WaaP of *Pseudomonas aeruginosa* Is a Novel Eukaryotic Type Protein-tyrosine Kinase as Well as a Sugar Kinase Essential for the Biosynthesis of Core Lipopolysaccharide*

WaaP of *P. aeruginosa* is a crucial sugar kinase that phosphorlates HepI in the inner core region of lipopolysaccharide (LPS). WaaP shares homology with eukaryotic protein kinases in the conserved functional motifs (I–IX), indicating that it is also a protein kinase. This interpretation is substantiated by several lines of evidence including the following: (i) site-directed mutagenesis on catalytic domain residues abrogated the protein kinase activity; (ii) positive reaction in immunoblotting with anti-phosphotyrosine monoclonal antibody PY20; (iii) matrix-assisted laser desorption/ionization time-of-flight (MALDI-TOF) mass spectrometry and proteolytic peptide mapping showing excess mass equivalent to eight phosphate substituents on the tyrosine residues in WaaP; and (iv) WaaP is capable of catalyzing tyrosine self-phosphorylation as well as phosphorlyating an exogenous synthetic co-polymer poly(Glu, Tyr). Thus, WaaP possesses dual kinase functions, and it utilizes a catalytic mechanism similar to that of the eukaryotic protein kinases. WaaP was localized to the cytoplasm, suggesting that phosphorylation of the LPS core occurred prior to translocation to the periplasm and attachment of O-antigen. A chemiluminescence-based enzyme-linked immunosorbent assay (ELISA) was developed to measure the kinetics of the WaaP sugar kinase activity, and the results showed that the $K_{m}$ was 0.22 mM for ATP and 14.4 μM for hydrofluoric acid-treated LPS, $V_{\text{max}}$ was 408.24 pmol min$^{-1}$, and $k_{\text{cat}}$ was 27.23 min$^{-1}$.

In eukaryotes, protein-tyrosine kinases play important roles in biological regulation, i.e. signal transduction and growth control. Crystallography studies of protein kinases provided an insight into molecular recognition at the substrate and ATP binding sites as well as the mechanisms of action of these enzymes. At present, little is known about tyrosine kinases in prokaryotes, since they are regarded as rare and poorly defined (1–3). Recent reports that described a number of protein-phosphotyrosine kinases (PTKs) involved in polysaccharide biosynthesis include Wzc$_{esp}$ in *Escherichia coli* isolates with group 1 capsules (4), Wzc in *E. coli* K-12 (5, 6), Etk in *E. coli* (1), PTK in *Acinetobacter johnsonii* (7, 8), and CpsD in *Streptococcus pneumoniae* (9). Most of these enzymes are either proposed or identified to be involved in the transportation or regulation of the production of exopolysaccharides required for virulence (1, 8). Interestingly, none of them showed significant homology to the typical tyrosine kinases from eukaryotes (10). Also, no protein-tyrosine kinase has been reported to date to phosphorylate the core lipopolysaccharide of Gram-negative bacteria.

*Pseudomonas aeruginosa* is an opportunistic pathogen that can cause life-threatening infections in compromised patients including those with burn wounds or cystic fibrosis and individuals receiving chemotherapy (11). Lipopolysaccharide (LPS) located in the outer membrane of *P. aeruginosa* is one of the major virulent factors. It is composed of lipid A, core oligosaccharide (including inner core and outer core regions), and O-antigen (Fig. 1). The inner core LPS is composed of 1-glycero-d-manno-heptose and 3-deoxy-d-manno-octulosonic acid. LPS of *P. aeruginosa* is known to be the most highly phosphorylated among Gram-negative bacteria (12, 13). The multiple phosphoryl substituents in this region are essential for the outer membrane stability (14). Its inner core possesses three phosphate groups located on C-2, C-4, and C-6 of HepI (Fig. 1), respectively. These phosphate substituents contribute negative charges that are crucial in forming ionic bridges with divalent cations to stabilize the outer membrane.

The involvement of waaP in the phosphorylation of HepI of *P. aeruginosa* LPS has been investigated at the genetic and LPS structural levels by our laboratory (14). Mutation of this gene is lethal to the bacterium, and the knockout of the chromosomal waaP gene was accomplished only when another copy of waaP was added in trans (14), indicating that the presence of phosphate(s) on HepI is essential for the viability of *P. aeruginosa*. Furthermore, waaP$_{P_{o}}$ can complement a *Salmonella typhimurium* waaP mutant and restore resistance to SDS and novobivcin in this mutant. By performing two-dimensional $^{1}H$/$^{31}P$ NMR analysis, our group also demonstrated that waaP$_{P_{o}}$ can reconstitute the phosphate on C-4 of HepI. These data enabled us to conclude that waaP encodes a sugar kinase to phosphorylate C-4 on HepI (14). Importantly, since WaaP is crucial to *P. aeruginosa*, inhibitors of the kinase

---

* This work was supported by funding from the Canadian Bacterial Disease Network (to J. S. L.). The MALDI-TOF mass spectrometry equipment at the University of Guelph was acquired through a grant jointly funded by the Canadian Foundation of Innovation and the Ontario Research and Development Challenge Fund (to Krasimir Yankulov (principal investigator), J. S. L., and others). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Recipient of a Marsha Morton Scholarship from the Canadian Cystic Fibrosis Foundation. To whom correspondence should be addressed. Tel.: 519-824-4120 (ext. 3823); Fax: 519-837-1802; E-mail: jlam@uoguelph.ca.

1 The abbreviations used are: PTK, phosphotyrosine kinase; LPS, lipopolysaccharide; HepI, heptose I; ELISA, enzyme-linked immunosorbent assay; IMAC, immobilized metal ion affinity chromatography; MALDI-TOF, matrix-assisted laser desorption/ionization-time-of-flight; BSA, bovine serum albumin; mAb, monoclonal antibody; PBS, phosphate-buffered saline; HF, hydrofluoric acid; MIC, minimum inhibitory concentration.
function may have therapeutic value. Therefore, this protein is an attractive target for the development of novel drugs to control infection by *P. aeruginosa*, which is intrinsically resistant to a wide range of antibiotics. This requires an in depth understanding of the biochemical properties of this enzyme and development of an assay that can be automated for screening large numbers of potential inhibitors.

