Main title:
Purification and characterization of WaaP from *Escherichia coli*, a lipopolysaccharide kinase essential for outer membrane stability

Running title: Purification and properties of WaaP, an LPS kinase

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SUMMARY

In *Escherichia coli*, *Salmonella enterica*, and *Pseudomonas aeruginosa*, the *waaP* (*rfaP*) gene product is required for the addition of phosphate to O-4 of the first heptose residue of the lipopolysaccharide (LPS) inner core region. This phosphate substitution is particularly important to the biology of these bacteria: it has previously been shown that WaaP is necessary for resistance to hydrophobic and polycationic antimicrobials in *E. coli*, and that it is required for virulence in invasive strains of *S. enterica*. WaaP function is also known to be essential for the viability of *P. aeruginosa*. The predicted WaaP protein shows low levels of similarity (10-15% identity) to eukaryotic protein kinases, but its kinase activity has never been tested. Here we report the purification of WaaP and the reconstitution of its enzymatic activity *in vitro*. The purified enzyme catalyzes the incorporation of $^{33}$P from [$\gamma$-$^{33}$P]ATP into acceptor LPS purified from a defined *E. coli waaP* mutant. Enzymatic activity is dependent upon the presence of Mg$^{2+}$, and is maximal from pH 8.0 to 9.0. The apparent $K_m$ (determined at saturating concentrations of the second substrate) is 0.13 mM for ATP and 76 µM for LPS. These data are the first proof that WaaP is indeed an LPS kinase. Further, site-directed mutagenesis of a predicted catalytic residue suggests that WaaP shares a common mechanism of action with eukaryotic protein kinases.
INTRODUCTION

The outer membrane of a gram-negative bacterium is a barrier to many antibiotics and host defense factors (1, 2). This barrier function is due in large part to structural features of the lipopolysaccharide (LPS) molecules that make up the outer leaflet of the outer membrane bilayer. In *Escherichia coli*, *Salmonella enterica*, and a variety of other gram-negative pathogens including members of the families *Pseudomonadaceae* and *Vibrionaceae*, the LPS molecule is conceptually divided into three distinct regions: 1) a hydrophobic membrane anchor designated lipid A; 2) a short, branched chain of sugar residues with multiple phosphoryl substituents, referred to as the core oligosaccharide; and 3) a structurally diverse polysaccharide composed of repeating oligosaccharide units, termed the O antigen (3) (Fig. 1). The presence of phosphoryl substituents on the inner (lipid A proximal) region of the LPS core oligosaccharide is a key structural feature required for the formation of a stable outer membrane in these bacteria (4-6). These phosphoryl substituents are postulated to be critical to outer membrane integrity because their negative charge allows neighbouring LPS molecules to be crosslinked by divalent cations (1, 2).

The genes involved in core phosphorylation have only recently been identified with certainty. In *E. coli*, *waaP* was shown to be required for phosphate addition to HepI (4), and this reaction was found to be a prerequisite for the addition of the HepIII residue by the *waaQ* gene product, which in turn was required for the *waaY*-mediated addition of a second phosphate at HepII (see Fig. 1). Core phosphorylation in *S. enterica* proceeds in similar fashion (5). Given the sequential action of the *waaP, waaQ*, and *waaY* gene products, mutation of *waaP* alone is
therefore enough to eliminate all phosphate from the heptose region of the LPS inner core, resulting in a strain which is hypersensitive to detergents and hydrophobic antibiotics. Although mutation of \textit{waaY} does reduce the amount of core phosphate, this reduction is not sufficiently serious to cause hypersensitivity to such compounds (4).

Mutants of \textit{E. coli} and \textit{S. enterica} with highly truncated core oligosaccharides, such that they lack the inner core heptose residues that serve as the sites for phosphorylation (see Fig. 1), exhibit a pleiotropic phenotype called "deep-rough". Characteristics of the deep-rough phenotype include 1) hypersensitivity to detergents and hydrophobic antibiotics, 2) the appearance of phospholipid bilayer patches in the outer membrane, 3) leakage of periplasmic proteins into the culture medium, and 4) a marked decrease in the protein content of the outer membrane (reviewed in 7, 8). It has also been shown that the LPS from deep-rough mutants cannot support the proper folding of some outer membrane proteins (9). It was thought originally that all these characteristics could be explained simply by loss of core phosphoryl substituents. However, this was recently shown to be somewhat of an oversimplification, at least in the case of the outer membrane protein defect; a defined \textit{waaP} mutant was shown to have wild-type levels of outer membrane proteins despite a complete lack of core phosphate (10). So while the loss of core phosphate undoubtedly plays an important role in the manifestation of the deep-rough phenotype, clearly other factors must also be involved.

