

## Natural Selection in the Chicken Host Identifies 3-Deoxy-D-manno-Octulosonic Acid Kinase Residues Essential for Phosphorylation of *Pasteurella multocida* Lipopolysaccharide<sup>∇</sup>

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***Pasteurella multocida* is the causative agent of a number of diseases in animals, including fowl cholera. *P. multocida* strains simultaneously express two lipopolysaccharide (LPS) glycoforms (glycoforms A and B) that differ only in their inner core structure. Glycoform A contains a single 3-deoxy-D-manno-octulosonic acid (Kdo) residue that is phosphorylated by the Kdo kinase, KdkA, whereas glycoform B contains two unphosphorylated Kdo residues. We have previously shown that *P. multocida* mutants lacking the heptosyltransferase, HptA, produce full-length glycoform B LPS and a large amount of truncated glycoform A LPS, as they cannot add heptose to the glycoform A inner core. These *hptA* mutants were attenuated in chickens because the truncated LPS made them vulnerable to host defense mechanisms, including antimicrobial peptides. However, here we show that birds inoculated with high doses of the *hptA* mutant developed fowl cholera and the *P. multocida* isolates recovered from diseased birds no longer expressed truncated LPS. Sequencing analysis revealed that the *in vivo*-derived isolates had mutations in *kdkA*, thereby suppressing the production of glycoform A LPS. Interestingly, a number of the spontaneous KdkA mutant strains produced KdkA with a single amino acid substitution (A112V, R123P, H168Y, or D193N). LPS structural analysis showed that complementation of a *P. multocida* *kdkA* mutant with wild-type *kdkA* restored expression of glycoform A to wild-type levels, whereas complementation with any of the mutated *kdkA* genes did not. We conclude that in *P. multocida* KdkA, the amino acids A112, R123, H168, and D193 are critical for Kdo kinase function and therefore for glycoform A LPS assembly.**

*Pasteurella multocida* is a Gram-negative pathogen that is the causative agent of a wide range of diseases in animals, including bovine hemorrhagic septicemia, fowl cholera, and porcine atrophic rhinitis (4). The major virulence determinants in *P. multocida* include the capsule and lipopolysaccharide (LPS), both of which vary in composition and structure between strains (2, 5, 7–8, 11, 21–23). The LPS of *P. multocida* is composed of an inner and an outer core region, and like the LPS produced by species within the *Neisseria* and *Haemophilus* genera, *P. multocida* LPS lacks the polymeric O antigen that is attached to the distal end of the LPS structure in most other Gram-negative bacteria (6, 9, 17, 20, 23). Structural analysis of the LPS isolated from a number of *P. multocida* strains revealed that most simultaneously produce two LPS glycoforms that differ only in their inner core structure (23; A. Cox, unpublished observations). The key difference between the two structures is that the glycoform A inner core contains a single phosphorylated 3-deoxy-D-manno-octulosonic acid (Kdo) residue (lipid A-Kdo<sub>1</sub>-P), with the phosphate group frequently

being substituted with phosphoethanolamine (PEtn), whereas the glycoform B inner core contains two Kdo residues (lipid A-Kdo<sub>1</sub>-Kdo<sub>2</sub>) (22).

The enzymes required for assembly of the LPS inner core have been identified and include a Kdo transferase, KdtA, that is predicted to add both the first and the second (glycoform B only) Kdo residues; a Kdo kinase, KdkA, that adds the phosphate group to the lipid A-Kdo<sub>1</sub> for assembly of glycoform A; and two heptosyltransferases, HptA and HptB, specific for the addition of the first heptose to glycoform A and glycoform B, respectively (Fig. 1) (10). Previous data suggest that the amount of each glycoform produced is primarily dependent upon the phosphorylation activity of the glycoform A-specific Kdo kinase: if the lipid A-Kdo<sub>1</sub> acceptor molecule is phosphorylated by KdkA, the molecule is recognized by HptA and glycoform A is assembled, whereas if lipid A-Kdo<sub>1</sub> remains unphosphorylated, an additional Kdo is added by the Kdo transferase (KdtA) and glycoform B is assembled (Fig. 1) (10).

In previous studies with *P. multocida* strain VP161, we systematically inactivated each of the LPS transferase genes and the Kdo kinase gene (*kdkA*) and analyzed the effects of these mutations on the LPS structure and virulence in chickens (3, 10). In *kdkA* mutants, there is no phosphorylation of lipid A-Kdo<sub>1</sub>, so all lipid A-Kdo<sub>1</sub> acceptor molecules are used to