WaaP of *E. coli* (WaaPEc) shares 52% homology with WaaPpa. The kinase activity of WaaPpa was determined by an assay using [γ-32P]ATP to phosphorylate the LPS from the waaP knockout mutant of *E. coli* (15). In that study, the authors focused on the purification of the enzyme and characterization of the enzyme kinetics. However, a *k*ₐₐₜ value was not obtained, and there were no data to link WaaPpa to the family of tyrosine kinases.

In this paper, we report the overexpression and purification of WaaP and also provide evidence to show that this protein is a eukaryotic-type protein tyrosine kinase. We also developed an enzyme-linked immunosorbent assay (ELISA) based on the Basic Local Alignment Searching Tool (BLAST) and the amino acid sequence of WaaP was aligned with the protein kinase—protein tyrosine kinases in the subdomains stated in the nomenclature of Hanks and Quinn (10). The alignment of WaaPpa and WaaPEc was accomplished by using the Basic Local Alignment Searching Tool (BLAST) and the nonredundant GenBank™ CDS data base (16).

Site-directed Mutagenesis and in Vivo Complementation Assay—waaP was amplified by PCR from pCOREc1 (17) with the flanking forward and reverse primers 5'-ATATAGGACGCTGCTGGTGCTGG-3' and 5'-TATATAGGCTTGCTGGCTTCCGG-3' containing BamHI and HindIII, respectively. The PCR product was cloned into pUCP26 (18) as a positive control for complementation assay. Mutations of waaP were constructed by the method of “overlapping extension” as described by Horton (19) using PCR with the flanking primers as well as the primers shown below. The K89A mutation was introduced into the gene with the forward and reverse primers 5'-GCTACGCACGCCGTCCGGCTGGTGCTGG-3' and 5'-GCTACGCACGCCGTCCGGCTGGTGCTGG-3'; K69R was introduced with 5'-CAACCATGGCCTGCTGCACTATGTC-3' and 5'-CAACCATGGCCTGCTGCACTATGTC-3'; and D163A was introduced with 5'-GCTACGCACGCCGTCCGGCTGGTGCTGG-3'. The underlined nucleotides indicate the mutations. The PCR products were then cloned into pUCP26 at BamHI and HindIII sites, respectively, and transformed into *E. coli* F470 waaP (20). Constructs containing the mutations of waaP were confirmed by nucleotide sequencing (performed at the Laboratory Services Division, University of Guelph, Ontario, Canada). In *vivo* complementation was tested by assessing the minimum inhibitory concentration (MIC) of SDS and novobiocin, respectively, according to Walsh et al. (14). *E. coli* F470 waaP (15) was used as the negative control.

### EXPERIMENTAL PROCEDURES

**Amino Acid Alignment Analysis of WaaP with WaaPpa and Protein Kinases**—Amino acid sequence of WaaP was aligned with the protein kinases in the subdomains stated in the nomenclature of Hanks and Quinn (10). The alignment of WaaPpa and WaaPEc was accomplished by using the Basic Local Alignment Searching Tool (BLAST) and the nonredundant GenBank™ CDS data base (16).

**Site-directed Mutagenesis and in Vivo Complementation Assay—**waaP was amplified by PCR from pCOREc1 (17) with the flanking forward and reverse primers 5'-ATAATAGGACGCTGCTGGTGCTGG-3' and 5'-TATATAGGCTTGCTGGCTTCCGG-3' containing BamHI and HindIII, respectively. The PCR product was cloned into pUCP26 (18) as a positive control for complementation assay. Mutations of waaP were constructed by the method of “overlapping extension” as described by Horton (19) using PCR with the flanking primers as well as the primers shown below. The K89A mutation was introduced into the gene with the forward and reverse primers 5'-GCTACGCACGCCGTCCGGCTGGTGCTGG-3' and 5'-GCTACGCACGCCGTCCGGCTGGTGCTGG-3'; K69R was introduced with 5'-CAACCATGGCCTGCTGCACTATGTC-3' and 5'-CAACCATGGCCTGCTGCACTATGTC-3'; and D163A was introduced with 5'-GCTACGCACGCCGTCCGGCTGGTGCTGG-3'. The underlined nucleotides indicate the mutations. The PCR products were then cloned into pUCP26 at BamHI and HindIII sites, respectively, and transformed into *E. coli* F470 waaP (20). Constructs containing the mutations of waaP were confirmed by nucleotide sequencing (performed at the Laboratory Services Division, University of Guelph, Ontario, Canada). In *vivo* complementation was tested by assessing the minimum inhibitory concentration (MIC) of SDS and novobiocin, respectively, according to Walsh et al. (14). *E. coli* F470 waaP (15) was used as the negative control.

**Cloning of waaP into an Expression Vector—**waaP was amplified by PCR using pCOREc1, as the template, that contains the core gene cluster of *P. aeruginosa* (14). The forward and reverse primers were nucleotide 5'-GAACCATGGGATCCATTTAAGCT-3' and 5'-GAACCATGGGATCCATTTAAGCT-3', containing NdeI and HindIII restriction endonuclease sites, respectively. The reverse primer also contains the mutation (underlined) to change the stop codon of waaP from TAG to TCT. This PCR product was cloned into pET30a expression vector (Novagen, Madison, WI) at NdeI and HindIII sites to be in frame with the His tag at the C terminus of the protein. The construct was then introduced into *E. coli* JM109 by CaCl₂ transformation procedure (21), and the transformants were selected on Luria agar (Fisher) containing 30 mg/liter kanamycin. Both strands of DNA were sequenced to verify the sequence of the cloned waaP and the in-frame His tag. The resultant construct waaP was overexpressed in *E. coli* BL21(DE3)pLysS (Novagen). All of the chemicals used in this paper were from Sigma unless stated.