The importance of LPS core phosphorylation and of WaaP activity in particular extend beyond the obvious membrane defects. For example, mutation of
waaP in *S. enterica* serovar Typhimurium has been shown to cause a complete loss of virulence in mouse infection models (5). In addition, WaaP activity is intuitively a prerequisite for the functioning of the currently unidentified enzyme responsible for 2-aminoethyl phosphate (PEtN) modification of the *E. coli* and *S. enterica* inner core heptose region (see Fig. 1). PEtN modification of the LPS inner core is correlated with resistance to polymyxin in these bacteria (11, 12), and may provide a means by which these pathogens can modulate their surface charge. Inhibition of WaaP would thus hinder the ability of these bacteria to adapt their surfaces to particular microenvironments during pathogenesis.

The predicted WaaP protein shares limited similarity with eukaryotic protein kinases (4), but kinase activity has never been demonstrated. Here, we report the purification of a His$_6$-tagged derivative of WaaP, and the development of an *in vitro* assay that demonstrates unequivocally that WaaP is an LPS core heptose kinase. We also give the first characterization of the catalytic properties of the WaaP enzyme, and provide site-directed mutagenesis data which suggest that the enzyme's mechanism of action is similar to that of eukaryotic protein kinases.
EXPERIMENTAL PROCEDURES

Materials and Bacterial Strains - Materials and kits were purchased from the following suppliers: PCR primers (Guelph Molecular Supercentre, University of Guelph); restriction enzymes (New England Biolabs and Boehringer Mannheim); QIAprep Spin Miniprep Kit and QIAquick PCR Purification Kit (Qiagen); antibiotics (Sigma); nickel-nitrilotriacetic acid agarose (Ni-NTA agarose) (Qiagen); PD-10 desalting columns (Amersham Pharmacia); \([\gamma-33P]ATP\) (3000 Ci/mmol) (NEN Life Science Products) and other assay reagents (Tris, MgCl2, Triton X-100, dithiothreitol) (Sigma); EcoLite scintillation fluid (ICN). LPS acceptor for the WaaP assay was purified from defined \(waaP\) mutant strain CWG296 (4) by hot phenol/water extraction of cells (13). \(E. coli\) strain BL21(DE3) (\(F^-ompT\ gal\ [dcm]\ [lon]\ hsdS\_\_\_ (rB\_\_\_mB\_\_\_)

\(\lambda\)DE3) is from Novagen, and \(E. coli\) strain DH5\(\alpha\) (\(\supE\44\Delta lac\U169\ (\phi80lac\Delta M15)\ hsdR\17\ recA1\ endA1\ gyrA96\ thi-1\ relA1\) is from Life Technologies, Inc.

Cloning and Expression of \(waaP\) - Oligonucleotide primers for the amplification of \(waaP\) from the \(E. coli\) F470 chromosome (R1 core prototype strain, see reference 14) were designed to introduce appropriate restriction sites for cloning. The forward primer (5’-\(\underline{TGTTGATc}AAATAGTGCCACTCA\_3’\)) introduced a \(BamHI\) site (underlined) 32 base pairs downstream of the \(waaP\) stop codon, and the reverse primer (5’-\(\underline{GGTGATC}catATGGTGAACTTAA\_3’\)) introduced an \(NdeI\) site (underlined) overlapping the \(waaP\) ATG start codon (bases in lower case indicate mismatches between the primer and chromosomal sequences). PCR amplification was performed with \(PwoI\) DNA polymerase (Boehringer Mannheim), used as recommended by the manufacturer. The coding region for \(waaP\) was subsequently
isolated as a *Bam*HI-*Nde*I fragment, then cloned between the *Bam*HI and *Nde*I sites of pET-28a(+) (Novagen), generating plasmid pWQ910, and sequenced to ensure error-free amplification. Plasmid pWQ910 introduces an N-terminal His$_6$-tag onto the WaaP protein and provides high-level, IPTG-inducible expression from the T7 promoter.