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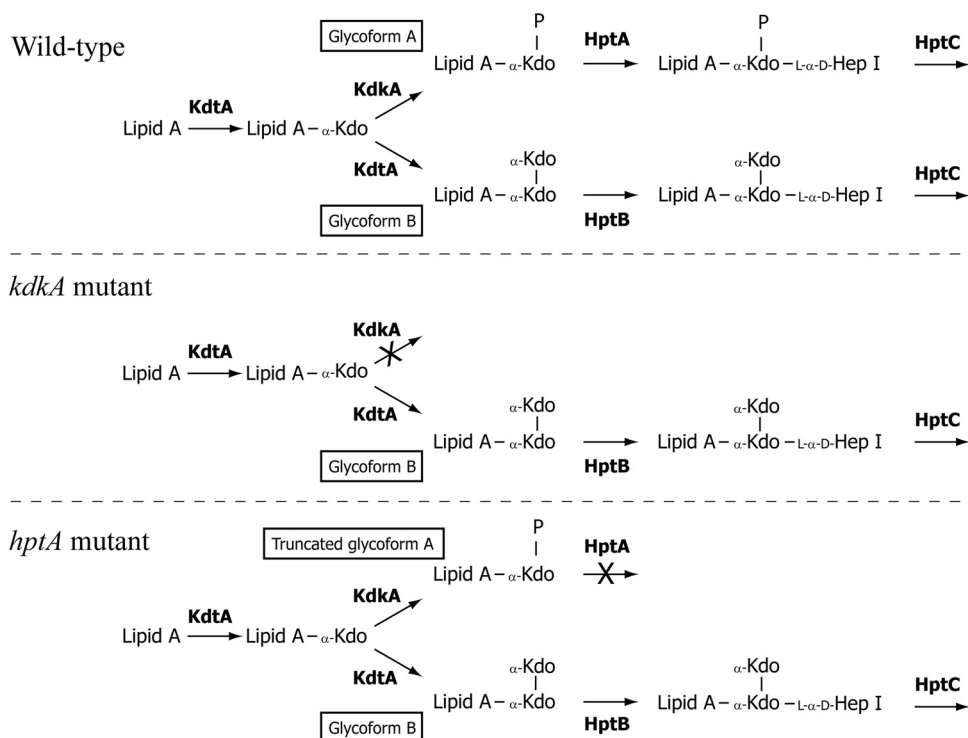


FIG. 1. Schematic representation of LPS inner core assembly in the *P. multocida* wild-type (VP161), *kdkA* mutant (AL721), and *hptA* mutant (AL836). Two LPS inner core structures, glycoforms A and B, are observed. The enzymes required for selected steps in the biosynthesis of each glycoform are shown in boldface. Residues are heptose (Hep), 3-deoxy-D-manno-octulosonate (Kdo), and phosphate (P).

produce full-length glycoform B (Fig. 1). These *kdkA* mutants are fully virulent in chickens infected by the intramuscular (i.m.) route (10). In contrast, *P. multocida hptA* mutants produce full-length glycoform B but lack the first heptosyltransferase (HptA) required for assembly of glycoform A beyond lipid A-Kdo<sub>1</sub>-P (Fig. 1) (10). These *hptA* mutants are fully attenuated in chickens due to the production of a large amount of truncated LPS, rendering them vulnerable to host defense mechanisms, such as antimicrobial peptides (10).

In the present study, we show that under *in vivo* selective pressure, avirulent *P. multocida hptA* mutants can spontaneously revert to full virulence by way of secondary *kdkA* suppressor mutations. These virulent isolates produce full-length glycoform B LPS and no longer produce any truncated LPS. Sequencing analysis of the mutated *kdkA* genes amplified from each of the recovered *in vivo hptA* mutants demonstrated that they all contained single nucleotide substitutions or deletions. Importantly, four of the mutated *kdkA* genes were intact, but each encoded a single amino acid substitution. Further analysis confirmed that each amino acid substitution resulted in the loss of Kdo kinase activity. This is the first report that defines the amino acids essential for bacterial Kdo kinase activity, a critical enzyme in LPS assembly.

#### MATERIALS AND METHODS

**Bacterial strains, plasmids, media, and growth conditions.** The bacterial strains and plasmids used in this study are listed in Table 1. *Escherichia coli* was grown routinely in Luria-Bertani broth. *P. multocida* strains were grown in brain heart infusion (BHI) broth. Solid media were obtained by the addition of 1.5%

agar. When required, the media were supplemented with spectinomycin (100  $\mu$ g/ml), kanamycin (50  $\mu$ g/ml), or tetracycline (2.5  $\mu$ g/ml).

**DNA manipulations.** Restriction digests and ligations were performed according to the manufacturers' instructions using enzymes obtained from NEB (Ipswich, MA) or Roche Diagnostics (Mannheim, Germany). Plasmid DNA was prepared using Qiagen plasmid minikits (Hamburg, Germany), while genomic DNA was prepared using the cetyltrimethylammonium bromide (CTAB) method (1). PCR amplification of DNA was performed using *Taq* DNA polymerase or an Expand high-fidelity PCR system (Roche Diagnostics) and purified using a QIAquick PCR purification kit from Qiagen GmbH (Hamburg, Germany). The oligonucleotides used in this study are listed in Table 1. Sequencing reactions were performed using the Applied Biosystems Prism BigDye Terminator mix, version 3.1. Electropherograms were generated on an Applied Biosystems 3730S genetic analyzer and were analyzed using the Vector NTI Advance, version 10, program (Invitrogen, Carlsbad, CA).

