Identification of *Pasteurella multocida* tryptophan synthase β -subunit by antisera against strain P1059

Peter E. Jablonski,¹ Lynn M. Jablonski,^{2†} Oscar Pintado,³
Namalamar Sriranganathan³ and Carolyn J. Hovde²

Author for correspondence: Carolyn J. Hovde. Tel: +1 208 885 5906. Fax: +1 208 885 6518.
e-mail: cbohach@uidaho.edu

¹ Department of Biological Sciences, Northern Illinois University, DeKalb, IL 60115-2861, USA

² Department of Microbiology, Molecular Biology and Biochemistry, University of Idaho, Moscow, ID 83844-3052, USA

³ Department of Biosciences and Pathobiology, Virginia Maryland Regional College of Veterinary Medicine, Virginia Polytechnic Institute and State University, Blacksburg, VA 24061-0442, USA

***Pasteurella multocida* strain P1059 is a highly virulent bacterium which causes fowl cholera in turkeys and chickens. A genomic library of *P. multocida* P1059 DNA was constructed using pUC19, expressed in *Escherichia coli* DH5 α , and screened with chicken antisera generated against *P. multocida* P1059. Twelve out of the 4100 clones screened were immunoreactive. Plasmids isolated from these twelve clones were transformed into *E. coli* CSR603 for maxicell analysis. Five proteins, with molecular masses of 34, 37, 43, 46 and 55 kDa, were expressed. Further work focused on the 43 kDa protein because it was expressed at levels detectable by SDS-PAGE and immunoblot analysis. The nucleotide sequence of the 1.8 kbp insert containing the gene encoding this protein was determined. The sequence contained three open reading frames (ORFs). The first ORF (ORF1) did not appear to code for any known protein. The second ORF (ORF2) encoded a protein of 403 amino acids (43 662 Da). The deduced amino acid sequence showed 77% identity (84% similarity) with the tryptophan synthase β subunit (TrpB) of *Salmonella typhimurium* and *Vibrio parahaemolyticus*. The eight conserved regions of TrpB are observed in the *P. multocida* enzyme, including the conserved lysine (Lys-88) and consensus sequence (GGGSNA) implicated in pyridoxal phosphate binding. The expression and identity of the *P. multocida* TrpB were confirmed by complementation studies using *E. coli* W3110 *tnaA2 trpB9578*. The third ORF (ORF3) consisted of the first 77 nucleotides of the gene encoding the α -subunit of tryptophan synthase (*trpA*), and overlapped the 3'-end of *trpB* by 14 nucleotides. The deduced amino acid sequence of the 77 nucleotides of the *P. multocida* TrpA had 68% identity (92% similarity) with the analogous region of TrpA from *Klebsiella aerogenes* (*K. pneumoniae*).**

Keywords: *Pasteurella multocida*, tryptophan synthase, nucleotide sequence analysis

INTRODUCTION

Pasteurella multocida P1059 is one of a number of strains which can cause fowl cholera, a highly contagious disease in chickens and turkeys (Rimler & Rhoades, 1989). The molecular basis of the pathogenesis of *P. multocida* is not well understood, although many putative virulence

factors such as capsule (Hansen & Hirsh, 1989; Snipes *et al.*, 1987), outer-membrane proteins (Lu *et al.*, 1991a, b; Truscott & Hirsh, 1988), lipopolysaccharide (Rhoades & Rimler, 1987; Wijewardana *et al.*, 1990), siderophores (Hu *et al.*, 1986) and toxins (Rhoades & Rimler, 1988) have been reported. Several *P. multocida* genes have been cloned in recent years, including adenylate cyclase (Mock *et al.*, 1991), *P. multocida* toxin (*toxA*) (Lax *et al.*, 1990; Petersen, 1990; Petersen & Foged, 1989), a repressor of *toxA* termed TxaR (Petersen, 1990), the *aroA* gene (Homchampa *et al.*, 1992), and most recently two genes, one of which encodes for a protein antigenically related to porin H (Manoha *et al.*, 1994). Despite this progress, gene

† Present address: Department of Biological Sciences, Northern Illinois University, DeKalb, IL 60115-2861, USA.

