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Molecular and Functional Characterization of *Salmonella enterica* Serovar Typhimurium *poxA* Gene: Effect on Attenuation of Virulence and Protection

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Salmonella enterica poxA **mutants exhibit a pleiotropic phenotype, including reduced pyruvate oxidase activity; reduced growth rate; and hypersensitivity to the herbicide sulfometuron methyl, α-ketobutyrate, and amino acid analogs. These mutants also failed to grow in the presence of the host antimicrobial peptide, protamine. In this study, PoxA$^-$ mutants of *S. enterica* serovar Typhimurium (*S. typhimurium*) were found to be 10,000-fold attenuated in orally inoculated BALB/c mice and 1,000-fold attenuated in intraperitoneally inoculated BALB/c mice, compared to wild-type *S. typhimurium* UK-1. In addition, *poxA* mutants were found to be capable of colonizing the spleen, mesenteric lymph nodes, and Peyer's patches; to induce strong humoral immune responses; and to protect mice against a lethal wild-type *Salmonella* challenge. A 2-kb DNA fragment was isolated from wild-type *S. typhimurium* UK-1 based on its ability to complement an isogenic *poxA* mutant. The nucleotide sequence of this DNA fragment revealed an open reading frame of 325 amino acids capable of encoding a polypeptide of 36.8 kDa that was confirmed in the bacteriophage T7 expression system. Comparison of the translated sequence to the available databases indicated high homology to a family of lysyl-tRNA synthetases. Our results indicate that a mutation of *poxA* has an attenuating effect on *Salmonella* virulence. Further, *poxA* mutants are immunogenic and could be useful in designing live vaccines with a variety of bacterial species. To our knowledge, this is the first report on the effect of *poxA* mutation on bacterial virulence.**

The pyruvate oxidase of *Escherichia coli* is a peripheral membrane protein that catalyzes the oxidative decarboxylation of pyruvate to acetate and CO_2 (19). Under laboratory conditions, this enzyme is not essential and conversion of pyruvate to acetate is considered a waste of energy, compared with its conversion to acetyl coenzyme A (18). Pyruvate oxidase has been of interest primarily as a model for studying protein-lipid interaction. The enzyme is a water-soluble tetramer of identical 62-kDa subunits (18). It requires thiamine pyrophosphate, flavin adenine dinucleotide, and Mg^{2+} as cofactors (2, 3, 19). In the presence of the substrate and cofactors, the enzyme undergoes conformational changes and binds to *E. coli* membrane vesicles and to phospholipid vesicles (38, 43). This peripheral membrane binding is necessary for the terminal transfer of electrons to ubiquinone-8, which is dissolved in the lipid bilayer (20, 28).

To study protein-lipid interactions by genetic means, mutations in two genes affecting pyruvate oxidase activity have been identified. The structural gene for pyruvate oxidase, *poxB*, has been located at 18.7 min on the *E. coli* genetic map (5), and a regulatory gene, *poxA*, has been located at 94 min (4). Enzymatic and immunological data indicated that mutations in *poxA* result in a 6- to 10-fold decrease in pyruvate oxidase levels (4, 5). Chang and Cronan reported that *poxA* mutants grew more slowly than the isogenic parent in both minimal and rich media, while *poxB* mutants exhibited normal growth.

In their efforts to elucidate the mechanism of inhibition of acetolactate synthase II by the herbicide sulfometuron methyl (SM) {*N*-[(4,6-dimethylpyrimidin-2-yl)aminocarbonyl]-2-methoxycarbonyl-benzenesulfonamide} in *Salmonella enterica* serovar Typhimurium (*S. typhimurium*), Van Dyk and LaRossa (48) isolated 15 mutant strains sensitive to SM, following Tn*10* mutagenesis. Among these SM-hypersensitive mutations, a *poxA* mutation was identified and mapped to the 94-min region of the *S. typhimurium* genetic map (49), a location analogous to that of *poxA* in *E. coli*. Like the *E. coli* counterpart, the *S. typhimurium poxA* mutant exhibited reduced pyruvate oxidase activity and growth rate (49). Furthermore, the *E. coli* and *S. typhimurium poxA* mutants shared several additional phenotypes including hypersensitivity to SM; α-ketobutyrate (AKB); and a wide range of bacterial growth inhibitors such as antibiotics, amino acid analogs, and dyes (49). Since mutants defective in *poxB* did not exhibit these phenotypes, it was concluded that a mutation within the *poxA* regulatory locus of *E. coli* and *S. typhimurium* would result in pleiotropic effects not due solely to decreased *poxB* expression.

Although the *poxA* gene was identified 16 years ago, molecular and functional data on the gene and gene product are lacking. In this study, we cloned and determined the nucleotide sequence of the *poxA* gene and characterized the *poxA* gene product of *S. typhimurium*. Our results show for the first time that *S. typhimurium* mutants with deletions of the *poxA* gene are attenuated and immunogenic.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. The bacterial strains used in this study and their sources are listed in Table 1. Bacteriophage P22HT*int* was used for transduction of markers into *Salmonella* strains (41). Strains were grown in Luria broth (LB), Lennox medium (32), or antibiotic no. 2 (AB2) agar (Difco, Detroit, Mich.). When required, antibiotics were added to the growth media at the following concentrations: kanamycin, 50 μg/ml; ampicillin, 100 μg/ml; streptomycin, 100 μg/ml; tetracycline, 12.5 μg/ml.

