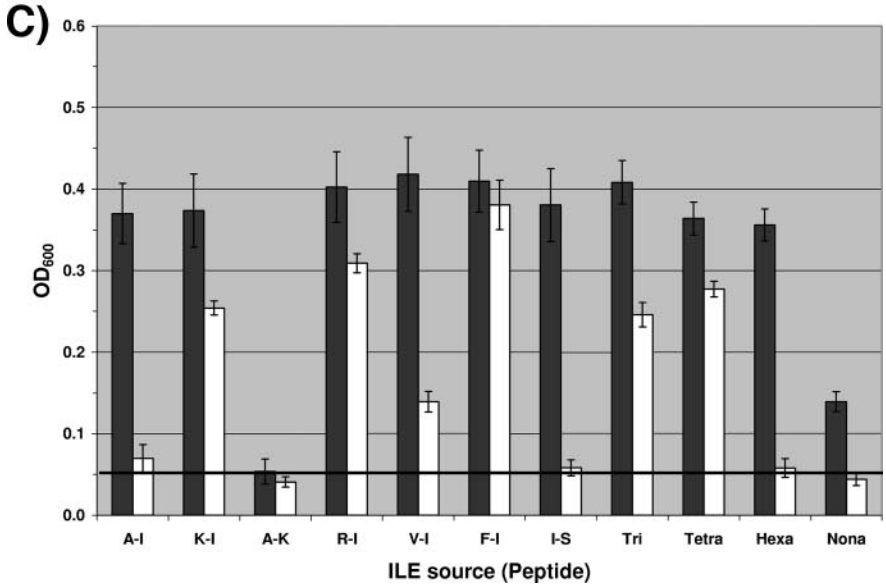
FIG. 3. Growth curves for mutants versus the wild type in CDM. Cells were grown in several variations of CDM containing Ile in either its single-amino-acid form (A), as the dipeptide Ala-Ile (B), or as the dipeptide Lys-Ile (C). Samples were taken every 4 hours over a total growth time of 28 h. Growth curves were performed for the wild-type strain 43504 (filled squares) and the *oppA* (filled triangles), *oppB* (open triangles), *oppC* (filled diamonds), *dppA* (filled circles), *dppB* (open circles), and *dppA oppA* (open diamonds) mutants.

24 h, this was the time frame used to assess comparative growth yields for subsequent Ile-dependent growth of the peptide transport mutants. Growth curves for these strains grown in peptide-supplemented medium are discussed below.

Ile-dependent growth of peptide transport mutants in CDM. Each mutant strain was compared to the wild-type parent strain 43504 for the ability to utilize polypeptides of various lengths as an Ile source (Fig. 4; see a summary of these results in Table 3). Each strain was grown in several variations of CDM in which the only source of the essential amino acid Ile was polypeptides ranging from two to nine amino acids in length (Table 2). The inclusion of glucose in the CDM did not significantly affect the growth of any of these strains, regardless of the Ile source (data not shown), indicating that *H. pylori* can utilize amino acids as its sole carbon source. The non-Ile-containing dipeptide Ala-Lys was used as a negative control, as none of the strains would be expected to grow without a source of Ile. Similarly, the nonapeptide was used as a negative con-







trol, since gram-negative bacteria do not readily transport oligopeptides longer than eight amino acids across their outer membranes (23). As expected, the parent strain was able to grow well in all variations of the medium (OD_{600}, >0.35 at 24 h) and it did not grow in the dipeptide negative control (Ala-Lys) (Fig. 4, black bars). Growth of the parent unexpectedly occurred on the nonapeptide, but the growth yield at 24 h was much less (OD_{600}, ~0.15) than that under other conditions, where full growth was observed (OD_{600}, >0.35).