**Cloning and Expression of the Chaperones encoded by groES and groEL** - The GroES and GroEL chaperones of *E. coli* were overexpressed simultaneously with the recombinant His$_6$-WaaP in order to increase the yield of soluble WaaP protein (based on the work of 15). The operon containing *groES* and *groEL* was PCR-amplified from the *E. coli* W3110 chromosome (16). Again, primers were designed to introduce appropriate restriction sites for cloning; the forward primer (5′-GGAGAGTTAcatATGAATATTG-3′) introduced an *Nde*I site (underlined) overlapping the *groES* start codon, and the complementary reverse primer (5′-AGGTGCAGGAAgcTTACATCATG-3′) introduced a HindIII site (underlined) overlapping the *groEL* stop codon (bases shown in lower case indicate mismatches between the primer and chromosomal sequences). The coding region for *groESL* was subsequently isolated as an *Nde*I-HindIII fragment, and cloned between the *Nde*I and HindIII sites of pET-30a(+) (Novagen). The *groESL* coding region was then excised from the pET-30a(+) derivative as an *Xba*I-HindIII fragment (along with the optimally positioned ribosome binding site), and cloned between the *Xba*I and HindIII sites of pBAD33 (17). The resulting plasmid, pWQ911, allows for high-level chaperone expression from the arabinose-inducible P$_{BAD}$ promoter.
Growth Conditions and Preparation of Cell-free Extracts - Luria-Bertani broth containing chloramphenicol (30 µg/ml) and kanamycin (50 µg/ml) was inoculated (1:20) from an overnight culture of BL21(DE3)pWQ910/pWQ911, and grown to an OD$_{600}$ ≅ 0.8 at 37°C with shaking at 200 rpm. L-arabinose (0.02%) was then added to induce GroES and GroEL expression, and cells were grown for an additional 45 min, at which point IPTG (0.5 mM) was added to induce expression of WaaP. After 4.5 h, cells were harvested by centrifugation (15 min, 5000 $\times$ g, 4°C), then frozen and stored overnight at -20°C. The cell pellet was then thawed on ice, and resuspended in a minimal volume (4 ml/g wet weight) of lysis buffer (50 mM sodium phosphate, pH 8, 350 mM NaCl, 10 mM imidazole, 2% Triton X-100, 10 mM $\beta$-mercaptoethanol). Lysozyme (1 mg/ml) was added to initiate lysis, and both DNase I (5 µg/ml) and RNase A (10 µg/ml) were added to reduce viscosity. After 45 min, the sample was sonicated on ice ($6 \times 10$ sec bursts, with 20 sec cooling periods) to ensure complete lysis, then centrifuged (30 min, 25000 $\times$ g, 4°C) to remove cellular debris.

Immobilized Metal-Affinity Chromatography - One ml of Ni-NTA agarose (50% slurry, Qiagen) was added to every 6 ml of cleared lysate, and batch binding was allowed to proceed for 1 h (4°C, with shaking at 200 rpm). The lysate/Ni-NTA agarose mixture was loaded into a disposable plastic column (5 ml) and, once settled, the cleared lysate was allowed to flow through. The column was washed with $3 \times 5$ ml of wash buffer (50 mM sodium phosphate, pH 8, 350 mM NaCl, 30 mM imidazole, 0.5% Triton X-100), then the His$_6$-tagged WaaP was eluted in $4 \times 0.5$ ml of elution buffer (50 mM sodium phosphate, pH 8, 250 mM NaCl, 200 mM imidazole,
0.5% Triton X-100). The second 0.5 ml elution typically contained the highest concentration of WaaP.

**High Performance Anion Exchange Chromatography** - The enzyme eluted from the Ni-NTA agarose column was further purified by using subtractive anion exchange chromatography to bind the remaining contaminating proteins. Protein eluted from the Ni-NTA agarose column was first exchanged into a lower ionic strength loading buffer (25 mM sodium phosphate, pH 8, 150 mM NaCl, 0.5% Triton X-100) using PD-10 columns (containing Sephadex G-25) as recommended by the manufacturer (Amersham Pharmacia). The sample was then applied (0.8 ml/min) to a Beckman Q HyperD 20 column (equilibrated in 40 mM sodium phosphate, pH 8, 50 mM NaCl), and the WaaP-containing flow-through was collected in 0.5 ml fractions.

**Assay Conditions and Analysis** - Unless otherwise indicated, reaction mixtures contained 50 mM Tris, pH 8.5, 17.5 mM MgCl$_2$, 0.5% Triton, 0.5 mM dithiothreitol, 1 µCi [$\gamma$-$^{33}$P]ATP diluted to 1 µM with cold ATP, and 1 mM LPS. The enzyme source was added last to initiate the reaction, and comprised 10% of the reaction volume. Reactions were incubated at 35°C for 30 min, then stopped by the addition of an equal volume of stop solution (1 M acetic acid, 0.1 M sodium phosphate monobasic, 25 mM MgCl$_2$). LPS was collected in a pellet by centrifugation (15 min, 14000 rpm), and washed three times in stop solution to remove unincorporated radiolabel. (For each wash, the LPS pellet was completely resuspended by vortexing for about 5 min.) After the third wash, the LPS pellet was resuspended in water, and the incorporated radiolabel was quantified in EcoLite scintillation fluid (ICN) using a Packard TRI-CARB Liquid Scintillation Analyzer (Model 2000CA).
Analysis of Reaction Products by Gel Filtration Chromatography - Radiolabelled LPS was pooled from 10 reaction mixtures, and resuspended in 250 µL of 2% acetic acid. The LPS/acetic acid suspension was then incubated at 100°C for 90 min in order to cleave the acid-labile ketosidic linkages involving the anomeric carbon of Kdo (i.e. between KdoI and the non-reducing GlcN of lipid A, and between KdoII and KdoI). Insoluble lipid A was removed by centrifugation (15 min, 14 000 rpm). The water-soluble core oligosaccharides were then applied to a column of Sephadex G-25 (1 × 40 cm) with water as eluent. The column void volume and included volume were determined using Blue Dextran and KCl, respectively. Eluted fractions (0.85 ml) were collected and analyzed for radioactivity by liquid scintillation counting (described above).