Recombinant DNA, genetic techniques, and nucleotide sequencing. Recombinant DNA techniques were performed according to standard procedures (40). Total DNAs from *Salmonella*, *Shigella*, *Klebsiella*, *Pseudomonas*, *Pasteurella*, and *Borrelia* species were isolated according to a published protocol (34). Total

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TABLE 1. Bacterial strains used in this study

Strain	Relevant genotype	Reference or source
S. typhimurium LT2 strain SMS401	*poxA401*::Tn*10*	49
S. typhimurium UK-1		
χ3761	Wild-type UK-1	R. Curtiss' collection
MGN-791	*poxA401*::Tn*10*	This work
MGN-816	Δ*poxA402*	This work
MGN-939	Δ*poxA402* (pMEG-250)	This work
MGN-1036	Δ*poxA270*	This work
MGN-1154	Δ*poxA270* (pMEG-274)	This work
E. coli		
BL21(DE3)	F^- *ompT* [*lon*] *hsdS* (r^- m^-; an *E. coli* B strain) with DE3, λ T7 RNA polymerase	46
MGN-617	SM10 λ*pir* derivative, *thi thr leu tonA lacY supE* λ*pir recA*::RP4-2-Tc::Mu (Km^r) Δ*asdA1*	37a
CC118 λ*pir*	*araD139* Δ(*ara-leu*)*7697* Δ*lacX74 galK* Δ*phoA20 galE recA1 rpsE argE*(Am) *rpoB thi* λ*pir*	10

DNAs of *Mycobacterium tuberculosis* and *Erysipelothrix rhusiopathiae* were kindly provided by J. Clark-Curtiss and T. K. Ball, respectively. Total DNA was digested with *Cla*I or *Eco*RV and resolved on a 0.8% agarose gel. Southern blotting and hybridization were performed according to standard procedures (40). DNA probes were fluorescein labeled (Amersham, Arlington Heights, Ill.) according to the manufacturer's instructions. Transformation of plasmid DNA into *E. coli* and *Salmonella* strains by electroporation was carried out as described elsewhere (36) by using an *E. coli* Gene Pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.). The *poxA401*::Tn*10* insertion (48, 49) was moved into *S. typhimurium* UK-1 by P22HT*int* transduction as previously described (42). Expression and [^{35}S]methionine labeling of plasmid-encoded polypeptides in a bacteriophage T7 expression system were carried out as described elsewhere (47), with *E. coli* BL21(DE3) as host (46) for the expression vectors derived from plasmid pBluescript II (Stratagene, La Jolla, Calif.). Nucleotide sequence determination was performed by ACGT, Inc. (Northbrook, Ill.), with double-stranded DNA as the template, and both strands were sequenced. Nucleotide sequence analyses were performed with the MacVector software package (version 5). Comparison of translated and nontranslated nucleotide sequences with those in the available databases was carried out with the BLAST program at the server of the National Center for Biotechnology Information at the National Library of Medicine (1).

Construction of Δ*poxA270* defined deletion strains. A defined deletion in *poxA* was constructed by removing the last 270 amino acids of PoxA encoded by a 1,018-bp *Bst*BI-*Xho*I fragment of pMEG-273, filling in the termini with the large fragment of DNA polymerase I, and religating, yielding plasmid pMEG-279. A 1-kb *Xho*I-*Xba*I fragment of pMEG-279, carrying the mutated Δ*poxA270* allele and flanking sequences, was cloned into the *Sal*I-*Xba*I sites of the R6K-derived replicon pKNG101 (24) to give the suicide plasmid pMEG-280. The defined mutation Δ*poxA270* was subsequently recombined into the *S. typhimurium* UK-1 chromosome by bacterial mating according to the procedure described elsewhere (23) with the following modification: 100 μl of fresh overnight culture of the universal donor *E. coli* MGN-617 (Table 1) harboring the suicide plasmid pMEG-280 and 100 μl of the recipient *S. typhimurium* χ3761 were mixed in 5 ml of buffered saline gelatin. The solution was filtered through a 0.45-μm-pore-size-filter, which was then placed on the surface of an L-agar plate containing 100 μg of diaminopimelic acid per ml and incubated at 37°C for at least 8 h. Bacteria bound to the filter were then resuspended in 5 ml of buffered saline gelatin, and 10-fold dilutions were plated onto LB agar containing 100 μg of streptomycin per ml. Single recombinants that had the plasmid integrated into the chromosome were grown in LB in the absence of antibiotic selection, and 10-fold dilutions were plated on AB2 agar medium containing 5% sucrose to select for strains that had undergone resolution of the cointegrate by recombination. Double-crossover recombinants were tested for streptomycin sensitivity due to loss of the suicide plasmid, for the $PoxA^-$ phenotype on AB2 plates, and for sensitivity to 1.25 mg of protamine sulfate per ml.

Animal experiments. Oral and intraperitoneal inoculations of 6- to 7-week-old female BALB/c mice with the different *S. typhimurium* strains were carried out as previously described (17).

Analysis of the humoral immune responses. The humoral immune responses to *S. typhimurium poxA* mutants were assessed by enzyme-linked immunosorbent assay (ELISA). Microtiter plates were coated with 250 ng of *S. typhimurium* lipopolysaccharide (LPS) (Sigma, St. Louis, Mo.) per well in 0.2% trichloroacetic acid (pH 7.4) for 2 h at 37°C. Free binding sites were blocked with 3% bovine serum albumin and 0.1% Tween in phosphate-buffered saline (PBS) for 30 min at room temperature. Washes were performed with PBS-Tween between incubations. Plates were incubated with mouse sera diluted in PBS-Tween for 1 h at 37°C, followed by a 1-h incubation at 37°C with the secondary antibody goat anti-mouse immunoglobulin A (IgA)-, IgM-, or IgG-conjugated alkaline phosphatase (Kirkegaard & Perry Laboratories, Inc., Gaithersburg, Md.) diluted 1:500 in PBS-Tween. Plates were developed with *p*-nitrophenylphosphate (Sigma), and the optical density at 405 nm was measured after a 30-min incubation at 37°C.