The GenBank accession number for the nucleotide sequence reported in this paper is U22344.

structure in *Pasteurella* has not been well investigated. For example, a conserved consensus sequence for the ribosome-binding site is unknown. Further characterization of a variety of *P. multocida* genes will undoubtedly reveal patterns to help elucidate gene regulation and identify potential new virulence factors.

The purpose of this study was to clone and express *in vivo*-expressed antigenic proteins of *P. multocida* P1059 in the hope of identifying potential virulence factors. A number of the 4100 clones analysed reacted with chicken antiserum directed against *P. multocida* P1059. We focused on a 43 kDa protein because of its high expression in an *Escherichia coli* maxicell system. The gene encoding this protein was cloned, sequenced, and identified as encoding for the β subunit of tryptophan synthase (TrpB) based on sequence analysis and complementation studies with an *E. coli* *trpB* auxotroph.

Tryptophan synthase was one of the first enzymes of the tryptophan pathway to be extensively studied. The enzyme catalyses the terminal step in the biosynthesis of tryptophan from L-serine and indoleglycerol phosphate and is a tetramer comprised of two α subunits coded for by the *trpA* gene and a β dimer coded for by the *trpB* gene. The β subunit has two binding sites for pyridoxal phosphate and also two independent sites to which the α subunit can bind (Creighton & Yanofsky, 1966; Wilson & Crawford, 1965). This enzyme has been used to investigate evolutionary relationships between micro-organisms as there is a high degree of similarity among tryptophan synthases from diverse micro-organisms, especially within the β subunit. Here we report the cloning and nucleotide sequence analysis of the *trpB* locus from *P. multocida* and show that it, too, is similar to other *trpB* genes sequenced to date.

METHODS

Bacterial strains. *P. multocida* strain P1059 (ATCC 15742) was obtained from the collection of Dr Gordon Carter (Virginia Tech, Blacksburg, VA, USA). *E. coli* strains DH5 α and CSR603 were obtained from the collection of Dr Scott Minnich (University of Idaho, Moscow, ID, USA). *E. coli* W3110 *tnaA2 trpB9578* was a kind gift of Dr Charles Yanofsky (Stanford University, Stanford, CA, USA).

Preparation of *P. multocida*. *P. multocida* P1059 was grown for 18 h at 37 °C with shaking (150 r.p.m.) in brain heart infusion broth (BHIB). An aliquot (0.1 ml) was used to inoculate 10 ml fresh BHIB. Cells were harvested in late-exponential phase (approximately 425 Klett units, filter no. 54, green) by centrifugation at 7000 g for 10 min at 22 °C, and were washed twice in phosphate-buffered saline (PBS) (10 mM potassium phosphate, pH 7.0, containing 10 mM NaCl). The cell pellet was resuspended in 10 ml PBS and inocula were made by serial dilutions in the same buffer.

Preparation of chickens and chicken antisera. Ten-week-old specific pathogen-free (SPF) white leghorn chickens were purchased from SPAFIS (Norwich, CT, USA). Each group was kept in isolation. However, within a group, birds were free to interact for the duration of the experiment. Birds were housed in temperature-controlled rooms at 20 °C and provided water

and feed *ad libitum*. Chickens were immunized in the wing vein three times, 10 d apart, with an average dose of 3.6×10^8 c.f.u. of live *P. multocida* P1059. Two birds in each group received 200 μ l of PBS as a negative control. To validate infection by *P. multocida*, samples were taken aseptically from the liver, heart, and pericardial fluid. To validate septicaemia, samples were taken from the bone marrow of the femur. The presence of *P. multocida* was confirmed by Gram staining, characteristic colony morphology on 5% (v/v) blood agar, lack of growth on MacConkey agar, and nonfermentative API test strips (bioMérieux Vitek).