Site-Directed Mutagenesis - The gene encoding the His₆-tagged derivative of WaaP (together with its ribosome binding site) was excised from the WaaP expression plasmid pWQ910 (described above) as an XbaI-HindIII fragment, and cloned between the XbaI and HindIII sites of pBAD18 (17). The resulting plasmid, pWQ912, was used as the template for PCR-based site-directed mutagenesis. It was necessary to move waaP out of plasmid pWQ910 for these experiments to allow for regulated expression of WaaP and its mutated versions in strain CWG296 (waaP) (4), which is not lysogenic for λDE3 expressing the T7 RNA polymerase. Complementary oligonucleotide primers were designed containing the desired mutation (resulting in the replacement of Asp 162 with Ala in WaaP): 5′ -

CAGATATAACAGGCACGTCCTGTTAATGCC - 3′; 5′ -
GGGCATTACACCCGCGTCTGTTATATCTG - 3′ (the underlined bases indicate the
mismatch between primer and template sequences.) PCR amplification of the entire pWQ912 plasmid was performed with PwoI DNA polymerase (Boehringer Mannheim) as follows: 1) one cycle at 95°C for 2 min; 2) 16 cycles at 95°C for 30 sec, 55°C for 1 min, and 68°C for 11 min. The amplified plasmid was purified using the QIAquick PCR Purification Kit (Qiagen), then digested with DpnI (specific for the methylated parental DNA template, thus selecting for plasmid containing the mutation.) The DpnI-digested sample was transformed into electrocompetent E. coli DH5α cells, and plated on LB-agar plates containing ampicillin (100 µg/ml). Plasmid purified from the resulting colonies was sequenced to ensure only the desired mutation had been introduced into the waaP coding region.

**In vivo Complementation** - The plasmids encoding the parental and mutated (D162A) versions of WaaP (pWQ910 and pWQ913, respectively) were transformed into CWG296 (waaP) by electroporation, and WaaP expression was induced by the addition of 0.02% L-arabinose. To determine the ability of WaaP (D162A) to complement the antibiotic supersensitivity of the waaP mutation in CWG296, SDS and novobiocin sensitivity testing were performed as described previously (4).
RESULTS

Expression of WaaP - The addition of an N-terminal His$_{6}$-tag onto WaaP was shown to have no effect on its ability to complement the CWG296 waaP mutant phenotype (data not shown), thus validating the use of the His$_{6}$-tag for purification. Expression of the His$_{6}$-tagged WaaP derivative from plasmid pWQ910 resulted in a significant amount of protein, as seen by the appearance of an intense band located at ~33 kDa in Coomassie Blue-stained SDS-PAGE gels (Fig. 2). The predicted molecular weight of WaaP is 31049 kDa, and of His$_{6}$-tagged WaaP is 33212 kDa, a difference which could be observed by SDS-PAGE (data not shown). The identity of the overexpressed protein was further confirmed by Western immunoblotting with monoclonal antibodies specific for the His$_{6}$-tag (data not shown). Reactivity with the His$_{6}$-tag-specific monoclonal also demonstrated that the N-terminus of the protein was not processed post translation, consistent with the prediction from the waaP sequence.

Unfortunately, when the WaaP-expressing cells were lysed and fractionated by differential centrifugation (into insoluble matter, membranes, and cytosol), the recombinant protein was found almost exclusively in insoluble inclusion bodies.

Many standard variables were tested to increase the yield of soluble WaaP (e.g. growth medium, growth temperature, time of induction, duration of induction, inducer concentration, removal of the His$_{6}$-tag, expression from different promoters, and compatible solute accumulation), however, none of these treatments provided substantial improvement in the yield of soluble protein. We therefore created a plasmid which would allow for the co-expression of the E. coli GroES and GroEL chaperones (based on the work of 15), with the hope that this
would increase the yield of soluble WaaP. Since the WaaP expression plasmid pWQ910 had a kanamycin resistance marker and a pBR origin of replication, we based the chaperone expression plasmid on pBAD33, which has a chloramphenicol resistance marker and a compatible origin of replication from pACYC (17). Not only can these two plasmids be maintained simultaneously in a bacterial cell, but expression from each is controlled independently (expression from pBAD plasmids requires L-arabinose induction, while expression from pET plasmids requires IPTG induction). The ability to express chaperones independently of WaaP allowed for accumulation of chaperones prior to the induction of WaaP. Using this co-expression system, it was possible to achieve sufficient soluble protein expression to allow for the purification of the protein under native conditions, although the majority of protein still remained insoluble (Fig. 2). Subcellular fractionation also indicated a tendency for the recombinant His$_6$-tagged WaaP to associate with membranes, so Triton X-100 was added to the lysis buffer to help extract any membrane-associated WaaP and achieve the highest possible yield of soluble protein. The association of recombinant WaaP with membranes was not unexpected; indeed the enzymes involved in LPS core assembly are predicted to function as peripheral membrane proteins at the cytoplasmic face of the inner membrane (8) where they have access to both their cytoplasmic substrates (sugar nucleotides or ATP) and their lipid acceptor molecules.