Nucleotide sequence accession number. The nucleotide sequence described in this paper has been deposited in GenBank under accession no. AF001831.

RESULTS

Construction of *S. typhimurium poxA* mutants and cloning of the *poxA* gene by genetic complementation. The *poxA401*::Tn*10* allele was introduced into wild-type *S. typhimurium* UK-1 strain χ3761 by P22 transduction (see Materials and Methods) by using a P22 lysate grown on *S. typhimurium* SMS401, to generate strain MGN-791 (Table 1). Although *poxA* mutants grow slower than the parent (4), this differential growth rate does not appear to be a strong phenotype. Unlike the LT2 derivative strain SMS401, which exhibits hypersensitivity to AKB (48, 49), we found that *S. typhimurium* UK-1 *poxA401*::Tn*10* showed only marginal sensitivity to AKB (data not shown). Therefore, we screened a range of bacteriological media for substantial difference in growth rates between *poxA* mutants and the isogenic parent. We found that MGN-791, a *poxA401*::Tn*10* derivative of *S. typhimurium* UK-1, produced microcolonies on MacConkey and AB2 agar, compared to the parental strain, which produced large colonies. A fusaric-acid-resistant derivative of MGN-791 was selected following Tn*10* deletion to generate MGN-816, which also produced microcolonies on AB2 agar (Fig. 1). Under the same conditions, the parental strain χ3761 produced normal-size colonies on both AB2 agar and LB agar plates.

The *poxA* gene was cloned based upon its ability to restore normal growth to a *poxA*-defective strain, MGN-816, on AB2 agar medium. A genomic library of wild-type *S. typhimurium* UK-1 strain χ3761 in a pUC19-derived vector, pNEB193, was used to select a clone expressing PoxA. This library was introduced into *S. typhimurium* MGN-816 by electroporation and selection on an AB2 agar plate containing ampicillin. Among ~5,000 ampicillin-resistant microcolonies, one transformant, MGN-939, exhibited a large colony morphology (Fig. 1), indicating that strain MGN-939 had acquired a DNA fragment capable of fully complementing the growth defect of the *poxA* mutant. The plasmid, designated pMEG-250, was purified and introduced into *E. coli* CC118 λ*pir*. A partial restriction map of the 2-kb DNA insert in pMEG-250 was established (Fig. 2). To

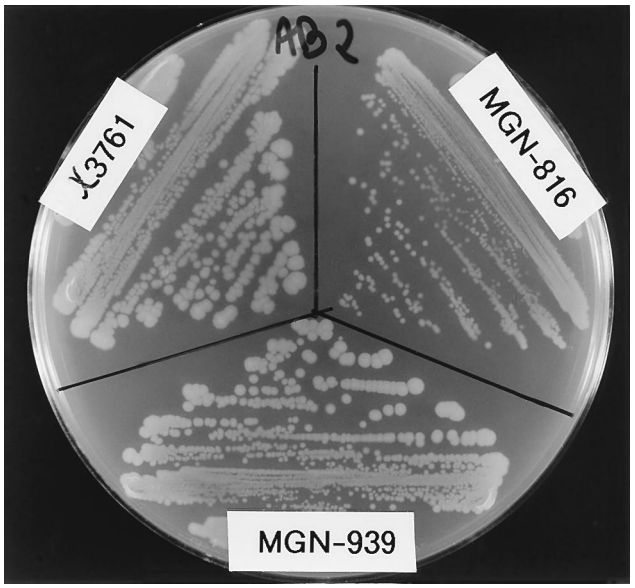

FIG. 1. Phenotype of *S. typhimurium poxA* mutants on AB2 agar medium. $_{\chi}$3761, wild-type UK-1; MGN-816, Δ*poxA402*; MGN-939, MGN-816(pMEG-250). The plate was photographed after 17 h of incubation at 37°C.

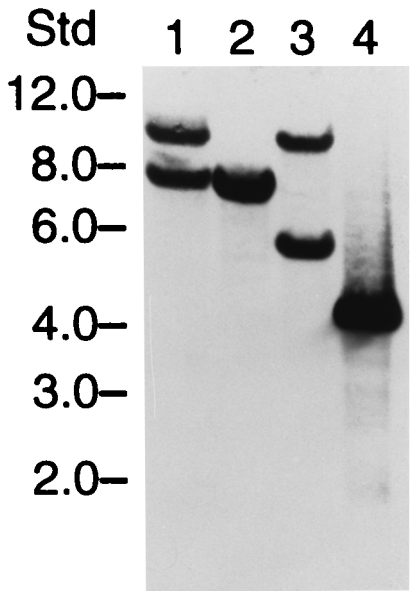

FIG. 3. Southern blot analyses of *S. typhimurium poxA* transposon insertion mutant. Chromosomal DNA from mutant and isogenic wild-type strains was digested with *Cla*I or *Eco*RV and transferred to a GeneScreen Plus nylon membrane. The blot was hybridized to a fluorescein-labeled 2-kb DNA probe containing the *poxA* gene. Std, 1-kb DNA ladder; lane 1, *Cla*I-digested MGN-791 (*poxA401*::Tn*10*); lane 2, *Cla*I-digested $_{\chi}$3761 (parent); lane 3, *Eco*RV-digested MGN-791 (*poxA401*::Tn*10*); lane 4, *Cla*I-digested pMEG-250.