The *oppA* mutant phenotype for Ile utilization was similar to that of the parent (Fig. 4A). The *oppC* mutant also failed to exhibit any deficiency in peptide utilization (Table 3). A double mutant deficient in both of these domains remained as able to utilize peptides as the wild type, making it unlikely that these domains are redundant for each other (data not shown). In contrast, the *oppB* mutant had less-than-wild-type growth on most dipeptides as well as on tri- and tetrapeptide Ile sources (Fig. 4B). This rules out a downstream polar effect from an *oppA* mutation on *oppB*. Based on other mutations made with the KAN cassette, this lack of polarity is an expected result (25, 34). There is no gene immediately downstream of *oppB*, making it likely that the observed phenotype is caused by the mutation. The final growth yield for the *oppB* mutant was <50% that of the wild type for two dipeptides (Arg-Ile and Val-Ile) and 40% and 30% less than that of the wild type for tri- and tetrapeptides, respectively (Fig. 4B). The most surprising phenotypes were apparent when the hexa- and nonapeptide Ile sources were used; a compromised oligopeptide transport system was expected to have an obvious effect on utilization of these longer peptides, but growth yields were either similar to (hexapeptide; OD_{600}, ~0.35) or even better than (nonapeptide; OD_{600}, ~0.22) those seen for the wild type (~0.35 and 0.15, respectively). The *oppB oppC* double mutant mirrored the peptide utilization profile of the *oppB* mutant (Table 3), further indicating a limited (if any) peptide utilization role for *oppC*. The *H. pylori* Opp system is apparently not used for peptides larger than four amino acids.

While only two of the oligopeptide transport mutants showed subtle differences from the wild type, *dppA*, *dppB*, *dppC*, and *dppB dppC* strains showed a definite inability to use some dipeptides as well as the longer oligopeptides (six or more amino acids). Growth yields for the *dppA* strain on Ala-Ile and Ile-Ser were <15% those for the wild type, and those on hexapeptides were <10% those for the wild type (Fig. 4C). Even the unexpected growth by the parent strain on the nonapeptide (OD_{600}, ~0.15) was completely abolished in the *dppA* mutant (~0.07). Although the growth on Ala-Ile and Ile-Ser was abolished, the *dppA* mutant was able to utilize other dipeptides, albeit to a lesser extent than that for the wild type (for example, Arg-Ile and Phe-Ile, achieving growth yields of ~70% of the wild-type yields, and Lys-Ile and Val-Ile, achieving ~50% of the wild-type yields). With a few exceptions (see below), other dipeptide transport mutant strains (*dppB*, *dppC*, and *dppB dppC* mutants) showed similar peptide utilization phenotypes (Table 3).

The only cases where growth on the tripeptide was seriously affected were the *dppC* and *dppB dppC* mutants (Table 3). The *dppC* phenotype may indicate a specialized role in the transport of certain peptides. The other dipeptide transport mutants showed only minor decreases in the ability to utilize the tripeptide Ile source for growth compared to the wild type. The *dppA* mutant also showed a minor decrease in the ability to make use of the tetrapeptide Ile source (70 to 85% of wild-type yields), and the other strains (*dppB*, *dppC*, and *dppB dppC* mutants) had no growth impairment at all.

The double mutants in which like domains of both systems were disrupted in *cis* (*dppA oppA* and *dppB oppB* mutants) behaved like their corresponding dipeptide transport single mutants (*dppA* and *dppB* mutants, respectively). The *dppA oppA* mutant (Table 3) had a slight decrease in the ability to utilize the tri- and tetrapeptide Ile sources, but overall, its phenotype agreed with the data for the *dppA* mutant (Fig. 4C). The results for the *dppB oppB* strain agreed for the most part with data for the *dppB* mutant (Table 3), with the following exceptions. The cell yield after 24 h on the Phe-Ile dipeptide dropped dramatically in the double mutant (OD_{600}, ~0.35 to 0.11). Growth utilizing the tri- and tetrapeptide Ile sources was slightly less for the double mutant (for both tri- and tetrapeptides, from an OD_{600} of ~0.35 for the *dppB* mutant to one of ~0.23 for the *dppB oppB* mutant) than for the *dppB* strain. This may indicate a role for the oligopeptide system in the transport of these peptides. However, the double mutants did not lose the ability to grow on any other peptide Ile sources, indicating that other unidentified peptide transporters are responsible for the residual transport. A summary of the Ile-dependent growth yield assay results is displayed in Table 3.