Serum was collected from each chicken prior to the first immunization (prebleed) and 10 d after the final immunization. To separate the serum from other components of the blood, the samples were centrifuged at 3000 g for 25 min at 4 °C. Serum was removed, aliquoted, and stored at -80 °C. Once thawed, serum was stored at 4 °C.

Preparation of *in vivo*-grown *P. multocida* P1059. *In vivo*-grown *P. multocida* P1059 cells were harvested from the blood of chickens by differential centrifugation, using a modification of the method of Rimler & Rhoades (1981). Briefly, 14–16-week-old white leghorn chickens were inoculated intravenously with 10^4 c.f.u. of *P. multocida* in the wing vein. At 20–24 h post-inoculation they were bled aseptically via the jugular vein into a syringe containing appropriate amounts of an anticoagulant solution (ACD solution, Rimler & Rhoades, 1981). Plasma was collected by centrifugation (600 g for 30 min at 4 °C). The process was repeated three times. The final supernatant was aspirated and 20 ml was dispensed into sterile round-bottom Correx tubes (25 ml), and centrifuged at 10000 g for 60 min at 4 °C. The supernatant was discarded and the bacterial pellet was resuspended in 20% sucrose/Tris solution [0.025 M Tris/HCl (pH 7.0) containing 20% (w/v) sucrose]. The suspension was layered onto ice-cold 30% sucrose/Tris solution in new Correx tubes and centrifuged at 200 g for 30 min at 4 °C. The bacteria remained in the upper 20% sucrose layer and were recovered by centrifugation at 10000 g for 10 min at 4 °C in 1.5 ml micro-centrifuge tubes. The cells were confirmed to be *P. multocida* as described above and were stored at -80 °C.

Construction and screening of a *P. multocida* P1059 genomic library. Genomic DNA was isolated from *P. multocida* P1059 according to Murray & Thompson (1980). The DNA was digested with *Sau3A* and resolved by electrophoresis through 0.7% (w/v) low-melting-point agarose. Fragments 1–10 kb in size were excised, heated for 15 min at 65 °C, and mixed with *Bam*HI-linearized, dephosphorylated pUC19 at a vector to insert ratio of 1:1. The mixture was incubated at 37 °C for 15 min and then ligated using T₄ DNA ligase overnight at 22 °C. The mixture was used to transform *E. coli* DH5 α by the method of Hanahan (1983). A total of 4100 clones were selected on LB plates containing 100 μ g ml⁻¹ stock of Blue-gal (GIBCO/BRL). Each clone contained an average insert size of 4 kbp, representing over 99% of the genome (based on a genome size of $1.2\text{--}2.2 \times 10^6$ bp; Pohl, 1981). Individual clones were grown overnight at 37 °C in Terrific Broth (TB; Ausubel *et al.*, 1989), harvested, and stored at -80 °C in TB containing 15% (v/v) glycerol. The genomic library was also stored as a pooled plasmid preparation at -20 °C. The library was screened with chicken anti-*P. multocida* P1059 antisera (that had been adsorbed with *E. coli* DH5 α in order to distinguish cross-reactive colonies from those expressing recombinant proteins) by colony blot analysis (Sambrook *et al.*, 1989). Clones which reacted three times were considered positive for the *in vivo* expression of antigens from *P. multocida* P1059. Plasmids were isolated from antibody-reactive clones as described by Sambrook *et al.* (1989).

To confirm that clones originated from *P. multocida*, Southern blotting was performed by standard procedures (Ausubel *et al.*, 1989).