**Overexpression of the Plasmid-encoded waaP**—Terrific broth (22) supplemented with 3 mg/liter kanamycin and 3.4 mg/liter chloramphenicol was used for the overexpression of WaaP. The cells were first cultivated with shaking at 37 °C to 0.6 at A₆₀₀. The overexpression of recombinant protein was induced with 1 mM isopropyl-b-D-thiogalactopyranoside for 5 h. Cells were harvested by centrifugation at 5000 × g and pellets were frozen at −20 °C. The overexpression was also used as the control for comparison with the overexpression of WaaP.

**Purification of WaaP**—Two grams of frozen cell pellet was suspended in 20 ml Tris buffer (20 mM Tris-Cl, 0.5 mM NaCl, pH 8.0) containing 5 mM imidazole and 10 mM b-mercaptoethanol. A protease inhibitor mixture of 20 μl/liter that contains 4-(2-aminoethyl)benzenesulfonyl fluoride, bestatin, pepstatin A, trans-epoxysuccinyl-l-leucylamido(4-guanidino)butane (E-64), and N-bis(homophenyl)glycine was added. Cells were broken by sonication on ice with a macroprobe at a power setting of 4 for 2 min (Ultrasonic Processor XL 2020; MANDEL Scientific Company Ltd., Guelph, Ontario, Canada) followed by centrifugation at 10,000 × g for 4 min at 20 °C. The supernatant containing the soluble WaaP protein was mixed with 3 ml of cobalt-based immobilized metal ion affinity chromatography (IMAC) resin (TALON metal affinity resin; CLONTECH Laboratories, Palo Alto, CA) and incubated at 4 °C for 1 h with gentle shaking. Then the mixture was loaded onto a 1.6-cm diameter column and washed with 20 bed volumes of 5 mM imidazole/Tris buffer. The column was further washed with 10 bed volumes of 50 mM imidazole/Tris buffer, and WaaP was eluted with 1 mM imidazole/Tris buffer.

The eluted protein was dialyzed extensively at 4 °C against 20 mM Tris-Cl, pH 8, using dialysis tubing with a 3500 molecular weight cut-off (Spectrum Laboratories, Inc., Rancho Dominguez, CA), and concentrated with polyethylene glycol 8000.

**Protein Assay—**Protein concentration was determined by the BCA method (23) following the procedure described by the manufacturer (Pierce). Bovine serum albumin (BSA) was used as the standard.

**SDS-PAGE and Western Immunoblotting—**Purified WaaP protein was analyzed by a standard discontinuous SDS-polyacrylamide gel electrophoresis method using 12.5% resolving gels (24) and stained with...
P. aeruginosa WaaP Is a Self-phosphorylated PTK

Coomassie Blue R-250. SeeBlue™ prestained standards (NOVEX, Scarborough, Ontario, Canada) were used as the molecular weight marker. Western immunoblotting following SDS-PAGE was performed using nitrocellulose membrane according to Burnette (25) using Penta-Star™ (Applied Biosystems, Bedford, MA), diluted 1:5 (v/v) in diethanolamine buffer. The kinase reactions were performed in a 96-well microtiter plate as described above. The kinase reactions were performed in a 96-well microtiter plate as described above.

For self-phosphorylation reactions using additional WaaP in solution, 5 µg of IMAC-purified WaaP and the ATP mixture were added to each well that was precoated with WaaP and incubated at 37 °C for 1 h before proceeding with the chemiluminescence detection as described above.

For determining protein kinase activity of WaaP on exogenous tyrosine-containing substrate, 10 µg of polyGlu, Tyr 4:1 (Sigma) in 100 µl of 20 mM Tris-HCl, pH 7.5, was coated on the opaque 96-well microtiter plate. After the reaction was incubated at 37 °C for 30 min, the mixture was transferred to the scintillation counter (Packard, Downer's Grove, IL) and counted. Each sample was assayed in triplicate.

Preparation of Polyclonal Antibody against WaaP—Rabbit anti-WaaP antiserum was raised against purified WaaP using protocols described by our group (28). The polyclonal antibodies were purified by immunoaffinity adsorption according to the method of Olmsted (29) with modifications. Briefly, purified WaaP was electrophoresed and transferred to nitrocellulose membrane. The WaaP protein band was stained with Ponceau S (0.1% Ponceau S in 5% acetic acid) and cut out. The membrane was then blocked with 5% skim milk, 0.1% Tween 20 in PBS for 30 min and incubated with 1:5000 diluted solution containing 10% BSA/Tris-buffered saline, was used as the secondary antibody. For the Western immunoblotting, this antibody was used at a dilution of 1:2000 in 3% BSA/Tris-buffered saline, was used as the secondary antibody. The membranes were washed three times in PBS-Tween 20 and one time with PBS and cut into small pieces. The antibody was eluted by incubating with 0.7 ml of 0.2 M HCl-glycine buffer (pH 2.2) at room temperature for 15 min. The pH of the eluate was brought up to 7 with 0.3 M of 1 M K₂HPO₄, and the antibody was dialyzed against PBS at 4 °C for 24 h on a rocking platform. Then the membrane was washed three times in PBS-Tween 20 and one time with PBS and cut into small pieces. The antibody was eluted by incubating with 0.7 ml of 0.2 M HCl-glycine buffer (pH 2.2) at room temperature for 15 min. The pH of the eluate was brought up to 7 with 0.3 M of 1 M K₂HPO₄, and the antibody was dialyzed against PBS at 4 °C for 24 h on a rocking platform. Then the membrane was washed three times in PBS-Tween 20 and one time with PBS and cut into small pieces. The antibody was eluted by incubating with 0.7 ml of 0.2 M HCl-glycine buffer (pH 2.2) at room temperature for 15 min. The pH of the eluate was brought up to 7 with 0.3 M of 1 M K₂HPO₄, and the antibody was dialyzed against PBS at 4 °C for 24 h on a rocking platform. Then the membrane was washed three times in PBS-Tween 20 and one time with PBS and cut into small pieces.
P. aeruginosa WaaP Is a Self-phosphorylated PTK