**In trans complementation of the *P. multocida kdkA* mutant.** We previously constructed *P. multocida kdkA* mutant AL721 and showed that complementation of this strain with the wild-type *kdkA* gene, encoded on plasmid pAL462, restored expression of glycoform A LPS (10). For the complementation experiments with the mutated *kdkA* genes, two independent plasmid constructs were generated for each mutation, as follows. The *kdkA* gene was amplified directly from each of the *P. multocida hptA kdkA* double mutants (Table 1), using oligonucleotides BAP3914 and BAP3915. Each amplified product was then ligated into *Sal*I- and *Bam*HI-digested pAL99 (Table 1), such that transcription of *kdkA* would be driven by the constitutive *P. multocida tpiA* promoter. The nucleotide sequence of each of the recombinant plasmids was determined to check the fidelity of the cloned gene and the *tpiA* promoter region, and then each plasmid was introduced into *P. multocida kdkA* mutant AL721, generating complemented strains AL1799 through AL1806 (Table 1). As a control, the pAL99 vector was introduced separately into the *kdkA* mutant, generating strain AL839 (Table 1).

**Structural analyses of LPS.** LPS was isolated from plate-grown cells as described previously (3, 10). Sugars were identified as their alditol acetate derivatives and linkage analysis determined following methylation analysis by gas-liquid chromatography-mass spectrometry (MS), as described previously (22). Capil-

TABLE 1. Bacterial strains, plasmids, and primers used in this study

Strain, plasmid, or primer	Relevant description	Source or reference
<b>Strains</b>		
<i>E. coli</i> DH5 $\alpha$	<i>deoR endA1 gyrA96 hsdR17</i> (r $\kappa$ <sup>-</sup> m $\kappa$ <sup>+</sup> ) <i>recA1 relA1 supE44 thi-1</i> ( <i>lacZYA-argFV169</i> ), $\phi$ 80 <i>dlacZ</i> AM15 F <sup>-</sup>	Bethesda Research Laboratories
<i>P. multocida</i>		
VP161	Serotype A:1, Vietnamese isolate from chickens	27
AL435	VP161 carrying a Tn916 insertion in gene <i>pm1417</i> , fully virulent	10
AL721	<i>kdkA</i> mutant of AL435, Spec <sup>r</sup>	10
AL836	Nonpolar <i>hptA</i> mutant of AL435, Spec <sup>r</sup>	10
AL839	AL721 containing pAL99, Spec <sup>r</sup> Kan <sup>r</sup>	10
AL840, AL1808	AL721 containing pAL462, Spec <sup>r</sup> Kan <sup>r</sup>	10
AL1774, AL1775	Spontaneous A112V <i>kdkA</i> mutants of AL836, Spec <sup>r</sup>	This study
AL1777, AL1778	Spontaneous R123P <i>kdkA</i> mutants of AL836, Spec <sup>r</sup>	This study
AL1780, AL1781	Spontaneous D193N <i>kdkA</i> mutants of AL836, Spec <sup>r</sup>	This study
AL1782, AL1783	Spontaneous H168Y <i>kdkA</i> mutants of AL836, Spec <sup>r</sup>	This study
AL1799	AL721 containing pAL856, Spec <sup>r</sup> Kan <sup>r</sup>	This study
AL1800	AL721 containing pAL857, Spec <sup>r</sup> Kan <sup>r</sup>	This study
AL1801	AL721 containing pAL858, Spec <sup>r</sup> Kan <sup>r</sup>	This study
AL1802	AL721 containing pAL860, Spec <sup>r</sup> Kan <sup>r</sup>	This study
AL1803	AL721 containing pAL861, Spec <sup>r</sup> Kan <sup>r</sup>	This study
AL1804	AL721 containing pAL862, Spec <sup>r</sup> Kan <sup>r</sup>	This study
AL1805	AL721 containing pAL863, Spec <sup>r</sup> Kan <sup>r</sup>	This study
AL1806	AL721 containing pAL864, Spec <sup>r</sup> Kan <sup>r</sup>	This study
<b>Plasmids</b>		
pAL99	<i>P. multocida</i> expression plasmid (Kan <sup>r</sup> ), constitutive <i>tpiA</i> promoter upstream of cloning site	11
pAL462	Wild-type <i>kdkA</i> , cloned into pAL99	10
pAL856, pAL857	<i>kdkA</i> A112V, cloned into pAL99, independent clonings	This study
pAL858, pAL860	<i>kdkA</i> R123P, cloned into pAL99, independent clonings	This study
pAL861, pAL862	<i>kdkA</i> H168Y, cloned into pAL99, independent clonings	This study
pAL863, pAL864	<i>kdkA</i> D193N, cloned into pAL99, independent clonings	This study
<b>Primers</b>		
BAP2782	GCCCTACACAAATTGGGAGA, pUA826-specific primer	10
BAP3327	GCACGCCTGCTGTGCTTTAG, reverse primer in <i>kdkA</i> gene	This study
BAP3914	AAAGGGATCCGTGGCCATTGCTATCTCTG, forward primer flanking <i>kdkA</i> for expression, contains BamHI site	10
BAP3915	TATGTGTCGACCTTATGCTTGATATCCCGC, reverse primer flanking <i>kdkA</i> for expression, contains Sall site	10
BAP3966	CACACAGGATCCATTGGACGAGATAATGATGCCG, forward primer flanking <i>hptA</i> , contains BamHI site	10
BAP3967	ATAACAGTCGACGGTTCATCATTGAACGTC, reverse primer flanking <i>hptA</i> , contains Sall site	10

lary electrophoresis (CE)-electrospray (ES)-MS analysis was performed as described previously (22).