ensure that the complementation was due solely to cloned *poxA* and not to an unknown secondary mutation acquired during the selection, pMEG-250 was reintroduced into MGN-816. All transformants were complemented to the large colony phenotype on AB2 agar, suggesting that the 2-kb DNA fragment of UK-1 in pMEG-250 encodes an active PoxA protein. Alternatively, the DNA fragment could also encode a suppressor of *poxA*, as the complementing pMEG-250 is a high-copy-number plasmid. To address that possibility, we performed a Southern blot analysis of *Cla*I-digested total DNA from the parental strain $_{\chi}$3761 and the transposon Tn*10* insertion in *poxA* strain MGN-791, with the labeled 2-kb insert from pMEG-250 as the probe. The data in Fig. 3 show that the probe hybridized to a single 7.5-kb *Cla*I fragment in the parent (lane 2) and to two *Cla*I fragments (7.5 and 9.5 kb) in *poxA401*::Tn*10* (lane 1). These data are consistent with the size of transposon Tn*10* (9.3 kb) and the internal *Cla*I site (27). The results confirmed that the 2-kb DNA fragment of pMEG-250 encodes the *poxA* gene.

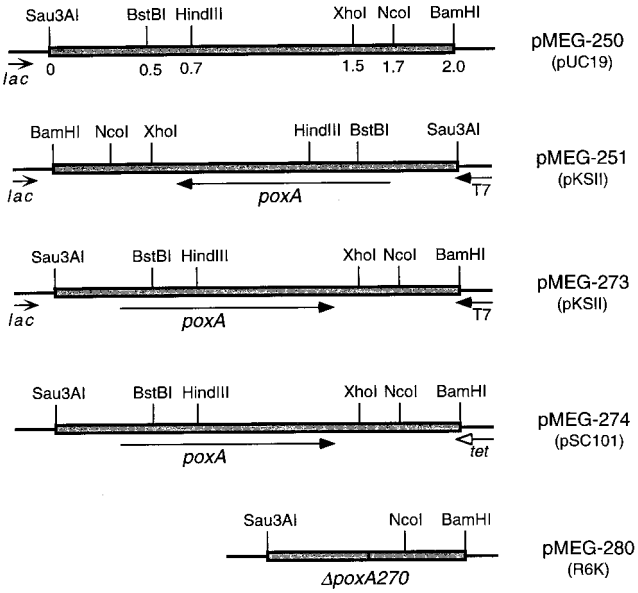

FIG. 2. Partial restriction endonuclease maps of the *poxA* insert on relevant plasmids. The positions of relevant restriction endonucleases are shown. The direction of transcription of the *poxA* gene is indicated by the arrow. The plasmid replicon is indicated in parentheses.

We next wondered whether the cloned *poxA* gene was expressed from its natural promoter. The *poxA*-encoding fragment was cloned in both orientations with respect to the plasmid-encoded *lac* promoter, generating pMEG-251 and pMEG-273 (Fig. 2). Both pMEG-251 and pMEG-273 were able to complement *poxA401*::Tn*10* mutation (data not shown), indicating that the 2-kb DNA fragment carries a promoter sequence necessary for the expression of *poxA*. In the absence of antibiotic selection, the high-copy-number plasmids, pMEG-250, pMEG-251, and pMEG-273, were unstable and were lost at a high frequency (data not shown). Therefore, the *poxA* gene was subcloned into the low-copy-number pLG339 vector (45), a pSC101 derivative, to give pMEG-274 (Fig. 2). On this plasmid, *poxA* was in opposite orientation in regard to the *tet* promoter. This construct was stable in *S. typhimurium* and was used in subsequent complementation experiments.

Nucleotide sequence of the *poxA* gene: *poxA* encodes the second lysyl-tRNA synthetase in *Salmonella*. The 2-kb DNA fragment of plasmid pMEG-250 was sequenced on both strands (see Materials and Methods). The nucleotide sequence (GenBank accession no. AF001831) was analyzed with Mac Vector 5.0 software. Searches for homology in the available databases were performed with the BLAST program at the National Center for Biotechnology Information (1). At the nucleotide level, the 2,008 bp showed 81% identity to the 94-min region of the *E. coli* chromosome, including *yjeA* and

yjeM, and 61% identity to a segment in the *Haemophilus influenzae* chromosome including the *yjeA* and *yjbM* genes. This is in agreement with the map position of *poxA401*::Tn*10* determined in *S. typhimurium*. *yjeA* and *yjeM/yjbM* were identified during the automated sequencing of the genome of these organisms. No function has been assigned to their corresponding gene products. One complete open reading frame (ORF) and one truncated ORF were found in the 2,008-bp sequence. The first ORF, preceded by a putative Shine-Dalgarno sequence, starts at nucleotide 345 and encodes a polypeptide of 325 amino acids with a predicted pI of 5.1 and molecular mass of 36.8 kDa. This complete ORF was assigned to the PoxA protein. Hydrophobicity analysis using the algorithm of Kyte and Doolittle (30) indicated that PoxA does not have a signal sequence or membrane-spanning domain, suggesting that PoxA is a cytoplasmic protein. PoxA showed 91 and 65% identity to GenX (YjeA) of *E. coli* (29) and *H. influenzae*, respectively. GenX (YjeA) has not been characterized at the molecular level, and no function has been ascribed to this protein in either organism. However, PoxA and GenX appear to belong to a family of class II lysyl-tRNA synthetases. This family of enzymes is characterized by two motifs: signature 1, FRNEEMG RHHNPEFTMLE, and signature 2, ALGVDRLVML. The second ORF, preceded by a putative Shine-Dalgarno sequence, starts at nucleotide 1548 and was truncated at nucleotide 2,008. This ORF was found to be homologous to *E. coli* YjeM (88% identity and 96% similarity). Although the transposon Tn*10* insertion has not been mapped, it is reasonable based upon the genetic complementation and defined deletion (see below) to state that the insertion took place in the *poxA* coding region. The complementation of the *poxA401*::Tn*10* mutation by pMEG-274 suggests that the downstream gene does not have a role in the phenotypes ascribed to *poxA* in this study.