To further characterize the most surprising strain difference (i.e., that the dipeptide transport mutants retained the ability to transport some dipeptides), growth curves for several mutants were performed using Ala-Ile- and Lys-Ile-supplemented CDM. The supplemented dipeptides are those in which the dipeptide transport mutants showed "abolished" and "impaired" growth, respectively. As shown in Fig. 3B, oligopeptide mutants grew as well as the wild type when utilizing Ala-Ile as the sole Ile source. However, under these conditions, the *dppA*, *dppB*, and *dppA oppA* strains showed a prolonged lag time (~16 h, in contrast to ~8 h for the other strains) and a much lower growth rate once they achieved log phase. When using Lys-Ile as the sole Ile source, as for the Ala-Ile growth curves, the oligopeptide mutants compared favorably in growth ability to the parent strain (Fig. 3C). Interestingly, while the *dppB* mutant exhibited growth no different from that of the wild type or the oligopeptide mutants, the *dppA* and *dppA oppA* strains entered stationary phase much earlier (~16 h, in contrast to 24 h for the other strains) and reached a growth yield ~50% that of the wild type.

FIG. 4. Ile-dependent growth assays of mutants versus the wild type. Horizontal black bars indicate the initial culture OD_{600} (~0.05). Results shown are the average OD_{600} readings for at least three cultures for each condition after 24 h of growth. Each chart compares the *H. pylori* wild-type strain 43504 (black bars) to each individual mutant (white bars) (*oppA* [A], *oppB* [B], or *dppA* [C]). Peptides were present at 0.1 mg/ml, and sequences are indicated in Table 2. The A-K dipeptide and nonapeptide were used as negative controls. See Table 3 for statistical analysis.

TABLE 3. Summary of Ile-dependent growth assays with peptide transport mutants[a]