**Assessment of virulence.** Virulence trials with chickens were performed as described previously (10). To check the phenotypes of the strains recovered from infected chickens, muscle swab and blood samples were plated onto BHI agar and BHI agar containing spectinomycin. The genotypes of the recovered strains with respect to the *hptA* gene were assessed using two different PCR assays. To identify the *P. multocida hptA* mutant, a primer located outside *hptA* (primer BAP3327) was paired with a primer specific for the integrated plasmid located within *hptA* (primer BAP2782). The amplification of a 700-bp product indicated that the strain still contained an insertion within *hptA*. To identify *P. multocida* wild-type revertant strains, primers flanking the *hptA* gene (primers BAP3966 and BAP3967) were used. *P. multocida* wild-type revertant strains were identified by the PCR amplification of a 1,050-bp product, indicating the loss of the plasmid insertion within *hptA*. To determine the LPS phenotype of the recovered strains, proteinase K-treated whole-cell lysates of the bacteria recovered from each *in vivo* site were separated by SDS-PAGE and visualized by carbohydrate silver staining, as described previously (25).

**Bioinformatic analysis.** Amino acid sequence alignments were generated using COBAL, a constraint-based multiple-alignment tool that utilizes conserved domain and local sequence similarity information (18).

**Nucleotide sequence accession number.** The GenBank accession number for the nucleotide sequence of the *P. multocida* VP161 *kdkA* gene is HM448896.

## RESULTS

***P. multocida hptA* mutants isolated from the blood of chickens with fowl cholera express only full-length LPS.** We previously observed that birds injected i.m. with 70 CFU of the *P. multocida hptA* single-crossover insertion mutant AL836 displayed no signs of disease (10). To extend these data, a virulence trial was conducted with chickens to determine the 50% infectious dose of the *hptA* mutant. Three groups of seven birds each were injected i.m. with 70,  $7 \times 10^3$ , or  $7 \times 10^5$  CFU. As expected (10), there were no signs of disease in the birds injected with the lowest dose of the *hptA* mutant, but two birds injected with  $7 \times 10^3$  CFU and all birds injected with  $7 \times 10^5$  CFU developed acute fowl cholera. As the *hptA* mutant was known to be attenuated in chickens, we hypothesized that the causative agent of disease was a wild-type revertant strain that had lost the single-crossover insertion from within *hptA*. Indeed, we have reported in a previous study that under *in vivo* selective pressure, wild-type revertant strains can arise spon-

TABLE 2. Analysis of *in vivo*-derived *P. multocida* isolates<sup>a</sup>

Bird	Time of death (h) <sup>b</sup>	Muscle		Blood	
		% Spec <sup>c</sup>	No. positive for pUA826:: <i>hptA</i> /total no. tested <sup>d</sup>	% Spec <sup>c</sup>	No. positive for pUA826:: <i>hptA</i> /total no. tested <sup>d</sup>
18	28	50	3/3	40	10/10
21	37	100	3/3	100	5/5
25	27	100	3/3	100	3/3
53	>48	100	3/3	100	3/3
23	37	10	ND <sup>e</sup>	0	ND
24	33	20	ND	0	ND

<sup>a</sup> Spectinomycin-resistant colonies isolated from each tissue site were analyzed by PCR for the presence of pUA826 integrated into the *hptA* gene.

<sup>b</sup> All birds showing terminal signs of fowl cholera were euthanized, in accordance with animal ethics requirements.

<sup>c</sup> Percentage of spectinomycin-resistant *P. multocida* isolates in tissue sample.

<sup>d</sup> Number of spectinomycin-resistant colonies positive by PCR for pUA826 integrated into *hptA* gene.

<sup>e</sup> ND, not done.