RESULTS

WaaP Has Features Consistent with Eukaryotic Type Protein Kinases—Our group (14) has previously provided genetic evidence to show that WaaP is a sugar (heptose) kinase. To further investigate its kinase function and compare it with other kinases including protein kinases, alignment comparisons between the amino acid sequence of WaaP and those of a number of well characterized protein kinases from eukaryotes were performed (Fig. 2). Since WaaP<sub>Pa</sub> and WaaP<sub>Ec</sub> share 52% identity at the amino acid sequence level, both sequences were also aligned and compared with the protein kinases. Two members of protein kinases (protein kinase C- and SNF1) from serine/threonine kinase family and two (Src and EGFR) from tyrosine kinase family were investigated for its kinase function and compared it with other kinases including protein kinases, alignment comparisons between the amino acid sequence of WaaP and those of a number of well characterized protein kinases from eukaryotes were performed (Fig. 2). Since WaaP<sub>Pa</sub> and WaaP<sub>Ec</sub> share 52% identity at the amino acid sequence level, both sequences were also aligned and compared with the protein kinases. Two members of protein kinases (protein kinase C- and SNF1) from serine/threonine kinase family and two (Src and EGFR) from tyrosine kinase family were selected, respectively, for the alignment comparisons to WaaP<sub>Pa</sub> (Fig. 2). The sequences of these protein kinase families can be divided into 12 subdomains (I-XII) according to the nomenclature of Hanks et al. (10, 36). Only subdomains I–IX are shown in the figure. The results indicated that WaaP has significant identity on the conserved, functional residues of the protein kinases. Subdomain I is rich in glycine residues, and the GXXG or GXXGXXG (in which X can be any amino acid) (41) signature of the nucleotide binding. Lys<sup>69</sup> in subdomain II is the well characterized catalytic domain residue that is involved in the proton transfer in the phosphotransfer reaction (42). In the central core of the catalytic domain VI through IX, the invariant residues Asp<sup>36</sup>, Asp<sup>44</sup>, and Asp<sup>45</sup> have been implicated in ATP binding, and this is also the feature of other bacterial phosphotransferases that use ATP as the phosphate donor (36). Furthermore, Asp<sup>36</sup> and Asp<sup>45</sup> may interact with the phosphate groups of ATP through Mg<sup>2+</sup> salt bridges (10, 36, 44, 45). The presence of these protein kinase-like conserved motifs suggested that WaaP might contain the activity of a protein kinase in addition to being a sugar kinase.

In contrast, the protease sequence of WaaP<sub>Ec</sub> (from E. coli F470) (20) did not align well with the functional motifs of the protein kinases. It did not contain the signature of the nucleotide binding site itself. However, the catalytic domain in subdomain VI can be divided into 12 subdomains (I–XII) according to the nomenclature of Hanks et al. (10, 36). Only subdomains I–IX are shown in the figure. The results indicated that WaaP has significant identity on the conserved, functional residues of the protein kinases. Subdomain I is rich in glycine residues, and the signature of the nucleotide binding. Lys<sup>69</sup> in subdomain II is the well characterized catalytic domain residue that is involved in the proton transfer in the phosphotransfer reaction (42). In the central core of the catalytic domain VI through IX, the invariant residues Asp<sup>36</sup>, Asp<sup>44</sup>, and Asp<sup>45</sup> have been implicated in ATP binding, and this is also the feature of other bacterial phosphotransferases that use ATP as the phosphate donor (36). Furthermore, Asp<sup>36</sup> and Asp<sup>45</sup> may interact with the phosphate groups of ATP through Mg<sup>2+</sup> salt bridges (10, 36, 44, 45). The presence of these protein kinase-like conserved motifs suggested that WaaP might contain the activity of a protein kinase in addition to being a sugar kinase.

To validate the accuracy of the alignment comparisons in Fig. 2, site-directed mutagenesis of WaaP<sub>Pa</sub> was performed targeting Lys<sup>69</sup> and Asp<sup>45</sup>, respectively. The effect of the site-directed mutation was evaluated by testing whether the mutant constructs could complement waaP<sub>Ec</sub>. It is noteworthy that the complementation assay was performed using a waaP<sub>Ec</sub> mutant as a recipient, since waaP<sub>Pa</sub> mutation is lethal to P. aeruginosa. The complementation of waaP<sub>Ec</sub> by wild type waaP<sub>Pa</sub> increased the MICs of waaP<sub>Ec</sub> by 3 and 30 times to novobiocin and SDS, respectively. However, the MICs of waaP<sub>Ec</sub> complemented with the mutants of waaP<sub>Pa</sub> did not show any difference when compared with those of the wild type waaP<sub>Ec</sub> mutant (Table I). This indicated that Lys<sup>69</sup> or Asp<sup>45</sup> are essential residues for the kinase function of waaP in P. aeruginosa. Therefore, these results substantiated the significance of the alignment of WaaP<sub>Pa</sub> with the protein kinases shown in Fig. 2.

Purification of WaaP by IMAC—Results from SDS-PAGE and the corresponding Western immunoblotting with Penta-His™ antibody showed that the His<sub>6</sub> tag was expressed as part of WaaP, and a band with an apparent molecular mass of 33 kDa was observed. This is very close to the predicted molecular mass of 32.9 kDa (i.e., the mass of WaaP plus His<sub>6</sub> tag), and over 90% of this protein was expressed in the soluble form (data not

2 X. Zhao and J. S. Lam, submitted for publication.
shown). The IMAC purification of WaaP has been optimized, and the yield obtained was 0.5 mg of protein/liter of culture with over 95% purity (Fig. 3, A and B).