**Purification of WaaP** - The incorporation of an N-terminal His$_6$-tag onto the recombinant WaaP protein allowed for the use of immobilized metal-affinity chromatography as an initial purification step (Fig. 2). Given that the predicted pI of
the WaaP protein is very high (approximately 9.8), the next logical step was to use
cation-exchange chromatography. However, the protein tended to precipitate rapidly
during the buffer exchange required prior to loading on the cation-exchange
column. To circumvent this problem, the enzyme was further purified by using
subtractive anion exchange chromatography to bind the remaining contaminating
proteins, allowing WaaP to be collected in the flow-through (Fig. 2). In this way, the
protein could be kept in a higher ionic strength buffer to minimize the precipitation
problem. The purity of the protein collected in the flow-through from the anion-
exchange column was over 95% as judged by SDS-PAGE (Fig. 2). After this final step,
the specific activity of the His<sub>6</sub>-tagged WaaP was 25.8-fold higher than the crude
extract, with a total activity yield of 0.53% (Table I).

Of note, the presence of 10 mM β-mercaptoethanol in the lysis buffer (see
Experimental Procedures) was found to greatly increase the activity of the purified
protein (greater than 10-fold, data not shown). Further, the purified enzyme could
be stored in 50% glycerol at -20°C, without significant loss of activity for over 30 days.

Effect of Mg<sup>2+</sup> Concentration and pH on Kinase Activity - The kinase activity of
purified WaaP was measured using a range of Mg<sup>2+</sup> concentrations from 0 to 25 mM,
as shown in Fig. 3A. The enzyme displays an absolute requirement for Mg<sup>2+</sup>, which
cannot be substituted with Ca<sup>2+</sup> (data not shown). Maximal kinase activity was
observed at 17.5 mM MgCl<sub>2</sub>. The activity of WaaP was then tested over a pH range
from 4.5 to 9.0, as shown in Fig. 3B. The kinase was shown to be inactive at pH 4.5,
with maximal activity between pH 8.0 and 9.0.
**Kinetic Properties of Purified WaaP** - The activity of WaaP was shown to be linearly dependent on both time (from 0 to 30 min) and protein concentration (from 0-200 µg/mL) (data not shown). When the concentration of ATP in the reactions was held constant at 2.5 mM and the concentration of LPS was varied (Fig. 4A), the $K_m$ for the LPS acceptor was calculated to be 76 µM. Likewise, when the concentration of LPS acceptor was held constant at 1 mM and the concentration of ATP was varied (Fig. 4B), the $K_m$ for ATP was determined to be 0.13 mM. In both cases, the apparent $V_{max}$ for the reaction was ~ 3.7 nmol/min/mg.

**Characterization of the Reaction Products by Gel Filtration Chromatography** - When the acceptor LPS purified from our defined *waaP* mutant strain, CWG296, is run on an SDS-PAGE gel and stained with silver, two distinct molecular weight species are observed (see inset Fig. 5). The structure of the larger of these two species is known to consist of complete lipid A-core lacking the usual modifications (P, PEtN, and HepIII) on the heptose region of the core (see Fig. 1) (4). The smaller molecular weight species is a truncated form of the larger, terminated after GlcI (see Fig. 1) as determined by co-migration with LPS from a defined *waaO* mutant on SDS-PAGE (data not shown). To determine whether WaaP was capable of phosphorylating both forms of LPS from the *waaP* mutant strain, the LPS reaction products were first hydrolyzed in mild acid to remove the insoluble lipid A portion of the molecule. (Treatment with 2% acetic acid at 100°C for 2 h cleaves the acid-labile ketosidic linkages involving the anomeric carbon of Kdo: between KdoI and the non-reducing GlcN of lipid A, and between KdoII and KdoI.) After hydrolysis and centrifugation, approximately 95% of the radioactivity was recovered in the
supernatant as soluble core oligosaccharides (data not shown). The soluble core oligosaccharides were then separated on a column of Sephadex G-25 (Fig. 5). Two distinct peaks of radioactivity were observed within the fractionation range of the column ($M_r$ of 100-5000 for dextrans), indicating that WaaP is indeed capable of phosphorylating both the larger and smaller molecular weight LPSs isolated from the $waaP$ mutant strain (Fig. 5). The very small third peak which occurs at the column included volume likely corresponds to a small amount of $^{33}$P being hydrolyzed from the reaction products during the acid hydrolysis.