taneously within a population of *P. multocida* single-crossover mutants and cause disease (3). Virulent, wild-type revertant strains are no longer spectinomycin resistant, as they have lost the single crossover insertion that encodes the spectinomycin resistance gene *aadA*. Therefore, to determine the phenotype of the *P. multocida* strain responsible for disease, blood and muscle (from the site of injection) tissue samples were collected at necropsy from six of the diseased birds and cultured on BHI agar and BHI agar with spectinomycin. In all birds, the *P. multocida* organism recovered from the site of injection was able to grow on BHI agar with spectinomycin, indicating the presence of the *hptA* mutant in the muscle tissue. Furthermore, spectinomycin-resistant *P. multocida* was recovered from the blood of four of the six diseased birds, suggesting that the *hptA* mutant was able to cause systemic disease. To determine if both mutant and wild-type revertant *P. multocida* isolates were present in the samples, the bacteria on the nonselective plates were patched onto selective (spectinomycin, 50 µg/ml) and nonselective plates (Table 2). Four of the six birds (birds 18, 21, 25, and 53) had the *hptA* mutant present in the blood and at the intramuscular injection site. In addition, bird 18 also had wild-type revertant *P. multocida* present at both sites. Although there was evidence that the *hptA* mutant was present at the injection site in birds 23 and 24, only wild-type revertant *P. multocida* was detected in the blood and these bacteria were not investigated further (Table 2). The spectinomycin-resistant *P. multocida* isolates from the blood of four birds (birds 18, 21, 25, and 53) were chosen for further study.

The *hptA* region of the genome in six spectinomycin-resistant isolates from each bird (three per site) was analyzed by PCR. The results confirmed that the spectinomycin-resistant *P. multocida* isolates recovered from the blood and muscle tissue of birds 18, 21, 25, and 53 had retained pUA826 integrated into the *hptA* gene (Table 2). We have previously shown that the heptosyltransferase HptA is essential for addition of heptose to the glycoform A inner core. *P. multocida hptA* mutants are fully attenuated, as inactivation of *hptA* results in a large amount of truncated glycoform A (lipid A-Kdo<sub>1</sub>-P) present on the surface of these mutants (Fig. 1) (10). To determine if the *hptA* mutants recovered from the infected chickens still produced truncated LPS, we analyzed whole-cell lysates from a number of the *in vivo*-derived *hptA* mutants by SDS-PAGE and carbohydrate silver staining. The virulent *hptA* mutants recovered from both the blood and muscle of the

infected birds no longer produced truncated LPS at detectable levels (Fig. 2). This finding was confirmed by mass spectrometry analysis of the LPS isolated from six *P. multocida hptA* mutants derived from the blood of bird 18 and from two mutants derived from the blood of bird 21, which clearly showed the lack of glycoform A (Table 3). On the basis of previous analysis of the assembly of the LPS inner core in *P. multocida*, these data indicated that the *in vivo*-derived *hptA* mutants each had a compensatory mutation which prevented the phosphorylation of the first Kdo residue by KdkA.

**All virulent *hptA* mutants isolated from chickens have compensatory mutations in the *kdkA* gene.** Sequencing of the *kdkA* gene from 12 independent *in vivo*-derived *hptA* mutants lacking truncated glycoform A LPS revealed that 7 isolates contained a point mutation within the *kdkA* open reading frame which resulted in truncation of the KdkA protein (Table 4). Interestingly, the remaining *in vivo*-derived *P. multocida hptA* mutants expressed a complete *kdkA* gene with a single point mutation, resulting in one of the following amino acid substitutions: A112V, R123P, D193N, or H168Y (Table 4). These data indicate that all these amino acid substitutions result in the loss of KdkA kinase activity.

**Complementation of a defined *P. multocida kdkA* mutant with the R123P, H168Y, or D193N *kdkA* fails to restore production of glycoform A.** To determine if the KdkA transferases

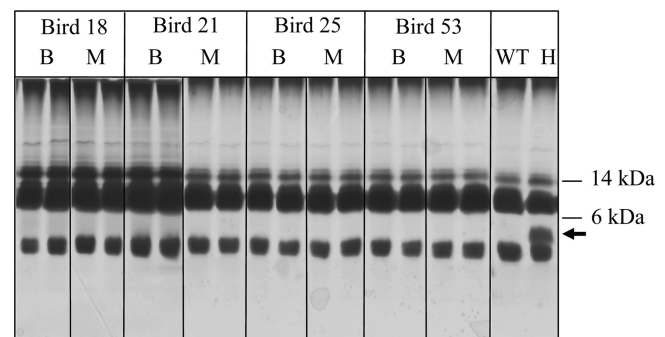


FIG. 2. Analysis of *P. multocida* LPS produced by *in vivo*-derived *hptA* mutants by PAGE and carbohydrate silver staining of whole-cell lysates. Truncated LPS is indicated by the arrow. WT, wild-type strain (VP161); H, *in vitro*-grown *hptA* mutant (AL836); B, blood isolate; M, muscle isolate. The positions of the relevant standard molecular mass markers (kDa) are shown on the right.