Maxicell analysis. Preparation of electrocompetent *E. coli* cells and transformation by electroporation with a Bio-Rad Gene Pulser apparatus were as described by Dower *et al.* (1988). We selected one of the isolated plasmids, pOP43-2G, for further analysis because it contained an insert encoding high levels of a 43 kDa protein. *E. coli* CSR603 was transformed with pOP43-2G and plated on LB-Amp plates. Ampicillin-resistant colonies were selected and the presence of the plasmid confirmed. A single colony was used to inoculate M63-glucose medium (Sambrook *et al.*, 1989) supplemented with 1% (w/v) Casamino acids (Difco Laboratories) and 100 µg ampicillin ml⁻¹. The culture was incubated at 37 °C overnight with shaking and used to inoculate 10 ml of the same medium and was grown until mid-exponential phase (approximately 100–110 Klett units), transferred to a plastic Petri plate (10 ml × 150 mm) and irradiated with a total of 5300 µJ of UV energy (Stratalinker UV Crosslinker; Stratagene). Irradiated cultures were allowed to recover for 30 min at 37 °C prior to adding cycloserine (final concentration 200 µg ml⁻¹), and were incubated overnight at 37 °C with shaking. Cells were harvested and resuspended in SDS-PAGE sample buffer and analysed on SDS polyacrylamide gels (12%, w/v, acrylamide) as described below.

Gel electrophoresis and Western blotting (immunoblotting). Denaturing SDS-PAGE was carried out as described by Laemmli (1970). Proteins were detected by staining with Coomassie Blue R-250. Immunoblot analysis was performed according to Towbin *et al.* (1979) using alkaline-phosphatase-conjugated anti-chicken rabbit IgG (Sigma), as described by Bollag & Edelstein (1991).

DNA sequencing and analysis. The sequence of both strands of the 1830 bp insert of pOP43-2G was determined from denatured double-stranded templates by the dideoxynucleotide chain-termination method (Sanger *et al.*, 1977) with Sequenase polymerase (version 2.0; USB) and 5'-[α-³⁵S]thio-dATP (New England Nuclear; 1156 Ci nmol⁻¹, 42.8 TBq nmol⁻¹). Oligonucleotides were purchased from USB (−40 and reverse primer for M13mp19) or synthesized by Oligos, Etc (Wilsonville, OR, USA) complementary to sequences internal to the insert. All reactions were carried out according to the manufacturer's protocols. DNA and protein sequences were compared with nonredundant DNA sequence and protein databases with the programs BLASTN and BLASTP (Altschul *et al.*, 1990), respectively, through the electronic mail server at the National Center for Biotechnology Information (NCBI). Sequence data were further analysed using the PC-Genie sequence analysis programs (Intelli-Genetics, Mountain View, CA, USA).

Complementation of *E. coli* trpB mutant strain. *E. coli* W3110 *tnaA2 trpB9578* was made electrocompetent and transformed with pOP43-2G by electroporation as described above. Transformants were plated on M63-glucose supplemented with 1% (w/v) Casamino acids and thiamin (1 µg ml⁻¹) with or without tryptophan (40 µg ml⁻¹) and ampicillin (100 µg ml⁻¹) and incubated overnight at 37 °C. Ampicillin-resistant, tryptophan-independent colonies were selected and assayed for the presence of pOP43-2G. Plasmid pUC19 served as a negative control.

RESULTS AND DISCUSSION

In an effort to detect *in vivo*-expressed antigens of *P. multocida* we created a plasmid-based genomic library and examined 4100 clones, of which 12 reacted with chicken

Fig. 1. Detection of *P. multocida* immunoreactive antigens by immunoblot analysis. Lanes: 1, *in vitro*-grown *P. multocida* P1059; 2, *E. coli* CSR603; 3, *E. coli* CSR603 containing pOP43-2G; 4, *in vivo*-grown *P. multocida* P1059; 5, normal chicken serum. The membrane was processed as described in Methods. The molecular mass standards were (kDa): lysozyme, 14.3; β-lactoglobulin, 18.4; carbonic anhydrase, 29.0; ovalbumin, 43.0; bovine serum albumin, 68.0. TrpB is indicated by an arrow (lane 3).