Strain	Growth (OD_{600}) with peptide[a]										
	AI	KI	RI	VI	FI	IS	AK	Tri[b]	Tetra[b]	Hexa[b]	Nona[b]
WT	0.37 ± 0.04	0.37 ± 0.04	0.40 ± 0.04	0.42 ± 0.05	0.41 ± 0.04	0.38 ± 0.04	0.05 ± 0.02	0.41 ± 0.03	0.36 ± 0.02	0.36 ± 0.02	0.14 ± 0.01
oppA	0.39 ± 0.05[c]	0.40 ± 0.04[c]	0.38 ± 0.02[c]	0.42 ± 0.01[c]	0.37 ± 0.03[c]	0.42 ± 0.06[c]	0.05 ± 0.01[c]	0.38 ± 0.03[c]	0.36 ± 0.03[c]	0.36 ± 0.06[c]	0.14 ± 0.00[c]
oppB	0.22 ± 0.04	0.26 ± 0.04	0.18 ± 0.04	0.18 ± 0.04	0.30 ± 0.01	0.27 ± 0.03	0.05 ± 0.01[c]	0.26 ± 0.01	0.29 ± 0.01	0.38 ± 0.03[c]	0.22 ± 0.01
oppC	0.42 ± 0.02[c]	0.36 ± 0.04[c]	0.37 ± 0.04[c]	0.44 ± 0.03[c]	0.40 ± 0.04[c]	0.38 ± 0.04[c]	0.06 ± 0.00[c]	0.43 ± 0.04[c]	0.42 ± 0.03	0.38 ± 0.05[c]	0.19 ± 0.01
oppB oppC	0.27 ± 0.03	0.29 ± 0.01	0.29 ± 0.03	0.26 ± 0.02	0.26 ± 0.01	0.25 ± 0.03	0.07 ± 0.00[c]	0.24 ± 0.02	0.30 ± 0.01	0.35 ± 0.01[c]	0.22 ± 0.04
dppA	**0.07 ± 0.02**	0.25 ± 0.01	0.31 ± 0.01	0.14 ± 0.01	0.38 ± 0.03[c]	**0.06 ± 0.01**	0.04 ± 0.01[c]	0.25 ± 0.02	0.28 ± 0.01	**0.06 ± 0.01**	**0.04 ± 0.01**
dppB	**0.10 ± 0.01**	0.28 ± 0.02	0.27 ± 0.02	0.19 ± 0.01	0.35 ± 0.04	**0.06 ± 0.01**	0.04 ± 0.01[c]	0.33 ± 0.03	0.37 ± 0.03[c]	**0.05 ± 0.01**	**0.04 ± 0.00**
dppC	**0.07 ± 0.01**	0.22 ± 0.01	0.32 ± 0.02	0.14 ± 0.01	0.25 ± 0.02	**0.05 ± 0.01**	0.06 ± 0.01[c]	0.14 ± 0.04	0.34 ± 0.01	**0.05 ± 0.00**	**0.05 ± 0.00**
dppB dppC	**0.06 ± 0.00**	0.21 ± 0.03	0.32 ± 0.04	0.19 ± 0.02	0.22 ± 0.02	**0.07 ± 0.01**	0.06 ± 0.00[c]	0.10 ± 0.01	0.32 ± 0.03	**0.06 ± 0.01**	**0.05 ± 0.00**
dppA oppA	**0.07 ± 0.01**	0.27 ± 0.01	0.33 ± 0.02	0.14 ± 0.01	0.35 ± 0.01	**0.07 ± 0.01**	0.04 ± 0.01[c]	0.23 ± 0.02	0.24 ± 0.01	**0.05 ± 0.00**	**0.05 ± 0.00**
dppB oppB	**0.06 ± 0.00**	0.23 ± 0.02	0.16 ± 0.04	0.18 ± 0.01	0.13 ± 0.01	**0.06 ± 0.00**	0.07 ± 0.00[c]	0.25 ± 0.02	0.23 ± 0.01	**0.06 ± 0.00**	**0.05 ± 0.00**

[a] Results shown are the average OD_{600} values for three independent assays with cells grown for 24 h in CDM supplemented with the indicated peptide as the sole Ile source. The initial culture OD_{600} was ~0.05. Growth of the mutants was statistically significantly less than that of the parent strain ($P < 0.05$) unless indicated otherwise. Conditions under which no growth occurred are indicated in bold.

[b] All amino acid sequences can be found in Table 2.

[c] Growth data statistically indistinguishable from that for the wild type ($P > 0.05$).

Growth of substrate binding domain mutants in EDTA-supplemented CDM. One explanation for the unexpected result that the oligopeptide mutants are normal for oligopeptide growth could lie in the production of extracellular peptidases by *H. pylori* (i.e., perhaps the longer peptides are degraded into dipeptides, confounding the interpretation of the dipeptide growth data). Indeed, an analysis of the cell-free spent medium identified several metalloproteases encoded in the *H. pylori* genome (T. Smith, personal communication). To determine if any of these peptidases digested peptide sequences, enabling the subfragments to be transported by the more accommodating dipeptide transport system, growth of several strains was compared on CDM supplemented with 10 μM EDTA (with either the Lys-Ile dipeptide or the hexapeptide as the Ile source). This working concentration inhibited growth of the wild-type strain in the CDM, and higher chelator concentrations were toxic to the wild-type strain under these conditions (data not shown). EDTA would be expected to inactivate any metalloproteases that may be present in the medium. However, while the addition did decrease the overall growth yield of each strain tested after 24 h, it did not appear to affect the growth patterns of the mutants (*dppA*, *oppA*, and *dppA oppA* strains). As shown in Fig. 5A and B, both the wild-type strain and the *oppA* strain were able to grow relatively as well as before, while the *dppA* and *dppA oppA* strains did not gain the ability to grow on these Ile sources in the presence of EDTA. Similar studies using 500 μM EGTA yielded similar results (data not shown). This makes it unlikely that the results from the Ile-dependent growth assays were due to the presence of any extracellular metalloproteases expressed in *H. pylori*. This is not surprising considering previous evidence showing that *H. pylori* does not exhibit extracellular protease activity (6, 30).