**Site-Directed Mutagenesis** – Alignment of the predicted WaaP proteins from *E. coli* and *S. enterica* shows greater than 80% identity (90% similarity), while the *Pseudomonas aeruginosa* WaaP homolog is approximately 55% identical (70% similar) to those of *E. coli* and *S. enterica* (data not shown). Of particular note, residues 159 to 171 (NHRDCYICHFLH) and 184 to 192 (SVIDLHRAQ) (*E. coli* residue numbers) are absolutely conserved among all of the WaaP homologs. On the other hand, alignment of the *E. coli* WaaP protein with eukaryotic protein kinases typically shows only about 10-15% identity (25-30% similarity) (Fig. 6). It is interesting, however, that the majority of conserved amino acids in Fig. 6 coincide with residues that are known in eukaryotic protein kinases to be important for catalysis, based on both sequence similarity (18) and crystal structure data (19,20). To test the biological significance of these sequence similarities, we engineered a nucleic acid change into the $waaP$ open reading frame in plasmid pWQ912 that resulted in the replacement of Asp 162 with Ala (plasmid pWQ913). We chose Asp 162 specifically because it is absolutely conserved among all the known WaaP homologs...
(within the sequence NHRDCYICHFLH, see above), and because sequence similarity predicts that it functions as the WaaP catalytic base. In this role, the WaaP Asp 162 residue would accept a proton from the 4-OH of HepI, thus leaving an oxyanion capable of nucleophilic attack at the γ-phosphate of ATP. Recent data have questioned the proposed general-base role of this residue in eukaryotic protein kinases (21), but there is no doubt that it is essential for catalysis. The replacement of Asp 162 with Ala would thus be expected to completely abrogate WaaP activity. When plasmid pWQ913 encoding WaaP (D162A) was introduced into our defined waaP mutant strain, CWG296, it was unable to complement the mutant's antibiotic and detergent supersensitivity (data not shown). Further, purified WaaP (D162A) showed negligible kinase activity in our in vitro assay system (data not shown), clearly indicating that Asp 162 is an essential residue.
DISCUSSION

Phosphorylation of the LPS core region is essential for outer membrane stability in *E. coli* and *S. enterica*, and has been studied over the course of many years (reviewed in 7, 8, 22). Indeed, the activity of an LPS core phosphorylating enzyme was reported as early as 1969, using acceptor LPS from an undefined phosphate-deficient mutant of *S. enterica* and crude lysates from wild-type bacteria as the enzyme source (23). Unfortunately, the kinase implied by these early studies was never actually identified, nor was it confirmed that ATP was the direct donor of phosphate. More recently, a single gene called *rfaP* (now *waaP*) was implicated in all the phosphoryl modifications of the *E. coli* LPS core (24). However, the conclusions from these studies were necessarily limited by their reliance on strains with polar mutations and also in hindsight by the strict sequential action of the two core kinases. The direct involvement of the *waaP* gene product in the phosphorylation of the *E. coli* LPS core at HepI was only recently established with confidence, based on the LPS core structures resulting from defined *waaP*, *waaQ*, and *waaY* mutations (4). Since then, mutation of *waaP* in *S. enterica* was shown to result in the same LPS defect (5), and the homolog of WaaP in *P. aeruginosa* was shown to complement a *waaP* defect in *S. enterica* (6).

The data presented here provide the first biochemical characterization of WaaP activity, and prove unequivocally that WaaP is indeed an LPS core heptose kinase. Purified protein was used to determine the optimal assay conditions and catalytic properties of WaaP. Optimal activity was observed between pH 8.0 and 9.0, and like most kinases, the enzyme displayed an absolute requirement for Mg$^{2+}$. The
apparent $K_m$ values for the WaaP-catalyzed reaction were calculated to be 0.13 mM for ATP, and 76 µM for LPS (determined at saturating concentrations of the second substrate). In this study, the site of WaaP phosphorylation was only localized to the soluble core oligosaccharide portion of the LPS that was released by mild acid hydrolysis. However, given the known structure of the *E. coli* core (25, 26) and the core structures resulting from defined *waaY* and *waaP* mutations (loss of phosphate on HepII and loss of phosphate on both HepII and HepI, respectively) (4), it is possible to conclude that WaaP is in fact a heptose-specific LPS core kinase, and is responsible for phosphorylation at HepI. Further, the WaaP enzyme is known to catalyze the addition of phosphate to only HepI (and not HepII), since LPS purified from a defined *waaY* mutant strain could not serve as an acceptor for the WaaP enzyme (data not shown).