antisera directed against this organism (data not shown). One of these clones contained a plasmid with a 1.8 kbp insert which was designated pOP43-2G. Using *E. coli* DH5α as a host, we were unable to detect the protein(s) encoded by the insert by SDS-PAGE and immunoblot analysis, suggesting that this strain was not suitable for expression of the cloned genes (data not shown). In addition, we were unable to detect the protein(s) encoded by the insert in *in vitro*-grown *P. multocida* P1059 when grown in BHI broth (Fig. 1, lane 1). However, when we used *E. coli* CSR603 maxicells transformed with pOP43-2G, we detected a 43 kDa protein by immunoblot analysis (Fig. 1, lane 3). This protein, subsequently identified as tryptophan synthase β-subunit (TrpB), was not expressed by *E. coli* CSR603 (Fig. 1, lane 2) nor by *in vivo*-grown *P. multocida* (Fig. 1, lane 4). While equal amounts of protein were loaded onto the polyacrylamide gel, there appeared to be fewer immunoreactive proteins in the *in vivo*-grown (Fig. 1, lane 4) versus *in vitro*-grown (Fig. 1, lane 1) cells. We have routinely observed this phenomenon following immunoblot analyses comparing protein extracts from cells grown under these conditions, although the cause is unknown. However, the antiserum used to develop these immunoblots was produced against *in vitro*-grown *P. multocida*, so one might expect to see stronger and more specific reactions with *in vitro*- rather than *in vivo*-grown cells. It has also been suggested that capsule, present only in the *in vivo*-grown cells, may mask potential antigenic determinants (Neera Galagedera, personal communication). These results suggest that expression of the *trpB* gene in *P. multocida* differs between *in vivo*- and *in vitro*-

```

1  GATTGCAGGGGACTTACCCATATCCGGGTGGCAATGTTTATGGCGCACAGAAGCCTTTATCAAACAATTAGCTTAACTTACGTGCTGATCTCGCAGGC 100
101 ACCGCATTCTGTGACCAATATTGAACCGGGATTATGCGGAGGGACGGAGTTTCCAATGTACGATTCAAAGGCGATGATCAACGCCGCCGCAAAATTATA 200
    ORF1 • M T N I E P G L C G G T E F S N V R F K G D D Q R A A K L Y
201 TGAAGATGTGACAGTTTGTACTCTGAAGATATTGCCAATATTGCTTGTGGGCAAACTCAACAGCCTGAACACGTCAACATTAACCCGATTGAAGTGATG 300
    E D V Q F V T P E D I A N I V L W A N Q Q P E H V N I N R I E V M
301 CCAACACGGGCAAGATGCGCGCCCACTCAATGTCGTCAAAACAAAGAAATAATTCTGCAATTCTTTTCATCATGCTAAAGTGCGAAGTCATTAGCACCT 400
    P T R Q R C A P T Q C R Q T N E I I P A I P F I M L R C E V I S T