Direct assay for extracellular peptidases, utilizing FITC-labeled hexapeptide. In addition to the indirect, chelator-based experiments testing for extracellular peptidases, a more direct assay utilizing an FITC-labeled hexapeptide and HPLC analysis was employed. After incubation at 37°C for 20 h and harvest of the culture supernatant containing the labeled hexapeptide, HPLC analysis showed three peaks (16, 23, and 58.5 min), two of which were also present in the supernatant of uninoculated medium without the labeled peptide (16 and 23 min [data not shown]). This indicates that the labeled hexapeptide elutes at 58.5 min. In the culture supernatants that were inoculated with various strains (wild type and *oppA*, *dppA*, and *oppA dppA* mutants), an extra minor peak at 52 min was observed, indicating some cell-dependent degradation of the labeled hexapeptide. Still, the amount of material eluting at 58.5 min (hexapeptide) remaining after 20 h of incubation with cells was 76% of the level initially added.

DISCUSSION

According to the genome annotation, *H. pylori* has two putative peptide transport systems (11). These are potentially important systems for obtaining carbon for the survival of the organism, as both genomic and biochemical data reveal a limited ability to utilize complex carbohydrates or CO_2 as a carbon source (11, 27, 28). The importance of these transport systems was investigated by disrupting several domains of each system and characterizing the resulting mutants for the ability to uti-