TABLE 3. Negative ion CE-ES-MS data and proposed compositions of O-deacylated LPS (LPS-OH) from the *P. multocida* parent (AL435), the *hptA* mutant (AL836), *in vivo*-derived *kdkA* and *hptA* double mutants, the *kdkA* mutant (AL721), and complemented *kdkA* mutant strains

Description, strain	Observed ions ( <i>m/z</i> )		Molecular mass (Da) <sup>a</sup>		Relative intensity	Proposed composition
	(M-2H) <sup>2-</sup>	(M-3H) <sup>3-</sup>	Observed	Calculated		
Parent, AL435	1,488.6	991.9	2,978.9	2,977.6	0.1	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH
	1,499.4	999.2	3,000.7	2,999.5	0.1	2PCho, 4Hex, 4Hep, Kdo-P, lipid A-OH
	1,548.9	1,032.9	3,100.7	3,100.6	0.2	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH-PEtn
	1,560.0	1,040.1	3,122.6	3,122.6	0.6	2PCho, 4Hex, 4Hep, Kdo-P-PEtn, lipid A-OH
	1,621.5	1,081.2	3,245.8	3,245.6	1.0	2PCho, 4Hex, 4Hep, Kdo-P-PEtn, lipid A-OH-PEtn
<i>hptA</i> mutant, AL836	616.8		1,235.6	1,252.2	0.1	Kdo-P, lipid A-OH
	678.4	451.6	1,358.8	1,375.2	0.2	Kdo-P-PEtn, lipid A-OH
	739.8	492.6	1,481.2	1,498.3	0.4	Kdo-P-PEtn, lipid A-OH-PEtn
	1,487.4	992.1	2,978.1	2,977.6	0.3	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH
	1,549.9	1,033.4	3,102.6	3,100.6	1.0	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH-PEtn
<i>In vivo</i> -derived <i>kdkA hptA</i> double mutants						
A112V KdkA, AL1774 (AL1775) <sup>b</sup> bird 18	1,487.4	992.1	2,978.1	2,977.6	0.3	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH
	1,548.9	1,032.9	3,100.7	3,100.6	1.0	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH-PEtn
R123P KdkA, AL1777 (AL1778), bird 18	1,487.7	992.1	2,978.3	2,977.6	0.4 (0.3) <sup>c</sup>	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH
	1,549.5	1,032.6	3,100.9	3,100.6	1.0	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH-PEtn
D193N KdkA, AL1780 (AL1781), bird 18	1,487.7	991.5	2,977.5	2,977.6	0.4 (0.5)	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH
	1,549.2	1,032.3	3,100.1	3,100.6	1.0	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH-PEtn
H168Y KdkA, AL1782 (AL1783), bird 21	1,487.7	991.5	2,977.5	2,977.6	0.4	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH
	1,549.2	1,032.3	3,100.1	3,100.6	1.0	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH-PEtn
KdkA complementation data						
<i>kdkA</i> mutant, AL721		992.1	2,979.3	2,977.6	1.0	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH
		1,032.9	3,101.7	3,100.6	1.0	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH-PEtn
<i>kdkA</i> mutant + vector, AL839 (AL1807)	1,487.7	991.5	2,977.5	2,977.6	0.4 (0.3)	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH
	1,548.9	1,032.9	3,100.7	3,100.6	1.0	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH-PEtn
<i>kdkA</i> mutant + wild-type KdkA, AL840 (AL1808)		991.8	2,978.4	2,977.6	0.1	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH
		1,032.9	3,101.7	3,100.6	0.3 (0.2)	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH-PEtn
	1,560.0	1,040.1	3,122.6	3,122.6	0.6 (0.5)	2PCho, 4Hex, 4Hep, Kdo-P-PEtn, lipid A-OH
	1,621.5	1,081.2	3,245.8	3,245.6	1.0	2PCho, 4Hex, 4Hep, Kdo-P-PEtn, lipid A-OH-PEtn
<i>kdkA</i> mutant + A112V KdkA, AL1799 (AL1800)	1,488.0	991.5	2,977.7	2,977.6	0.6	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH
	1,549.2	1,032.6	3,100.6	3,100.6	1.0	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH-PEtn
		1,040.1	3,123.3	3,122.6	0.3 (0.2)	2PCho, 4Hex, 4Hep, Kdo-P-PEtn, lipid A-OH
	1,621.5	1,080.9	3,245.4	3,245.6	0.4 (0.5)	2PCho, 4Hex, 4Hep, Kdo-P-PEtn, lipid A-OH-PEtn
<i>kdkA</i> mutant + R123P KdkA, AL1801 (AL1802)	1,488.0	991.5	2,977.7	2,977.6	0.3 (0.4)	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH
	1,548.9	1,032.6	3,100.4	3,100.6	1.0	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH-PEtn
<i>kdkA</i> mutant + H168Y KdkA, AL1803 (AL1804)	1,487.7	991.5	2,977.5	2,977.6	0.4 (0.5)	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH
	1,549.2	1,032.6	3,100.6	3,100.6	1.0	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH-PEtn
<i>kdkA</i> mutant + D193N KdkA, AL1805 (AL1806)	1,487.7	991.2	2,977.0	2,977.6	0.4 (0.3)	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH
	1,548.9	1,032.6	3,100.4	3,100.6	1.0	2PCho, 3Hex, 4Hep, 2Kdo, lipid A-OH-PEtn

<sup>a</sup> Molecular mass was determined from the observed ions or calculated from the proposed composition. Average mass units were used for calculation of molecular weight, based on the proposed composition, as follows: lipid A-OH, 952.00; hexose (Hex), 162.15; heptose (Hep), 192.17; Kdo-P, 300.13; Kdo, 220.18; PEtn, 123.05; phosphocholine (PCho), 165.05.