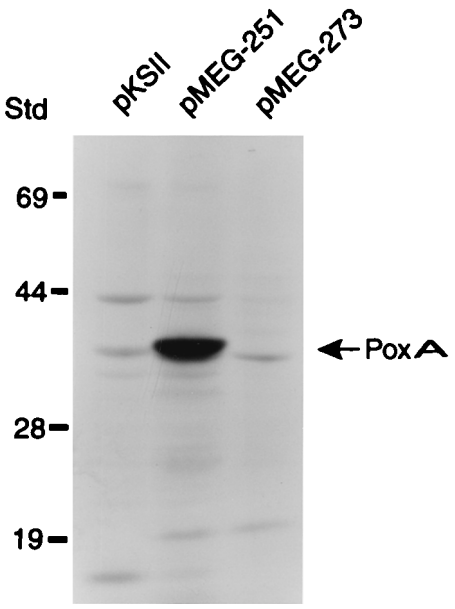

FIG. 4. Identification of *poxA* gene product by using the T7 expression system. *poxA* was cloned in both orientations under the control of the bacteriophage T7 promoter in the vector pKSII and introduced into *E. coli* BL21(DE3), which carries a bacteriophage T7 RNA polymerase gene under the control of the *lac* promoter. After induction, whole-cell lysate proteins were separated on an SDS-polyacrylamide gel as described in Materials and Methods. Numbers on the left indicate the positions of molecular mass standards (in kilodaltons). The arrow indicates a polypeptide of about 36 kDa specifically encoded by pMEG-251, the putative gene product of *poxA*. pMEG-251, *poxA* under the T7 promoter; pMEG-273, *poxA* in opposite orientation from the T7 promoter; pKSII, vector control.

Expression of *poxA* in a bacteriophage T7 RNA polymerase expression system. The 2-kb DNA fragment capable of complementing the *poxA* mutation was cloned in both orientations under the control of the bacteriophage T7 promoter in the vector pKSII, to generate pMEG-251 and pMEG-273 (Fig. 2). Plasmid-encoded polypeptides were examined as previously described (47). Cell lysates of *E. coli* BL21(DE3) carrying pMEG-251 (Fig. 4, lane 2) showed a polypeptide with a molecular mass of about 36 kDa which was absent from lysates of cells carrying either pMEG-273 (Fig. 4, lane 3) or the vector alone (Fig. 4, lane 1). The size of the expressed polypeptide is in complete agreement with the predicted size of PoxA. A 15-kDa polypeptide corresponding to the truncated ORF 2 was not detected under these experimental conditions.

Construction of Δ*poxA270* defined deletion strain of *S. typhimurium* UK-1. A defined deletion in *poxA* was constructed in *S. typhimurium* UK-1 as described in Materials and Methods, resulting in strain MGN-1036 (Table 1). In this strain, DNA coding for the last 270 amino acids of PoxA was deleted. MGN-1036 gave rise to microcolonies on AB2 agar medium and reduced growth rate in LB as expected. The deletion was confirmed by Southern blot analysis with the 2-kb DNA fragment encoding *poxA* as the probe (data not shown). Moreover, the defined Δ*poxA270* deletion was fully complemented by plasmid pMEG-274 (*poxA*$^+$) for normal growth rate and for virulence (see below). It has been reported elsewhere that *S. typhimurium* mutants sensitive to antimicrobial cationic peptides such as defensins and protamine also show reduced virulence (21). MGN-1036 failed to grow in the presence of 1.25 mg of protamine sulfate per ml, while the isogenic parent exhibited normal growth at that concentration. This phenotype was complementable by pMEG-274. MGN-1036 was characterized biochemically with API strips. The results showed no biochemical difference between the parent and the *poxA* mutant. The growth rate of the defined deletion strain MGN-1036 was identical to that of the previous *poxA* mutants MGN-791 and MGN-816. Taken together, the Δ*poxA270* defined mutant was phenotypically identical to the transposon-generated strains.

***S. typhimurium poxA* mutants are attenuated in virulence in mice.** In *S. typhimurium* and *Salmonella typhi*, mutations in several global regulatory loci including *cya*/*crp* (9, 26), *phoPQ* (17, 35), *rpoS* (8, 13, 37, 50), and *ompR* (11) have been associated with reduced virulence, without loss of immunogenicity in mice. *poxA* exhibits pleiotropic effects due to its global regulatory nature (49). To confirm a role of the *poxA* mutation in virulence, we compared the *poxA* mutant derivatives of *S. typhimurium* with the parental strain in the mouse typhoid model. Similar results were obtained in two independent studies. The data below are from the experiment conducted with Δ*poxA270* defined deletion strain MGN-1036, MGN-1154 (MGN-1036 carrying the *poxA*-complementing plasmid pMEG-274), and the χ3761 parental strain. Treatment groups of 7-week-old female BALB/c mice were orally inoculated with the doses indicated in Table 2. Eight days postinoculation, three mice were removed from the treatment group inoculated with 3.2×10^9 CFU of MGN-1036 and euthanized to determine the level of colonization in the spleen, mesenteric lymph nodes, and Peyer's patches. The results show on average 1.4×10^5 CFU in the spleen, 7.7×10^4 CFU in the mesenteric lymph nodes, and 2.2×10^5 CFU in the Peyer's patches. These data indicate that the Δ*poxA270* defined mutant of *S. typhimurium* UK-1 was capable of colonizing deep tissues in mice after oral inoculation. Following immunization, mice were monitored daily for 21 days. The oral and intraperitoneal 50% lethal