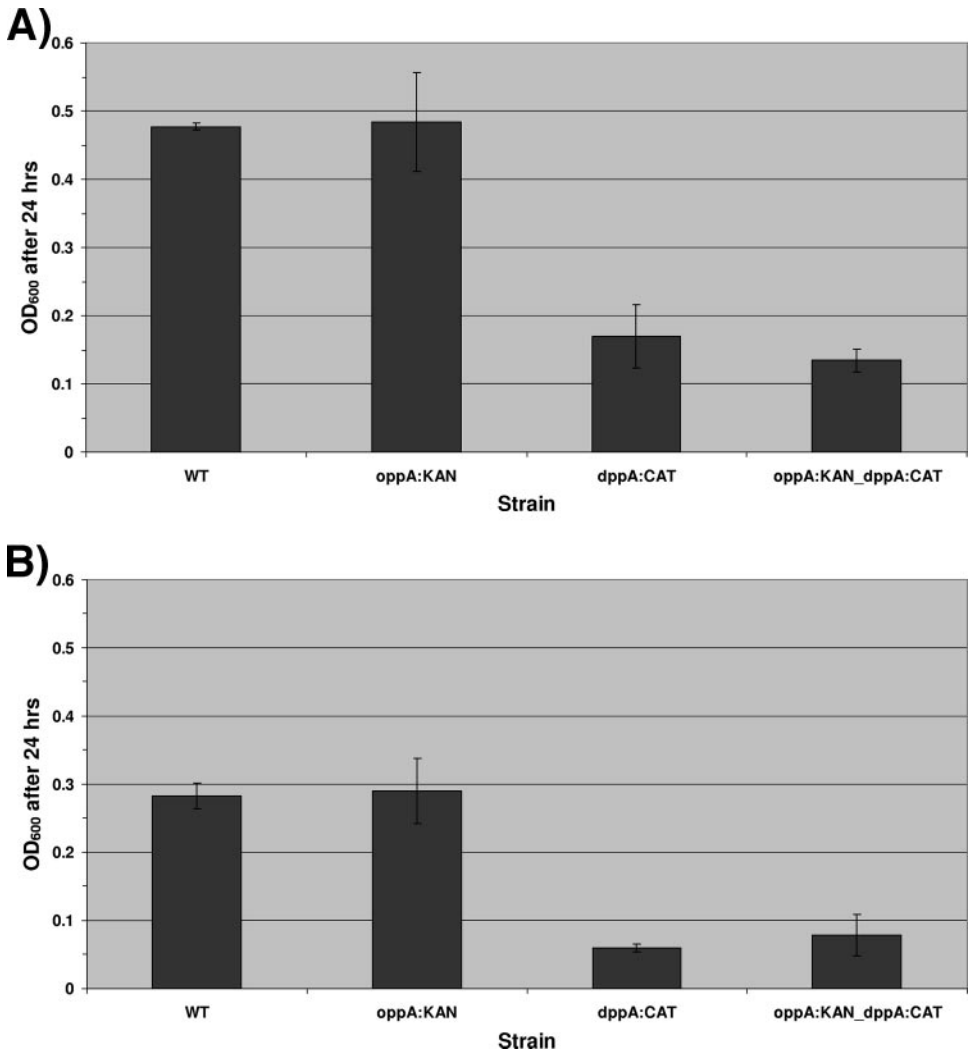


FIG. 5. Growth of mutants versus wild type in CDM supplemented with EDTA. Growth assays were performed as described in the legend to Fig. 4, except that 10 μM EDTA was added to the medium. Growth was determined for the wild type and the *oppA*, *dppA*, and *dppA oppA* mutant strains in CDM with either a Lys-Ile dipeptide Ile source (A) or a hexapeptide Ile source (B).

lize peptides varying in length from two to nine amino acids. The substrate binding domain of each system (*dppA* and *oppA* mutants), as well as one or both of the permease domains for each system (*dppB*, *dppC*, *oppB*, *oppC*, *dppB dppC*, and *oppB oppC* mutants), was inactivated. The *H. pylori* oligopeptide transport system has an unusual genomic makeup for an ABC-type transporter, being divided into two separate regions of the genome (Fig. 1B). The *oppC* mutant was therefore particularly important for addressing whether both regions (encoded by *oppAB* and *oppCD*) comprise a single transporter that is responsible for oligopeptide transport. The roles of the ATPase domains encoded by *dppD*, *dppF*, and *oppD* were not addressed within our study. As previously mentioned, a double permease mutant (*dppB dppC* or *oppB oppC* mutant) would theoretically render these ATPase domains inactive. Given the results presented herein and the possibility that an *oppC*- and *oppD*-encoded transporter system behaves as an alternate peptide transporter, these additional genes are currently under study to define their potential roles in peptide transport. Several double mutants were also constructed in which both systems were disrupted simultaneously in their putative substrate binding (*dppA oppA* mutant) or permease (*dppB oppB* mutant) domains.

Two of the oligopeptide transport mutants (*oppA* and *oppC* mutants) showed no significant differences from the wild type (Fig. 4A; Table 3), indicating either that these genes do not encode proteins having roles in peptide transport or that there is another protein able to fully support peptide use despite these mutations. An *oppA oppC* double mutant also behaved like the wild type, further displaying the lack of a role for each of these genes in peptide transport (data not shown). However, the *oppB* and *oppB oppC* mutants showed a measurable but subtle impaired ability to utilize di- and short oligopeptides (tri- and tetrapeptides) as Ile sources (Fig. 4B; Table 3). The likelihood of polar effects in this situation is minimal due to the *oppA* mutant behaving like the wild type, whereas the *oppB* mutant was deficient in peptide utilization. Growth yields were consistently less for these mutants (*oppB* and *oppB oppC* mu-

tants) than for the wild type (OD_{600}, ~0.25 versus ~0.35) on such peptides, so Opp appears to be involved in transport of a variety of short peptides. Nevertheless, growth was never completely abolished under any conditions assayed for these mutants, indicating the existence of another system capable of at least partially supporting peptide uptake.