<sup>b</sup> Duplicate strains were analyzed, but data for only one strain are shown.

<sup>c</sup> Where the relative intensity of each glycoform differed between duplicate strains, the value for the second strain is shown in parentheses.

containing the amino acid changes were functional, expression plasmids containing KdkA with one of the amino acid substitutions indicated above were constructed in duplicate. These plasmids, along with a plasmid containing wild-type *kdkA* (pAL462), were then introduced separately into the *P. multocida kdkA* mutant, AL721, which can no longer phosphorylate the first Kdo residue and which is therefore unable to produce

glycoform A LPS (Fig. 1) (10). CE-ES-MS analyses of isolated LPS showed that wild-type levels of both glycoform A and B LPS were produced when the *kdkA* mutant was complemented *in trans* with the wild-type *kdkA* gene (Table 3). However, complementation *in trans* with a *kdkA* gene encoding an R123P, H168Y, or D193N amino acid substitution failed to restore the expression of glycoform A LPS, confirming that

TABLE 4. Nucleotide changes observed in the *kdkA* gene amplified from *in vivo*-derived *P. multocida hptA kdkA* double mutants

Bird	Sample	Nucleotide change	Result	Strain designations
18	Muscle	T78 deleted	Frame shift after F26	
18	Blood	C64T	Stop	
18	Blood	C335T	A112V	AL1774, AL1775
18	Blood	G368C	R123P	AL1777, AL1778
18	Blood	C424T	Stop	
18	Blood	G577A	D193N	AL1780, AL1781
21	Muscle	C502T	H168Y	
21	Blood	C502T	H168Y	AL1782, AL1783
25	Muscle	C478T	Stop	
25	Blood	C538T	Stop	
53	Muscle	C466T	Stop	
53	Blood	C466T	Stop	

amino acids R123, H168, and D193 in KdkA are essential for kinase function (Table 3). Although LPS structural analysis showed that the A112V *kdkA* mutants AL1774 and AL1775 did not express observable amounts of the glycoform A inner core, complementation experiments showed that providing this gene in *trans* on the high-copy-number expression plasmid pAL99 allowed some expression of glycoform A LPS (Table 3, AL1799 and AL1800). However, the relative intensity values obtained from the CE-ES-MS data suggest that the amount of glycoform A produced by these strains was significantly less than the amount of glycoform A expressed when wild-type KdkA was provided in *trans* (approximately 30% and 82% of total LPS, respectively) (Table 3).

***P. multocida* KdkA is a member of the protein kinase superfamily.** Previous bioinformatic analyses indicated that bacterial LPS kinases are homologs of eukaryotic protein kinases, a large group of regulatory enzymes that are bilobal in structure, consisting of a small N-terminal subdomain and a large C-terminal subdomain separated by a linker or hinge region (18). The amino acids essential for function in the eukaryotic proteins of this superfamily are also highly conserved across the bacterial LPS kinases (15). Using the COBALT multiple-alignment tool (18), we compared the amino acid sequence of *P. multocida* KdkA with the amino acid sequences of Kdo kinases from a number of different bacterial species and the amino acid sequence of the catalytic subunit of the eukaryotic protein kinase A (cPKA) (Fig. 3). This alignment showed that 23 amino acids were conserved among all of the aligned kinases and revealed that three of the four substitutions (R123P, H168Y, and D193N) identified in the *P. multocida kdkA* mutants were in regions of the protein that aligned with critical residues within cPKA. The nonconservative substitution R123P (in the position equivalent to N115 in cPKA) is predicted to be positioned at the start of the 5th beta sheet immediately preceding the linker region (12). The relatively conservative H168Y substitution (in the position equivalent to Y164 in cPKA) is positioned in the predicted catalytic loop of the kinase where phosphate transfer from the ATP donor to the acceptor molecule occurs (Fig. 3). The D193N substitution in *P. multocida* KdkA aligns within the region predicted to form the Mg<sup>2+</sup> positioning loop and is situated only 2 amino

acids downstream from the essential D184 in cPKA. Interestingly, the fourth mutation, A112V, which resulted in a significant reduction in kinase activity (Table 3), does not appear to be located in a critical region of the kinase. Moreover, the equivalent residue that aligns with A112 in both cPKA and the *Haemophilus ducreyi* KdkA is also valine.

## DISCUSSION

*P. multocida* simultaneously produces LPS with two different inner core structures, termed glycoforms A and B. Glycoform A has a single phosphorylated Kdo residue attached to the lipid A (lipid A-Kdo<sub>1</sub>-P), whereas the glycoform B structure contains two unphosphorylated Kdo residues (lipid A-Kdo<sub>1</sub>-Kdo<sub>2</sub>) (10). In Gram-negative bacteria, glycoform B is the most commonly occurring LPS core structure, whereas glycoform A is produced by relatively few bacterial species, including *Haemophilus influenzae*, *Vibrio cholerae*, and the veterinary pathogens *Mannheimia haemolytica* and *Actinobacillus pleuropneumoniae*. The production of both the glycoform A and glycoform B inner core structures simultaneously has been detected only in *P. multocida* and *M. haemolytica*, both of which belong to the *Pasteurellaceae* family (16, 23). *H. influenzae*, which is also a member of the *Pasteurellaceae* family, can synthesize only a glycoform A inner core, as the *H. influenzae* Kdo transferase is monofunctional and therefore unable to add a second Kdo residue (26).