TABLE 2. Attenuation of *S. typhimurium* Δ*poxA270* mutant MGN-1036 and complementation in 7-week-old female BALB/c mice

Strain	Immunizing dose (CFU)	No. of survivor mice/total no. following:	
		Immunization[a]	Wild-type challenge[b]
χ3761	2.2×10^5	0/4	ND[c]
MGN-1036	3.2×10^8	4/4	4/4
MGN-1036	3.2×10^9	4/4	4/4
None	ND	ND	0/4
MGN-1154	2.2×10^5	0/4	ND
MGN-1154	2.2×10^6	0/4	ND
MGN-1154	2.2×10^7	0/4	ND
MGN-1154	2.2×10^8	0/4	ND

[a] Survivors were recorded 21 days postimmunization. All mice infected with 2.2×10^5 CFU of wild-type strain χ3761 or MGN-1154, a *poxA*-complemented strain, died within 10 days.
[b] Control and immunized mice were orally challenged with 2.6×10^8 CFU of wild-type UK-1 strain χ3761 (~10,000 × LD_{50}), 35 days postimmunization. Survival was assessed for an additional 16 days postchallenge. Nonimmunized control mice died within 10 days.
[c] ND, not done.

doses (LD_{50}) of wild-type *S. typhimurium* UK-1 are 7.1×10^4 and ~10 CFU, respectively (25a). All mice orally infected with 2.2×10^5 CFU of wild-type bacteria died within 10 days. In the MGN-1036 treatment groups, mice did not develop any clinical signs of typhoid fever (e.g., scruffiness) such as were seen in wild-type *Salmonella*-infected mice. Even mice receiving the highest oral dose, 3.2×10^9 CFU, of the defined Δ*poxA270* strain survived. Moreover, mice inoculated with the Δ*poxA270*-complementing strain, MGN-1154, died within 10 days irrespective of the doses (Table 2). These results confirmed that the attenuation of *S. typhimurium* virulence was due to the mutation in the *poxA* gene. In addition, this phenotype could be reversed to the parental virulence by using the low-copy-number plasmid, pMEG-274, carrying the functional *poxA* gene.

We determined whether the *S. typhimurium* Δ*poxA270* mutant could protect the immunized mice against lethal wild-type *Salmonella* challenge. Control and immunized mice were orally challenged with 2.6×10^8 CFU of virulent UK-1 strain $_{\chi}$3761 (~10,000 × LD_{50}), 35 days postimmunization. All control mice died within 10 days following challenge, and the experiment was terminated 16 days postchallenge. During that time, none of the vaccinated mice showed any clinical signs of disease, and all mice had survived ~10,000 × LD_{50} of wild-type *Salmonella* challenge after a single oral immunization (Table 2). Mice were then euthanized, and the spleen of each was examined for the presence of the immunizing and challenge strains. No bacteria were detected, suggesting that *S. typhimurium* Δ*poxA270* mutants were capable of protecting mice from the wild-type colonization of visceral organs.

Immunogenicity of *S. typhimurium poxA* mutants in mice. The immunogenicity of the defined deletion strain MGN-1036 was assessed, with the results for individual mice presented in Table 3. Humoral immune responses were measured by ELISA with purified *Salmonella* LPS as the coating antigen (see Materials and Methods). Sera were collected 29 days after a single oral immunization and 16 days postchallenge. High IgG, IgA, and, to a lesser extent, IgM titers were detected in orally immunized animals, indicating that the Δ*poxA270* mutant derivative of *S. typhimurium* was very immunogenic in mice. Mice receiving the highest oral dose, 3.2×10^9 CFU, were found to have higher IgG titers than mice receiving a log less bacteria (Table 3). However, both groups of mice were found to respond well to challenge as indicated by IgG titers postchallenge.

The data from this animal study demonstrate that *S. typhimurium* Δ*poxA270* mutants are attenuated and immunogenic. Moreover, *poxA* mutants are capable of protecting mice against a wild-type lethal challenge and colonization of deep tissues. Nearly identical results were obtained in another study using the Tn*10* deletion strain MGN-816 (data not shown). Furthermore, mice inoculated by the intraperitoneal route with 10^4 CFU of this Δ*poxA402* mutant, a dose equivalent to ~1,000 × LD_{50} of the parent, remained alive throughout the study (data not shown).

The *poxA* gene is present in other pathogenic microorganisms. Lastly, we questioned whether *poxA* was present in the genome of other bacterial species in addition to *Salmonella*, *H. influenzae*, and *E. coli*. To address this possibility, total DNA was purified from *S. typhimurium*, *Shigella flexneri*, *Yersinia enterocolitica*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Pasteurella multocida*, *Borrelia burgdorferi*, *M. tuberculosis*, and *E. rhusiopathiae*. Ten micrograms of *Cla*I-digested genomic DNA was subjected to Southern blot analysis (see Materials and Methods), with a labeled 1-kb *Bst*BI-*Xho*I internal frag-

TABLE 3. Immunogenicity of *S. typhimurium* Δ*poxA270* mutant MGN-1036 in BALB/c mice

Immunizing dose (CFU)	Mouse	Titer in serum[a]								
		IgG			IgM			IgA		
		After immunization[b]	After challenge[c]	Fold increase	After immunization	After challenge	Fold increase	After immunization	After challenge	Fold increase
3.2×10^8	1	1:100	1:204,800	2,048	1:800	1:1,600	2	1:800	1:6,400	8
3.2×10^8	2	1:200	1:409,600	2,048	1:200	1:800	4	<1:100	1:1,600	16
3.2×10^8	3	1:800	1:102,400	128	1:1,600	1:800	0.5	1:100	1:1,600	16
3.2×10^8	4	ND	1:102,400	ND	ND	1:800	ND	ND	1:1,600	ND
3.2×10^9	1	1:12,800	1:204,800	16	1:200	1:3,200	16	1:100	1:6,400	64
3.2×10^9	2	1:400	1:102,400	256	1:400	1:800	2	<1:100	1:400	4
3.2×10^9	3	1:6,400	1:12,800	2	1:1,600	1:1,600	1	1:200	1:400	2
3.2×10^9	4	1:6,400	1:25,600	4	1:200	1:100	0.5	<1:100	<1:100	1