These data thus help to clarify the sequence of events in core biosynthesis that result in the observed heterogeneity of phosphoryl modifications on HepI (see Fig. 1). It was previously speculated that all LPS molecules might initially be modified by the addition of PPEtN to HepI, with a subset of these molecules subsequently undergoing cleavage to liberate PEtN and leave just P (8). Now however, with the assignment of WaaP as the HepI kinase, the more likely scenario is clearly that WaaP catalyzes the transfer of P to HepI with a subset of molecules being further substituted at this P residue with PEtN by a currently unidentified enzyme (see Fig. 1). The determination of the gene responsible for this PEtN modification is of particular interest, given the potential role of PEtN core modification in resistance to polymyxin and modulation of surface charge (11, 12).
Given the importance of WaaP to membrane stability (4) and its requirement for virulence in vivo (5), inhibitors to the WaaP-catalyzed reaction could potentially function as novel antimicrobial compounds. As a starting point for the design of inhibitors, it would be useful to know the minimal structure that is capable of being phosphorylated by WaaP. The finding that WaaP is capable of phosphorylating both the high and low molecular weight LPS species isolated from a \textit{waaP} mutant strain (Fig. 6) helps to better define this minimal acceptor; apparently the outer core glycoses distal to GlcI are not strictly required (see Fig. 1). This finding is supported by the recent report that LPS isolated from a defined \textit{waaG} mutant strain (truncated after HepII, see Fig. 1) carries the phosphate modification on HepI, albeit at only 40 percent of wild-type levels (10). Interestingly, the HepII phosphate is completely absent in the \textit{waaG} mutant LPS (see Fig. 1), indicating that the putative kinase encoded by \textit{waaY} requires the presence of GlcI as part of its acceptor substrate (10).

Finally, it is noteworthy that WaaP was never assigned putative kinase function based on sequence homology alone, even though the sequence of the \textit{E. coli} \textit{waaP} gene has been known for some time (27), and homologs have since been identified in \textit{S. enterica} (28) and \textit{P. aeruginosa} (6). The complicating factor has always been that the primary sequences of these predicted proteins bear such limited semblance to characterized kinases that similarities cannot be detected with typical BLAST searches (29). However, with the advent of the PSI-BLAST search tool (30) it was observed that WaaP did show homology to kinases when considering only key residues throughout the length of the entire protein (4). We tested the biological significance of one of these key similarities by specifically mutating the WaaP Asp
162 residue, predicted by homology to function as the enzyme’s catalytic base. As expected for mutation of a residue essential to catalysis, the WaaP (D162A) protein showed no activity *in vivo* or *in vitro*. It is suggested therefore, given this conservation of catalytic residues, that WaaP may have a similar mechanism of action to other eukaryotic protein kinases. We are currently pursuing structural studies to garner further evidence in support of this hypothesis. Interestingly, the crystal structure of the APH(3')-IIIa aminoglycoside kinase from *Enterococcus*, which catalyzes the phosphorylation of a broad spectrum of aminoglycoside antibiotics, was recently solved (31). Like WaaP, the APH(3')-IIIa protein displays virtually no sequence similarity to eukaryotic kinases except at key residues, such as the conserved Asp mutated in this study. Yet despite this lack of sequence homology, the APH(3')-IIIa three-dimensional structure is strikingly similar to that of eukaryotic protein kinases (31).
ACKNOWLEDGMENTS

This work was supported in part through funding to C.W. by the Canadian Bacterial Diseases Network (Network of Centres of Excellence). J.A.Y. is the recipient of a Doctoral Research Award from the Medical Research Council of Canada. C.W. is a Medical Research Council of Canada Senior Scientist.
FOOTNOTES

1 The abbreviations used are the following: LPS, lipopolysaccharide; P, phosphate; PEtN, 2-aminoethyl phosphate; GlcN, D-glucosamine; Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid; Hep, L-glycero-D-manno-heptose; Glc, D-glucose; Gal, D-galactose; IPTG, isopropyl-1-thio-β-D-galactopyranoside; Ni-NTA agarose, nickel-nitrilotriacetic acid agarose; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; ATP, adenosine triphosphate; cAMP, cyclic adenosine monophosphate.
FIGURE LEGENDS

FIG. 1. **Structure of the LPS from *E. coli* strains with an R1-type core.** Core residues are designated by sugar abbreviation and number to facilitate identification. Abbreviations are as follows: GlcN, D-glucosamine; P, phosphate; Kdo, 3-deoxy-D-manno-oct-2-ulosonic acid; Hep, L-glycero-D-manno-heptose; PEtN, 2-aminoethyl phosphate; Glc, D-glucose; Gal, D-galactose. All sugars are in the pyranose configuration and the linkages are α unless otherwise indicated. The assignment of function to genes encoding core glycosyltransferases and phosphotransferases has been reported previously (4,32-34). The predicted activity of the *waaP* gene product is indicated by an asterisk.