While the *oppB*-specific oligopeptide transport knockout strains supported only a limited role for the gene in peptide transport, the dipeptide knockout strains yielded more information regarding peptide transport than anticipated (Table 3). This system was shown to play an essential role in the use of particular dipeptides (Ala-Ile and Ile-Ser) as well as hexa- and nonapeptides, indicating a wider-than-anticipated role for this transport system. While most or all dipeptide transport mutants were able to grow like the parent on a few Ile sources (Phe-Ile and the tetrapeptide), other peptides assayed (Lys-Ile, Arg-Ile, Val-Ile, and the tripeptide) were not utilized as well as they were in the wild-type strain. A polar effect has already been established for genes downstream of *dppA* when *dppA* is disrupted. Therefore, similar peptide utilization phenotypes are not surprising when these downstream genes are disrupted. Although a likely role for *oppB* in utilization of short peptides was shown (Fig. 4B), the resemblance of the *dppA oppA* and *dppB oppB* mutant phenotypes to the *dppA* and *dppB* phenotypes (Table 3), respectively, rules out the annotated oligopeptide transport system as the candidate responsible for the residual transport of some dipeptides (Phe-Ile, Lys-Ile, Arg-Ile, and Val-Ile) by the dipeptide transport mutants. It is possible that a functional *oppC-oppD* system is responsible for this residual activity. However, the fact that an *oppC* mutant showed no inhibition of peptide transport makes that unlikely.

The conclusions on transport described herein necessarily rely on Ile-dependent growth. While the parent strain is auxotrophic for Ile, a specific Ile-dependent transport assay is desirable to expand the conclusions to transport of the needed peptides per se. Although radioactively labeled amino acids are commercially available, similarly labeled peptides are more difficult to acquire. However, an FITC-labeled hexapeptide described previously (see Materials and Methods) was utilized to more directly assay peptide transport by growing the wild-type strain as well as the *oppA*, *dppA*, and *oppA dppA* mutant strains in CDM containing this labeled peptide. Unfortunately, *H. pylori* cells could not utilize this labeled peptide as a sole Ile source. Not surprisingly, the four strains (wild type and *oppA*, *dppA*, and *oppA dppA* mutants) grown with the amino acid Ile in addition to this labeled peptide all showed similar fluorescence accumulation (~150 arbitrary fluorescence units/mg protein) (data not shown). The conclusions herein are therefore based solely on growth phenotypes, not on peptide transport per se.

Taken altogether, these Ile-dependent growth data indicate that the annotated *opp* transport system of *H. pylori*, in contrast to its annotation, is primarily responsible for the transport of short peptides (four or fewer amino acids). On the other hand, the annotated *dpp* transport system of *H. pylori* appears to act more similarly to some described oligopeptide transport systems (9, 16, 35). The *H. pylori* Dpp system seems to be essential for the utilization of certain dipeptides (Ala-Ile and Ile-Ser) and longer peptides (six or more amino acids). It would seem to be highly unusual for a transport system to be essential for short (dipeptide) and long (hexapeptide or longer) peptide transport but not for transport of peptides of intermediate length (tri- and tetrapeptides). In light of this being the case for *H. pylori* and the fact that tri- and tetrapeptides were still utilized in the double mutants (*dppA oppA* and *dppB oppB* mutants), the data provide evidence of the existence of one or more additional peptide transport systems capable not only of tri- and tetrapeptide transport but also of the transport of some dipeptide sequences as well (namely, Lys-Ile, Arg-Ile, Val-Ile, and Phe-Ile). The fact that supplementation of the growth medium with chelators failed to have an effect on the growth of any mutant (Fig. 5A and B) makes it unlikely that sequence-specific proteases are responsible for the promiscuous phenotypes described. Previous studies have concluded that *H. pylori* lacks extracellular proteases (6, 30), making it even more unlikely that these growth phenotypes are due to peptide digestion. Extracellular peptidases would also have been expected to act on the nonapeptide as well, allowing subfragments to readily cross the outer membrane and result in growth similar to that seen with smaller Ile sources. The poor growth of all *H. pylori* strains on the nonapeptide Ile source makes it unlikely that extracellular peptidases were acting on these peptides. Finally, while the FITC-labeled hexapeptide showed a certain degree of degradation, the intact hexapeptide was still the major peptide in the medium (data not shown). In addition, there was only one contaminating peptide in the supernatants, eluting at 52 min (compared to 58.5 min for the intact labeled hexapeptide), showing that the breakdown product is similar in size to the full intact peptide. The *dpp* strains could not use this breakdown product (no growth on the hexapeptide), indicating that the subfragment cannot account for residual transport. Of course, detection of this breakdown product means that another subfragment of the original peptide (the remainder) was generated as well.