401 TTTTCTCTTTTTCACCACGTTTTCGTATTTTTCATAAAACACTTGTCTTTTATATTCTGAACCTAGTTTAATAGTTCAATATTTAAAAATGAAATAG 500
    F F L F F S P R F R Y F S
501 TTTCAATTTTATCCATCACACGATTATCATATTATTGTTTAAAGGAATAACCATGTCAAGAACCTTACTTAATCCCTATTTGCGCGAAATTTGGAGGAA 600
    TrpB • M S E T L L N P Y F G E F G G
601 TGTATGTCCCGAAATTCGTAGCCGCTGTGAAAAATCTCGAAAAAGCCTTGTGTGAGGCACAACAGATCCACATTTAAAGAAACCTTCTCGATT 700
    H Y V P E I L M P V L K N L E K A F V E A Q Q D P T F K E T F L D L
701 ATTGAAGAACTATGCAGTGTGCCCACTGCGCTGACTCGCTGTGCAATCTCACTCAAGGCAGTAAACCAAACCTGTACCTGAAGCTGAAGATTATTG 800
    L K N Y A G R P T A L T R C R N L T Q G S K T K L Y L K R E D L L
801 CATGGCGGTGCGCATAAAACCAATCAAGTATTAGGGCAAACTTATTAGCGAAACGGATGGGCAAAACCGTATTATTGCGGAAACAGGGGCGGGACAAC 900
    H G G A H K T N Q V L G Q I L L A K R M G K T R I I A E T G A G Q
901 ATGGCGTAGCAACGACAGCTCTCGCTTGCCATGCTCGGTATGCCATGCCAAATCTACATGGGCGCAAAAGATGTTGAACGTCAATGCCGCAACGTGTT 1000
    H G V A T T A L A C A M L G M P C Q I Y M G A K D V E R Q S P N V F
1001 TCGTATGCGTTTAATGGGGCGAATGTGACTGCGGTAAACGAAAGGCTCAGCCTCATTAAAGATGCCTGCTGTGAAGCATGCGCGATTGGGCGAGAAAC 1100
    R M R L M G A N V T A V T K G S A S L K D A C C E A M R D W A E N
1101 TATGAACACACCCATTATTTACTCGGCACGGCAGGCTCTCATCCCTTCTCTACTATCGTGGCTGAGTTCCAAAAATCATTGGTGAAGAAACCAAGC 1200
    Y E H T H Y L L G T A A G P H P F P T I V R E F Q K I I G E E T K
1201 AACAAATCTAGCACGCGAAGGGGCTTTACTGATGCAGTCATCGCTGCGGTTGGTGGAGGTTCAATGCCATTGGTATGTTCAATGACTTTATCGAGGA 1300
    Q Q I L A R E G R L P D A V I A A V G G G S N A I G M F N D F I E E
1301 AACTAGCGTACGTTTAATGGTGTGAACGAGCGGGCAAGGAATTGCCACTGGGCAACATGGCGCTCCACTAGGTGATGGCACAACGGGAATCTATTT 1400
    T S V R L I G V E P A G K G I A T G Q H G A P L G H G T T G I Y F
1401 GGTATGAAAGCGCCCTTGATGCAACACAGATGGACAAATGAAGAATCGTATTCGATTTCAGCAGGACTTGATTTTCTTCCGTTGGTCCACAACACG 1500
    G M K A P L M Q T P D G Q I E E S Y S I S A G L D F P S V G P Q H
1501 CACATTTACAAGCGATTGGGCGTGGGGAATATGAATCAATTACCGATGATGAAGCCTTATCCGCTTCGAAGCCTTAGCACGCGATGAAGGCATTATTCC 1600
    A H L Q A I G R A E Y E S I T D D E A L S A F E A L A R D E G I I P
1601 CGCATTAGAAAGTCCCCATGCCCTCGCTTATGCCCTAAAACGATTGAGCGCCACGCTGAAAAAGAACAACTCTTAGTGGTCAATTTATCTGGACGAGGT 1700
    A L E S A H A L A Y A L K L I Q R H A E K E Q L L V V N L S G R G
1701 GATAAGATATTTTACCCTAGATCGCATTTTATCGCAAAAGGAGTTTCACATCGCGCTTTTGATACCTCTTTTCACAATTACAACAAAAACAACA 1800
    D K D I F T V D R I L S Q R G V S H A P F ...
    trpA • M R R F D T L F S Q L Q Q K Q Q
1801 GGTGCATTGTCCCTTTTGTACGCTATGC 1830
    G A F V P F V T L C