Determining the Presence of Phosphotyrosine Residues in WaaP—To investigate if WaaP is a self-phosphorylated kinase, purified WaaP was examined by Western immunoblotting using anti-phosphotyrosine mAb PY20, and a single band was observed (Fig. 3C). This showed that WaaP contains phosphotyrosine, which is probably the result of self-phosphorylation. WaaP contains eight tyrosine residues; therefore, we proceeded to determine the number of tyrosine residues that are phosphorylated.

Assessment of the State of Phosphorylation among the Eight Tyrosine Residues of WaaP—Full-length WaaP protein with a C-terminal His tag (WaaPHisC) was subjected to MALDI-TOF mass spectrometry to determine the accurate molecular mass. The actual mass of WaaP from the MALDI-TOF analysis was 3.3544.618 Da (Fig. 4), which is larger than the predicted (nonphosphorylated) molecular mass of 32897.38 Da. The extra mass of 647.328 matched the value of 8.094 phosphate substituents (HPO₄⁻; mass = 79.969). This resulted provided evidence that all eight tyrosine residues in WaaP may be phosphorylated. We further performed ELISA using anti-phosphotyrosine mAb PY20 to interact with WaaP that had been dephosphorylated with phosphotyrosine-specific protease (protein-tyrosine phosphatase). The results showed that over 10% lower signal was detected from the protein-tyrosine phosphatase-treated WaaP than that of the nontreated WaaP (data not shown). This indicates that WaaP can be dephosphorylated by protein-tyrosine phosphatase, and therefore the extra mass of WaaP is due to the phosphorylation and not sulfation; the latter would have resulted in contributing approximately the same extra mass.

To identify the location of the phosphorylated amino acid residues in WaaP, purified WaaP was digested with trypsin and chymotrypsin, respectively. The peptides generated were analyzed by MALDI-TOF and compared with the predicted peptide map of WaaP digested by these two proteases, respectively. Each tyrosine-containing peptide from digested WaaP had extra mass corresponding to the addition of a phosphate group m/z 80 (Table II). This indicated that all eight tyrosine residues in WaaP can be phosphorylated.

Self-phosphorylation Activity of WaaP—To determine whether WaaP catalyzes tyrosine self-phosphorylation, purified WaaP was used in the self-phosphorylation assay with a sensitive, chemiluminescence-based ELISA using anti-phosphotyrosine PY20 antibody. The chemiluminescence signal of the self-phosphorylation of WaaP was 407.8 chemiluminescence units/µg min⁻¹, which was 21% higher than that of the control, 337.1 chemiluminescence units/µg min⁻¹ (Table III), indicating that WaaP exhibits self-phosphorylation activities. To further examine the mechanism of the self-phosphorylation (i.e. if the self-phosphorylation occurred within one molecule or between molecules), purified WaaP protein was coated on the 96-well plates, and self-phosphorylation assays were performed in two distinct ways. One approach was by the addition of an ATP mixture, and the other was by the addition of exogenous WaaP plus the ATP mixture (Table III). The use of exogenous WaaP did not improve the phosphorylation level of the coated WaaP (Table III), which indicated that the phosphorylation of WaaP probably occurs intramolecularly and not intermolecularly.

Determining the Kinase Activity of WaaP in Interaction with Exogenous Tyrosine-containing Substrate, Poly(Glu, Tyr)—We further investigated the ability of WaaP to phosphorylate in vitro an exogenous substrate, poly(Glu, Tyr) copolymer, which was used to precoat the 96-well microtiter plates. Phosphorylation was monitored by the chemiluminescence-based ELISA. The phosphorylation of poly(Glu, Tyr) gave a chemiluminescence response of 782 units/µg/min (Table III). This showed that WaaP could catalyze the phosphorylation of exogenous tyrosine substrates.

Cellular Localization of WaaP and Its State of Phosphorylation in the Cell Fractions—Cell fractionation was performed to localize WaaP in P. aeruginosa and the fractions were examined by SDS-PAGE and Western immunoblotting with purified polyclonal antibody against WaaP. WaaP can only be found in the cytoplasmic fraction of P. aeruginosa (Fig. 5B, lane 5). The overexpressed WaaP with the His tag exhibited higher molecular mass at about 33 kDa, which is close to the expected mass at 32.9 kDa (Fig. 5B, lane 2), and WaaP from P. aeruginosa (Fig. 5B, lane 5) showed smaller size at 31-kDa and is also close to the expected molecular mass at about 31.3 kDa. In Western immunoblotting of the different fractions using anti-phosphotyrosine mAb PY20, WaaP from P. aeruginosa (Fig. 5C, lane 5) and the overexpressed WaaP in E. coli (Fig. 5C, lane 2) were both strongly reactive with this antibody.

Determining the Sugar Kinase Activity of WaaP—To identify the reconstitution of phosphate on HF-LPS by WaaP enzymatic reaction, phosphate analysis was performed on the reconstituted HF-LPS as well as on wild type PAO1-LPS using the method of Ames and Dubin (32) and Zhao and Lam.2 Approximately 10% of phosphate was reconstituted on HF-LPS (20 nmol of P/ng of LPS) compared with 190 nmol of P/ng of LPS.
for PAO1-LPS. This indicated that the incorporation of the phosphate to the HF-LPS occurred due to the enzyme reaction; therefore, WaaP is also a sugar kinase in addition to a self-phosphorylated protein-tyrosine kinase. To develop a nonradio-labeling assay for determining the activity of WaaP, mAb 7-4 that specifically recognizes the phosphorylated LPS was used as the primary antibody. mAb 7-4 reacted with wild type LPS from strain PAO1 and did not react with HF-LPS that had been dephosphorylated. This antibody allowed the development of a highly sensitive, chemiluminescence-based ELISA (the details of the ELISA development are described elsewhere).2