[a] Serum IgG, IgM, and IgA titers were measured by ELISA with purified *Salmonella* LPS as coating antigen at 2.5 μg/ml. The titer is determined as the last serial dilution to have an optical density at 405 nm at or above 0.1. Negative control sera (from nonimmunized mice) had an optical density at 405 nm of <0.1 throughout the study. ND, not determined.
[b] Sera were collected at 29 days postimmunization with MGN-1036.
[c] Sera were collected at 16 days postchallenge with χ3761.

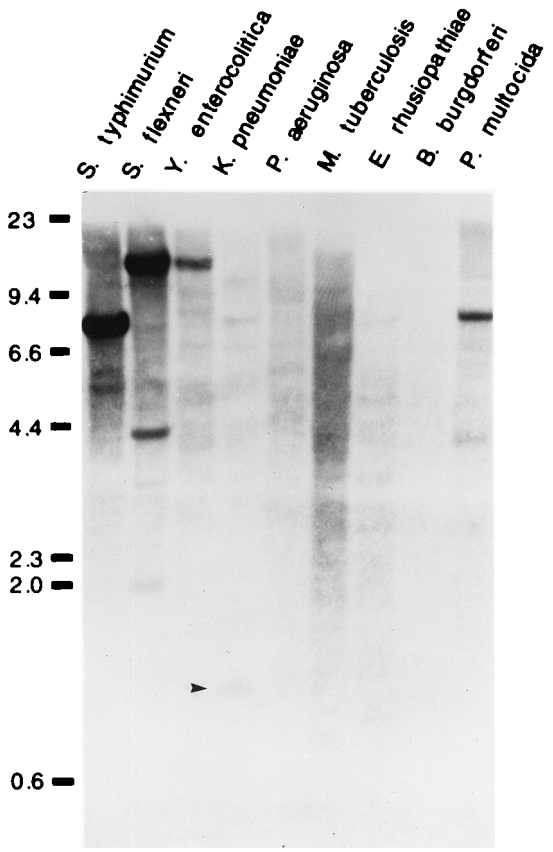

FIG. 5. Detection of the *poxA* gene in other bacterial species. Total DNA from *S. typhimurium* (5 μg), *S. flexneri* (10 μg), *Y. enterocolitica* (10 μg), *K. pneumoniae* (3 μg), *P. aeruginosa* (10 μg), *M. tuberculosis* (10 μg), *E. rhusiopathiae* (10 μg), *B. burgdorferi* (10 μg), and *P. multocida* (10 μg) was digested with the restriction enzyme *Cla*I. DNA fragments were separated on a 0.8% agarose gel and transferred to a GeneScreen Plus nylon membrane. The blot was hybridized to a fluorescein-labeled 1-kb *Bst*BI-*Xho*I internal fragment of *poxA* as a probe. The hybridization was carried out at 50°C with stringent washes in 1× SSC–0.1% SDS. The limited amount of genomic DNA available from *K. pneumoniae* could account for the weak signal. Numbers at left show molecular mass in kilodaltons.

ment of *poxA* as a probe. Despite the stringent hybridization and washing conditions (50°C, 1× SSC [1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate], 0.1% sodium dodecyl sulfate [SDS]), the results in Fig. 5 show that strong signals were detected in *S. flexneri* (three bands), *Y. enterocolitica* (one band), *K. pneumoniae* (one band), and *P. multocida* (two bands). Multiple hybridization bands in *S. flexneri* and *P. multocida* indicate the presence of other lysyl-tRNA synthetases (LysS and/or LysU) in these bacterial species. No hybridization bands were detected in *M. tuberculosis*, *B. burgdorferi*, *P. aeruginosa*, and *E. rhusiopathiae* (Fig. 5). These results indicate that *poxA* is present in other organisms in addition to *Salmonella*, *E. coli*, and *H. influenzae*.

DISCUSSION

We introduced a *poxA* mutation in *S. typhimurium* UK-1 by P22 transduction from an LT2 strain carrying a Tn*10* insertion in the gene (48). A clone from an *S. typhimurium* library was found to complement the effect of the *poxA* mutation and to hybridize to the *poxA* locus by Southern blot analysis (Fig. 3). Analysis of the 2-kb DNA sequence revealed one ORF capable of encoding a 36.8-kDa polypeptide, PoxA, which was confirmed in a bacteriophage T7 RNA polymerase expression system (Fig. 4). A homology search in the available databases showed that the *poxA* gene and encoded polypeptide were homologous to a family of class II lysyl-tRNA synthetases from several organisms. In bacteria, each of the 20 amino acids requires a specific cognate amino acyl-tRNA synthetase, except lysine. Two genes encoding functional lysyl-tRNA synthetases have been identified and characterized at the molecular level in *E. coli* K-12. *lysS* and *lysU* encode the constitutive LysS (25) and temperature-regulated LysU (22) lysyl-tRNA synthetases, respectively. Although mutations in *lysU* have no detectable phenotype, an alteration of *lysS* results in growth restriction at temperatures below 30°C (25). *E. coli* LysS and LysU have strong similarity over their entire length and migrate at similar positions on a two-dimensional electrophoresis gel (12, 33). Kong and colleagues (29) reported that *genX*, a gene located at min 94 on the *E. coli* genetic map, encodes a polypeptide with similarity to the carboxy terminus of LysS and LysU (7). It appears then that *E. coli* encodes three lysyl-tRNA synthetases, LysS, LysU, and GenX. Although the *lysS* gene has been mapped at 66.2 min on the *Salmonella* chromosome, a location similar to that of the *E. coli* counterpart (12), the *lysU* gene is absent from the *Salmonella* genome (39). To our knowledge, this is the first report showing that PoxA is the second putative lysyl-tRNA synthetase in *Salmonella* (the first being LysS) and that *genX* and *poxA* are allelic. We detected the presence of the *poxA* sequence in several bacterial species including *S. flexneri*, *Y. enterocolitica*, *K. pneumoniae*, and *P. multocida*, by Southern blot analysis (Fig. 5).