The kinase required for the phosphorylation of the first Kdo residue in *P. multocida* glycoform A is Kdo kinase (KdkA). Following Kdo phosphorylation, the first heptose residue is added by the glycoform A-specific heptosyltransferase, HptA (10). In a previous study, we showed that *P. multocida hptA* mutant AL836 produced full-length glycoform B LPS but was rendered avirulent by the large amount of truncated glycoform A LPS present on its surface. In contrast, *P. multocida kdkA* mutant AL721 was fully virulent; this mutant produced full-length glycoform B but did not express any truncated glycoform A, as it was unable to phosphorylate the first Kdo residue (10). In the present study, we found that introduction of the *hptA* mutant into chickens at high doses resulted in the natural selection and growth of spontaneous *kdkA hptA* double mutants that no longer assembled any truncated glycoform A.

Homologs of *P. multocida* KdkA are present in most species within the *Pasteurellaceae* family, including members of the genera *Pasteurella*, *Haemophilus*, *Actinobacillus*, and *Mannheimia*. Outside of the *Pasteurellaceae* family, KdkA homologs are also expressed by the human gastrointestinal pathogens *V. cholerae* and *Vibrio parahaemolyticus*, as well as by a number of species within the order *Alteromonadales*.

Previous bioinformatic analyses of the *P. multocida* Kdo kinase showed that KdkA is distantly related to eukaryotic protein kinases, of which cPKA is the best characterized (13–15, 24). In eukaryotic species, cPKA is required to catalyze the transfer of phosphate from MgATP to the target protein substrate. The structure of cPKA has been determined and consists of two lobes, one small and one large, with the catalytic site or ATP binding pocket being located in the active cleft between the two lobes (13–14). Most of the highly conserved amino acids identified in the cPKA structure are located in the





amino acids are brought into the correct conformation for ATP alignment and subsequent catalysis (12). Thus, the nonconservative R123P substitution in KdkA is likely to have a significant impact on the secondary structure of this kinase, and it is probable that the mutation affects the hinge action of the linker region.

The Kdo kinase expressed by AL1780 and AL1781 contained a D193N substitution that led to the complete loss of kinase activity. Analysis of the alignment with cPKA in this region showed that there are two aspartate residues in close proximity to each other in all the bacterial KdkA kinases examined. In the COBALT alignment, D191 aligns directly with the cPKA essential catalytic site amino acid D184, and the KdkA D193, present in all KdkA proteins examined, aligns 2 amino acids downstream. In cPKA, D184 is required to chelate the activating  $Mg^{2+}$  ion, which in turn binds to the  $\beta$ - and  $\gamma$ -phosphates of ATP (28). In the present study, we showed that the D193N substitution leads to a complete loss of KdkA kinase activity. This D193 residue may be critical for kinase function because of its close proximity to the predicted catalytic site amino acid D191, or alternatively, D193 rather than D191 may be the essential catalytic site amino acid in bacterial Kdo kinases.

The Kdo kinase mutants AL1774 and AL1775 expressed no glycoform A LPS as a result of an A112V substitution within KdkA. However, when this mutant *kdkA* gene was expressed from a high-copy-number expression plasmid, a low level of KdkA activity was observed. On the basis of the COBALT alignment of the bacterial Kdo kinases with the eukaryotic cPKA, the A112V substitution was not in a highly conserved or structurally critical region of the protein, and valine is also in the equivalent position in both cPKA and the *H. ducreyi* KdkA. Nevertheless, it is clear that in *P. multocida* KdkA, A112 is required for full enzyme activity.

In conclusion, the use of a fowl cholera natural host infection model has allowed us the unique opportunity to rapidly identify essential amino acids in the *P. multocida* Kdo kinase, KdkA, required for the addition of phosphate to the Kdo<sub>1</sub> of glycoform A LPS. Four of the *kdkA* mutants identified in this study encoded complete *kdkA* genes, but each had a single nucleotide change leading to an amino acid substitution. On the basis of analysis of the LPS produced by the *kdkA* mutants, we have shown that these changes in the KdkA protein either abolish (R123P, H168Y, and D193N) or result in the significant loss (A112V) of kinase activity on the lipid A-Kdo<sub>1</sub> acceptor molecule. Furthermore, on the basis of comparisons with the eukaryotic protein kinases, three of the mutations that abolish kinase activity are predicted to be in or near two important regions of the kinase molecule, namely, the linker region hinging the two lobes of the kinase molecule and the active cleft containing the ATP binding pocket. This is the first report of the identification of residues critical for the function of the bacterial Kdo kinase KdkA and will form a solid basis for future structural studies and the identification of inhibitors for this important LPS kinase.

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