ELISAs on the time course of WaaP reactions indicated that enzyme activities increased sharply in the initial 20 min and slowed down afterward (data not shown). Therefore, the reactions for the kinetic studies measured the phosphorylation within the initial 15 min. The enzyme reactions of WaaP were

for PAO1-LPS. This indicated that the incorporation of the phosphate to the HF-LPS occurred due to the enzyme reaction; therefore, WaaP is also a sugar kinase in addition to a self-phosphorylated protein-tyrosine kinase. To develop a nonradio-labeling assay for determining the activity of WaaP, mAb 7-4 that specifically recognizes the phosphorylated LPS was used as the primary antibody. mAb 7-4 reacted with wild type LPS from strain PAO1 and did not react with HF-LPS that had been dephosphorylated. This antibody allowed the development of a highly sensitive, chemiluminescence-based ELISA (the details of the ELISA development are described elsewhere).2

ELISAs on the time course of WaaP reactions indicated that enzyme activities increased sharply in the initial 20 min and slowed down afterward (data not shown). Therefore, the reactions for the kinetic studies measured the phosphorylation within the initial 15 min. The enzyme reactions of WaaP were

for PAO1-LPS. This indicated that the incorporation of the phosphate to the HF-LPS occurred due to the enzyme reaction; therefore, WaaP is also a sugar kinase in addition to a self-phosphorylated protein-tyrosine kinase. To develop a nonradio-labeling assay for determining the activity of WaaP, mAb 7-4 that specifically recognizes the phosphorylated LPS was used as the primary antibody. mAb 7-4 reacted with wild type LPS from strain PAO1 and did not react with HF-LPS that had been dephosphorylated. This antibody allowed the development of a highly sensitive, chemiluminescence-based ELISA (the details of the ELISA development are described elsewhere).2

ELISAs on the time course of WaaP reactions indicated that enzyme activities increased sharply in the initial 20 min and slowed down afterward (data not shown). Therefore, the reactions for the kinetic studies measured the phosphorylation within the initial 15 min. The enzyme reactions of WaaP were

for PAO1-LPS. This indicated that the incorporation of the phosphate to the HF-LPS occurred due to the enzyme reaction; therefore, WaaP is also a sugar kinase in addition to a self-phosphorylated protein-tyrosine kinase. To develop a nonradio-labeling assay for determining the activity of WaaP, mAb 7-4 that specifically recognizes the phosphorylated LPS was used as the primary antibody. mAb 7-4 reacted with wild type LPS from strain PAO1 and did not react with HF-LPS that had been dephosphorylated. This antibody allowed the development of a highly sensitive, chemiluminescence-based ELISA (the details of the ELISA development are described elsewhere).2

ELISAs on the time course of WaaP reactions indicated that enzyme activities increased sharply in the initial 20 min and slowed down afterward (data not shown). Therefore, the reactions for the kinetic studies measured the phosphorylation within the initial 15 min. The enzyme reactions of WaaP were

for PAO1-LPS. This indicated that the incorporation of the phosphate to the HF-LPS occurred due to the enzyme reaction; therefore, WaaP is also a sugar kinase in addition to a self-phosphorylated protein-tyrosine kinase. To develop a nonradio-labeling assay for determining the activity of WaaP, mAb 7-4 that specifically recognizes the phosphorylated LPS was used as the primary antibody. mAb 7-4 reacted with wild type LPS from strain PAO1 and did not react with HF-LPS that had been dephosphorylated. This antibody allowed the development of a highly sensitive, chemiluminescence-based ELISA (the details of the ELISA development are described elsewhere).2

ELISAs on the time course of WaaP reactions indicated that enzyme activities increased sharply in the initial 20 min and slowed down afterward (data not shown). Therefore, the reactions for the kinetic studies measured the phosphorylation within the initial 15 min. The enzyme reactions of WaaP were
also performed with varying concentrations of enzyme (0–15 µg), ATP (0–500 µM), and HF-LPS (0–50 ng), respectively (data not shown). The ELISA developed in this study could be successfully used to quantify the enzyme activity of WaaP in a 96-well microtiter plate. The kinetic parameters were determined from the above experiments and calculated based on the Michaelis-Menten equation. The $K_m$ was 0.22 mM for ATP and 14.4 µM for HF-LPS; $V_{max}$ for the enzyme reaction was 408.24 pmol min$^{-1}$; and $k_{cat}$ was 27.23 min$^{-1}$ (Table IV). Approximately 70% of enzyme activity remained after storage at −20 °C for 7 days.

**DISCUSSION**

Carbohydrates are probably the least understood of all classes of biologically important molecules (47), and much less is known about the properties of the sugar kinases involved in the biosynthetic pathway.

It is intriguing to observe that WaaP is a sugar kinase, showed significant amino acid identities in most of the functional motifs (subdomains I–IX) with the eukaryotic type protein kinases including members in both protein-tyrosine kinase and Ser/Thr kinase families. Importantly, we were able to validate this prediction on the conserved motifs by site-directed mutagenesis and the subsequent complementation assay. In subdomain I, WaaP has two glycine-rich regions, $X^4GXG$ and $X^5GXGXG$ (where X can be any amino acid), but so far no mutations have been made to show the importance of the space between the functional glycine region and the invariant lysine that lies 14–23 residues downstream (36). Therefore, in WaaP, either of these two glycine regions could be the nucleotide-binding site.

In prokaryotes, several protein-tyrosine kinases such as Wac in *E. coli* (5, 6), PTK in *A. johnsonii* (7, 8), and CpsD in *Streptococcus pneumoniae* (9) were reported to be involved in the transportation or regulation of LPS or capsule biosynthesis in bacteria. But they do not share sequence identities with WaaP or with the eukaryotic protein-tyrosine kinases in most of the functional motifs (36). Those proteins share the Walker A and Walker B consensus among their sequences, and the tyrosine residues in these sequences form a tyrosine-rich region and localize downstream of Walker B. However, in this study, we have shown that WaaP is a eukaryotic type protein-tyrosine kinase, and the tyrosine residues were found to scatter throughout the sequence of the protein.