The role of PoxA as a lysyl-tRNA synthetase in *S. typhimurium* remains unclear. In a preliminary study, we observed that the *poxA* mutation increases the levels of the Sip (Ssp) proteins in the culture supernatant. These proteins are secreted through the *S. typhimurium* type III protein secretion apparatus encoded in pathogenicity island I. The amount of culture supernatant proteins was partially reduced to the wild-type level when a functional *poxA* copy was introduced in the *poxA* mutant. In addition, we also observed that several foreign antigens are produced at much higher levels in *poxA* mutants compared to other attenuating mutations such as *cya*/*crp* and *phoP*, with the same promoter. These observations are somewhat intriguing, since the analysis of PoxA structure did not reveal the presence of a DNA binding motif or homology to known transcription factors. It is therefore unlikely that the effects of a *poxA* mutation could be at the transcription level. Based upon the homology of PoxA to lysyl-tRNA synthetases, the effect of the gene alteration is most likely at the translation level. Two codons (AAA and AAG) are associated with lysyl-tRNA. Therefore, it is possible that the two lysyl-tRNA synthetases, LysS and PoxA, constantly compete for substrates with different affinities. A mutation abolishing PoxA production would then result in a high translation of some mRNAs such as those encoding the Sips (Ssps) and low translation of others exemplified by PoxB (4, 5).

S. typhimurium poxA mutants were found to be attenuated for virulence in chicks (data not shown) and mice, although they retained the ability to colonize deep tissues and induce strong humoral immune responses. All immunized mice were protected against *Salmonella* lethal challenge, suggesting that the *poxA* mutation could be useful in designing live vaccines from *Salmonella* and possibly from other organisms. The exact mechanisms by which the *poxA* mutation is attenuated will require more investigation. It is known, however, that the *poxA* mutation has pleiotropic effects in *S. typhimurium* (49) including hypersensitivity to the herbicide SM and to AKB. The mutation reduces the levels of acetolactate synthase, an en-

zyme involved in the biosynthesis of branched amino acids such as valine and isoleucine. This results in a low turnover of AKB and hypersensitivity to both SM and AKB (49). SM hypersensitivity due to the lack of acetolactate synthase I has been reported for several other mutations in *S. typhimurium*. These include *ilvB* and *relA* genes (14, 31) and genes encoding integration host factor, adenylate synthase, and catabolic activator protein (15, 16). Van Dyk and LaRossa (48) reported that *poxA* mutants were also hypersensitive to a wide range of compounds of various hydrophobicities and molecular weights that inhibit many different cellular processes. These authors suggested that this phenotype was due to an alteration of membrane permeability. This in turn could lead to a constitutive production and export of Sip (Ssp) proteins in vivo, resulting in attenuation of virulence. However, the absence of immune responses to Sips (Ssps) in mice (unpublished results) argues against that hypothesis. Alternatively, the attenuation of virulence could be the result of reduced growth rate of *S. typhimurium poxA* mutants, compared to the wild-type bacteria. However, our recent study using other *S. typhimurium* SM-hypersensitive mutants (48) does not support this theory. Indeed, although some of these SM-hypersensitive mutants exhibit the same reduced growth rate as the *poxA* strains, they retain full virulence in mice. Therefore, it is unlikely that the attenuation of virulence is the sole result of reduced growth rate of *poxA* mutants compared to the parental strain.

PoxA controls the expression of *poxB*, the gene for pyruvate oxidase structural enzyme (4). Mutations in *poxA* result in a 6- to 10-fold decrease in pyruvate oxidase levels (4, 5), presumably due to an inefficient translation of PoxB mRNA. Although the effect of the *poxB* mutation on virulence has not been evaluated in *Salmonella*, it is noteworthy that the PoxB homolog in *Streptococcus pneumoniae* has been shown to be a virulence determinant in this organism (44). In addition to PoxA, the expression of *poxB* is also regulated by RpoS and cyclic AMP/cyclic AMP receptor protein (6), two global regulators with established roles in *Salmonella* virulence. Regulation of pyruvate oxidase by RpoS, cyclic AMP/cyclic AMP receptor protein, and PoxA strongly suggests that they belong to similar but yet different regulons.

We are currently investigating the mechanism of attenuation by *poxA* mutation by several approaches. In two-dimensional electrophoresis, several proteins are produced only in the Δ*poxA270* strain and not in the parental or the Δ*poxA270*-complemented strains when grown in LB. Such proteins produced only in the *poxA* strain could significantly contribute to the attenuation of bacterial virulence. The identification and characterization of genes encoding these proteins should provide a better understanding of the *poxA* phenotype, including the attenuation of *Salmonella* virulence. *P. multocida poxA* and *S. typhimurium poxB* mutants are also being investigated for reduced virulence in our laboratory. We are also investigating the humoral, cellular, and mucosal immune responses to foreign antigens expressed in the *S. typhimurium* Δ*poxA270* defined deletion strain, as well as to antigens in the carrier strain.

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