FIG. 2. **Coomassie Blue-stained SDS-PAGE gel of fractions generated during the purification of WaaP.** *Lane 1,* molecular weight standards; *lane 2,* pre-induced whole-cell lysate; *lane 3,* arabinose- and IPTG-induced whole-cell lysate; *lane 4,* insoluble material from cell lysate; *lane 5,* cleared lysate; *lane 6,* flow-through from Ni-NTA agarose affinity chromatography column; *lane 7,* protein eluted from Ni-NTA agarose affinity chromatography column; *lane 8,* flow-through from anion-exchange chromatography column.

FIG. 3. **Effect of Mg\(^{2+}\) concentration and pH on kinase activity.** *A,* WaaP activity was measured under standard assay conditions over a range of MgCl\(_2\) concentrations, as indicated. Mg\(^{2+}\) was absolutely required for activity, and optimal activity was observed at 17.5 mM MgCl\(_2\). *B,* The pH dependence of the reaction was demonstrated under standard assay conditions using 75 mM sodium acetate (pH 4.5, 5.0, 5.5), 75 mM sodium phosphate (pH 6.0, 6.5, 7.0, 7.5, 8.0), or 75 mM
diethanolamine-HCl (pH 8.0, 8.5, 9.0). The enzyme was inactive at pH 4.5, and optimally active between pH 8.0 and 9.0.

FIG. 4. **Kinetic properties of purified WaaP.** Standard assay conditions were used in these experiments, except that the substrate concentrations were varied as indicated. In A, the concentration of ATP was held constant at 2.5 mM and the concentration of LPS was varied. The apparent $K_m$ for the LPS acceptor was calculated to be 76 µM. In B, the concentration of LPS acceptor was held constant at 1 mM and the concentration of ATP was varied. The apparent $K_m$ for ATP was determined to be 0.13 mM. In both cases, the apparent $V_{max}$ for the reaction was ~ 3.7 nmol/min/mg.

Values were determined using EnzFitter v. 1.05 software (Biosoft), and lines were drawn using a non-linear least squares fitting to the following equation:

$$V = \frac{(V_{max} \times [S])}{(K_m + [S])}.$$

FIG. 5. **Separation of radiolabelled core oligosaccharides by chromatography on Sephadex G-25.** Oligosaccharides were generated by mild acid hydrolysis of the LPS reaction products from assays performed under standard conditions. The two peaks of radioactivity correspond to the core oligosaccharides from the two major LPS species that are evident in SDS-PAGE profiles of waaP mutant LPS (inset).

FIG. 6. **Alignment of WaaP from E. coli to cAMP-dependent protein kinase (cAPK) from Mus musculus (mouse).** Identical residues are indicated by single letter code, and similar residues are indicated by a "+". The WaaP and cAPK sequences show only 12% identity (23% similarity) over the length of the alignment, however the following list denotes potentially important similarities. 1) cAPK residues 50-55 contain the nucleotide-binding motif GXGXXG, of which Gly 52 (Gly 44 in WaaP) is
an invariant residue among eukaryotic protein kinases. 2) cAPK residue Lys 72 (Lys 58 in WaaP) is an invariant residue, that forms a salt bridge with the invariant residue Glu 91 (Glu 70 in WaaP). The cAPK Lys 72 residue is also involved in hydrogen bonding to the α and β phosphates of ATP. 3) cAPK residues Glu 121 and Glu 127 (Glu 114 and Glu 123 in WaaP) are involved in hydrogen bonding to adenosine. 4) cAPK residue Asp 166 (Asp 162 in WaaP) is an invariant residue that potentially functions as the enzyme's catalytic base, and is shown in this study to be essential for WaaP activity. 5) cAPK residue Asp 184 (Asp 187 in WaaP) is an invariant residue required to orient the γ-phosphate of ATP for efficient transfer to the substrate. The potential roles of conserved residues in cAPK were derived from crystal structure data (19, 20).
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| Sample           | Protein (mg/ml) | Total protein (mg) | Specific Activity (nmol/min/mg) | Total activity (nmol/min) | Purification (-fold) | Yield (%) |
|------------------|-----------------|--------------------|---------------------------------|---------------------------|----------------------|-----------|
| Cleared lysate   | 18.8            | 148                | 0.334                           | 49.5                      | 1                    | 100       |
| Ni-NTA           | 0.491           | 1.28               | 3.893                           | 4.97                      | 11.7                 | 10        |
| Anion-exchange   | 0.00395         | 0.0304             | 8.606                           | 0.261                     | 25.8                 | 0.53      |
Figure 1
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6
Purification and characterization of WaaP from Escherichia coli, a lipopolysaccharide kinase essential for outer membrane stability
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J. Biol. Chem. published online November 7, 2000

Access the most updated version of this article at doi: 10.1074/jbc.M008255200

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