Both WaaP from *P. aeruginosa* PAO1 and the overexpressed WaaP were found to be phosphorylated. This is different from most of the reported tyrosine kinases from bacteria in which the self-phosphorylation could only be detected in the overexpressed protein (1). The self-phosphorylation of WaaP in *P. aeruginosa* may contribute to its role as a dual functional kinase.

It is intriguing to observe that WaaP is a sugar kinase in addition to a protein kinase. Since the kinase functional domain in WaaP spanned over 200 amino acids, which is about 72% of the total WaaP sequence (276 amino acids), the enzyme probably utilizes the same functional domain to perform sugar phosphorylation and self-phosphorylation. Furthermore, our amino acid alignment analysis strongly suggested that WaaP might utilize a catalytic mechanism similar to that of the eukaryotic type PTKs. Crystallization of WaaP is under way to solve the mechanisms of the kinase activities of WaaP.

The localization of WaaP to the cytoplasmic cell fraction has shed some light on the events of core LPS substitutions during the biosynthesis of this region of the LPS. It is evident that the phosphorylation of HepI in *P. aeruginosa* LPS occurs before O-antigen units are attached to the core in the periplasm.

In contrast to WaaP$_{Pa}$, the amino acid sequence of WaaP$_Ec$ aligned rather poorly in regions corresponding to the conserved functional motifs in eukaryotic type protein kinases. Also, in the complementation assay (Table I), wild type waaP$_{Pa}$ could only partially complement the *E. coli* F470waaP$^{-}$ mutant, and the MIC values to the novobiocin and SDS were higher than in the waaP mutant but lower than in the wild type *E. coli* F470.
Kinetic studies were performed with various concentrations of enzyme (0–15 μg), ATP (0–500 μM), and HF-LPS (0–5 ng). The data were collected within the initial 15 min of the enzyme reaction. The kinetic parameters were determined by the Michaelis-Menten equation. Note that although 150 pmol of enzyme was added to the enzyme-substrate reaction mixture, because of the tendency on the enzyme to precipitate, only 10% (15 pmol) of the enzyme was left in the supernatant after centrifugation.

| K_{cat}(ATP) | K_{cat}(HF-LPS) | V_{max} | Enzyme | k_{cat} | k_{cat}/K_{m} |
|-------------|----------------|---------|--------|--------|--------------|
| mm          | μm             | pmol min⁻¹ |        |        | μm⁻¹ min⁻¹   |
| 0.22        | 14.4           | 408.24  |        | 15     | 27.23        |
|             |                |         |        |        | 1.93         |

These results indicated that WaaP_{Pa} and WaaP_{Ec} might be structurally different although they both have heptose kinase activity and similar kinetic properties. Importantly, in this study we were able to demonstrate that WaaP_{Pa} was a self-phosphorylated tyrosine kinase, whereas such a function has not been reported for WaaP_{Ec}. This implies that WaaP_{Pa} is an enzyme with dual functions, and it may also be involved in other functions such as transportation in the LPS biosynthesis like other tyrosine kinases (5, 7, 8, 9). This may account for the reason that waaP that encodes this enzyme is essential, since the mutation in this gene is lethal to P. aeruginosa (14), whereas mutation in waaP_{Ec} was not lethal to E. coli (15).

As a crucial enzyme to P. aeruginosa, WaaP is a rational drug target for developing new antibiotics. In recent years, enormous efforts have been made to develop protein-tyrosine kinase inhibitors for treatment of diseases such as cancer, psoriasis, and osteoporosis. Several new high throughput PTK assay technologies have been described, and a number of inhibitors have already been put through clinical trials (35). Most of the inhibitors (e.g. members of the 4-aminooquinazolinones family) are small molecules that are competitive at the ATP binding site (46). Since WaaP held such good identity with the typical protein kinases, the screening of inhibitors could begin by using these ATP competitors or analogues.

To develop a nonradiolabeling assay for the LPS phosphorylation, the identification of the phosphate as the epitope for mAb 7-4 was critical. Since mAb 7-4 only recognizes the phosphate by using these ATP competitors or analogues.

In conclusion, we have provided the evidence to show that WaaP_{Pa} possesses dual kinase functions. It is a novel eukaryotic type, self-phosphorylated PTK as well as a heptose kinase associated with the biosynthesis of the LPS core. We also demonstrated that the phosphorylation of LPS in P. aeruginosa occurred before the O-antigen was assembled onto the core.

Acknowledgments—We are grateful to Ravindra B. Kodali (Department of Molecular Genetics, University of Guelph), for performing the MALDI-TOF analysis; to Cory Wenzel for assistance in the preparation of HF-LPS; and to Yolanda I. Ho, a visiting scientist from the Chinese University of Hong Kong, for assistance in cell fractionation and affinity purification of the polyclonal anti-WaaP antibody. We thank Craig Daniels for providing helpful suggestions and critically reading the manuscript and Chris Whitfield for providing E. coli 470 waaP⁻.