Both peptide transport systems studied here are members of the large, highly conserved protein family of ABC transporters (15, 16, 20). All domains of each system were subjected to a BLAST search based on the predicted amino acid sequences from the 26695 genome (11). Not surprisingly, the amino acid sequences for the *dppA*, *dppB*, and *dppC* products showed the greatest homology to proteins annotated as dipeptide transporter domains. Although most BLAST results for the *dppD* and *dppF* sequences were actually annotated as general ABC transporter proteins, the fact that they were in the same operon as the *dppABC* genes (Fig. 1A) makes it likely that they all function in concert. However, based on the Ile-dependent growth assay results, it is obvious that the system acts as an oligopeptide transporter and is essential for the use of peptides that are at least six amino acids long.

All four domains of the oligopeptide transport system (*oppABCD*) showed significant homology to annotated oligopeptide transporters from other organisms. As previously stated, the putative oligopeptide transporter genes are separated into two regions of the genome, and the only evidence that the products of these two regions comprise a single transporter is based on sequence homology. A role in peptide transport was subtly indicated by knocking out *oppB*; however, separate knockouts of *oppA* and *oppC* failed to show any impairment of peptide usage. This indicates that either these two proteins (OppA and OppC) are functionally complemented by another

system in *H. pylori* or they have no role in peptide transport. The observed phenotype also argues against any downstream polar effects introduced from the *oppA* disruption. Experiments to identify possible substrates and to fully determine the relationship of the two regions are under way.

When the *dppABCDF* system is compromised, the remaining peptide utilization profile is altered considerably. This is displayed when either the substrate binding domain (*dppA* mutant) or one or both of the permease domains (*dppB*, *dppC*, and *dppB dppC* mutants) are inactivated. Since all strains were similarly growth deficient, it is likely that the transport system itself is compromised by each disruption, as opposed to some other effect of the individual mutations. Previous studies showed that a *dppA* knockout has a downstream effect on the rest of the operon (8), so it is unlikely that a partially functional dipeptide transport system is responsible for the residual dipeptide transport observed in the mutants in our study. The *dppA oppA* and *dppB oppB* strains exhibited similar growth patterns to those of the other dipeptide transport mutants (*dppA*, *dppB*, *dppC*, and *dppB dppC* mutants), supporting the idea that residual dipeptide transport is not due to the functioning of the *oppABCD* system; perhaps an unidentified transporter is responsible. There are many transport systems in the *H. pylori* genome that have yet to be characterized, some of which are predicted to transport amino acids (11). On the other hand, there are no alternative peptide transporting systems to the *dpp* and *opp* systems annotated in the *H. pylori* genome, such as the *dtp* system originally found in *Lactococcus lactis* (14). Current studies are focused on identifying a possible alternate peptide transport system(s) in *H. pylori*.

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