REFERENCES

1. Ilan, O., Bloch, Y., Frankel, G., Ullrich, H., Geider, K., and Rosenshine, I. (1999) EMBO J. 18, 3241–3248
2. Zhang, C. C. (1996) Mol. Microbiol. 20, 9–15
3. Cozzone, A. J. (1988) Biochimica (Parux) 80, 43–48
4. Wodstrich, T., Paiment, A., Hocking, J., Forrester, C., and Whitfield, C. (2000) J. Biol. Chem. 276, 2361–2371
5. Vincent, C., Doublot, P., Grangeasse, C., Vaganay, E., Cozzone, A. J., and Dudou, B. (1999) J. Bacteriol. 181, 3472–3477
6. Vincent, C., Dudou, C., Vaganay, E., Dudou, B., Ribertry, M., Cozzone, A. J., and Doublot, P. (2000) J. Mol. Biol. 304, 311–321
7. Duclou, B., Grangeasse, C., Vaganay, E., Ribertry, M., and Cozzone, A. J. (1996) J. Biol. Chem. 259, 891–895
8. Grangeasse, C., Doublot, P., Vincent, C., Vaganay, E., Ribertry, M., Dudou, B., and Cozzone, A. J. (1998) J. Mol. Biol. 286, 559–547
9. Morona, J. K., Puton, J. C., Miller, D. C., and Morona, R. (2000) Mol. Microbiol. 35, 1431–1442
10. Hanks, S. K., and Quinn, A. M. (1991) Methods Enzymol. 205, 38–62
11. Bode, G. P., Fainstein, V., Garcia, I., Rosenbaum, B., and Wong, Y. (1983) J. Infect. Dis. 148, 882–897
12. Wilkinson, S. G. (1983) Rev. Infect. Dis. Suppl. 5, 5941–5949
13. Sadowsky, N., Grangeasse, C., Vaganay, E., Riberty, M., Cozzone, A. J., and Whitfield, C. (1990) J. Biol. Chem. 265, 5498–5504
14. Walsh, A. G., Matewisch, M. J., Burrows, L. L., Monteiro, M. A., Perry, M. B., and Lam, J. S. (2000) Mol. Microbiol. 35, 718–727
15. Yethon, J. A., and Whitfield, C. (2001) J. Biol. Chem. 276, 5498–5504
16. Altschul, S. F., Madden, T. L., Schaffer, A. A., Zhang, Z., Miller, W., and Lipman, D. J. (1997) Nucleic Acids Res. 25, 3389–3402
17. de Kievert, T. R., Dasgupta, T., Schweiger, H., and Lam, J. S. (1995) Mol. Microbiol. 16, 556–574
18. West, S. E., Schweiger, H. P., Dall, C., Sample, A. K., and Runyan-Janecy, L. J. (1994) Gene (Amst.) 48, 81–86
19. Horeon, K. (1994) Mol. Biotechnol. 3, 93–99
20. Yethon, J. A., Heinrichs, D. E., Monteiro, M. A., Perry, M. B., and Whitfield, C. (1998) J. Biol. Chem. 273, 26310–26316
21. Hopkins, P. J., Grant, B. J., Penning, C. A., and Sullivan, K. F. (1990) BioTechniques 9, 570–572
22. Sambrook, J., Fritsch, E. F., and Maniatis, T. (1989) Molecular Cloning: A Laboratory Manual, 2nd Ed., p. A2, Cold Spring Harbor Laboratory, Cold Spring Harbor, New York
23. Smith, P. K., Krohn, R. I., Hermanson, G. T., Mallia, A. K., Gartner, F. H., Provenzano, M. D., Fujimoto, E. K., Goeke, N. M., Olson, B. J., and Klenk, D. C. (1985) Anal. Biochem. 135, 76–85
24. Laemmli, U. K. (1970) Nature 227, 680–685
25. Burnette, W. N. (1981) Anal. Biochem. 112, 195–203
26. Morona, R., Marpethson, D. F., Van Den Bosch, L., Carlin, N. I., and Manning, D. C. (1985) J. Biol. Chem. 260, 2589–2594
27. Kessler, E., and Saffrin, M. (1994) J. Biol. Chem. 269, 22726–22731
28. Huang, L., McGavin, M., Forsberg, C. W., Lam, J. S., and Cheng, K. J. (1990) J. Appl. Environ. Microbiol. 56, 1229–1234
29. Olmsted, J. B. (1986) Methods Enzymol. 134, 467–472
30. Westphal, O., and Jann, K. (1965) Methods Carbohydr. Chem. 5, 83–91
31. Kondo, S., Hasegawa, Y., and Hisatsune, K. (1992) Carbohydr. Res. 231, 55–64
P. aeruginosa WaaP Is a Self-phosphorylated PTK

4730

32. Ames, B. N., and Dubin, D. T. (1960) J. Biol. Chem. 235, 769–775
33. Bantoch, S., Buhler, T., and Lam, J. S. (1994) Clin. Diagn. Lab Immunol. 1, 55–62
34. de Kievit, T. R., and Lam, J. S. (1994) J. Bacteriol. 176, 7129–7139
35. al-Obeidi, F. A., Wu, J. J., and Lam, K. S. (1998) Biopolymers 47, 197–223
36. Hanks, S. K., Quinn, A. M., and Hunter, T. (1988) Science 241, 42–52
37. Parker, P. J., Coussens, L., Totty, N., Rhee, L., Young, S., Chen, E., Stabel, S., Waterfield, M. D., and Ullrich, A. (1986) Science 233, 853–859
38. Celenza, J. L., and Carlson, M. (1986) Science 233, 1175–1180
39. Anderson, S. K., Gibbs, C. P., Tanaka, A., Kung, H. J., and Fujita, D. J. (1985) Mol. Cell. Biol. 5, 1122–1129
40. Ullrich, A., Coussens, L., Hayflick, J. S., Dull, T. J., Gray, A., Tam, A. W., Lee, J., Yarden, Y., Libermann, T. A., and Schlessinger, J. (1984) Nature 309, 418–425
41. Wierenga, R. K., and Hol, W. G. (1983) Nature 302, 842–844
42. Kamps, M. P., and Sefton, B. M. (1986) Mol. Cell. Biol. 6, 751–757
43. Stryer, L. (1988) Biochemistry, pp. 178 and 220–221, W. H. Freeman and Co., New York
44. Brenner, S. (1987) Nature 329, 21
45. Madhusudan, Trafny, E. A., Xuong, N. H., Adams, J. A., Ten Eyck, L. F., Taylor, S. S., and Sowadski, J. M. (1994) Protein Sci. 3, 176–187
46. Garcia, P., Shoelson, S. E., George, S. T., Hinds, D. A., Goldberg, A. R., and Miller, W. T. (1993) J. Biol. Chem. 268, 25146–25151
47. Gervay, J., and McReynolds, K. D. (1999) Curr. Med. Chem. 6, 129–153