```

Fig. 2. Nucleotide and amino acid sequences of *P. multocida* *trpB* and the 5'-end of *trpA*. The DNA sequence was determined from both strands as described in Methods. The deduced amino acid sequence is given in the single-letter code below the nucleotide sequence. Start codons are single-underlined. ORF1 comprises nucleotides 112–438. The 5'-end of *trpB* (nt 555–1767) is indicated and the 3'-end of the gene is indicated by ●●●. The putative Shine–Dalgarno sequence upstream of the predicted ATG initiation codon is double-underlined. The 5'-end of the gene encoding *trpA* is indicated (nt 1753–1830). The putative Shine–Dalgarno sequence upstream of the predicted ATG initiation codon of the *trpA* gene is double-underlined.

grown cells. It is well known that TrpB is located in the cytoplasm, which raises the question as to why this intracellular protein is a prominent antigen in *P. multocida*. We can speculate that since *P. multocida* is cleared from the bloodstream and eventually lysed in the liver and other organs, intracellular proteins may be released and thus become suitable antigens despite their original cellular location.

The nucleotide sequence of the 1.8 kbp insert of pOP43-2G was determined on both strands, and revealed three potential open reading frames (ORFs) (Fig. 2). ORF1 (nt 12–438) is 327 nucleotides long and is upstream of ORF2. Computer analysis of the DNA and the deduced

amino acid sequences did not reveal significant similarity to any gene or protein in the nonredundant nucleic acid or protein databases of NCBI. ORF2 (nt 555–1767) is 1209 nucleotides long and could potentially encode a protein of 403 amino acids (Fig. 2). Analysis of the DNA sequence revealed high similarity with the tryptophan synthase (*trpB*) genes from several strains of bacteria. This ORF was thus designated *trpB*. The gene encoding TrpB starts with an ATG start codon and ends with a TGA termination codon. This termination codon overlaps the ATG initiation codon of ORF3 by 14 nucleotides. The putative product of the *P. multocida* *trpB* gene (43662 Da) showed 77% identity (84% similarity) with the tryptophan synthase β subunit of *Salmonella typhimurium* (Fig.

(a)	
<i>P. m.</i>	MSETLLNPYFGEFGMYVPEILMPVLKNLEKAFVEAQDPTFKETFLDLL -50
<i>S. t.</i>	M-TTLLNPYFGEFGMYVVPQILMPALNQLLEAFVRAQKDFEQAFADLL -49
<i>P. m.</i>	KNYAGRPTALTRCRNLTQGSKTKLYLKREDLLHGGAHKTNQVLGQILLAK -100
<i>S. t.</i>	KNYAGRPTALTKCQNITAGTRTTLYLKREDLLHGGAHKTNQVLGQALLAK -99
<i>P. m.</i>	RMGKTRIIAETGAGQHGVATTALACAMLGMPQIYMGAKDVERQSPNVFR -150
<i>S. t.</i>	RMGKSEIIAETGAGQHGVAT-SALASALLGLKCRIMGAKDVERQSPNVFR -148
<i>P. m.</i>	MRLMGANVTAVTKGSASLKDACCAMRDWAENYETHYLLGTAAGPHPPF -200
<i>S. t.</i>	MRLMGAIEIPVHSGSATLKDACNEALRDWGSYETAHYMLGTAAGPHPPY -198
<i>P. m.</i>	TIVREFQKIIGEETKQILAREGLRDPDAVIAAVGGGSNAIGMFNDFIET -250
<i>S. t.</i>	TIVREFQRMIGEETKAQILDKEGLRDPDAVIACVGGGSNAIGMFADFINDT -248
<i>P. m.</i>	SVRLIGVEPAGKGIATGQHGAPLGHGTTGIYFGMKAPLMQTPDGGIEESY -300
<i>S. t.</i>	SVGLIGVEPGGHGIIETGEHGAPLKHGRVGIYFGMKAPMMQADGGIEESY -298
<i>P. m.</i>	SISAGLDFFPSVGPQHAHLQAIGRAEYESITDDEALSAFEALARDEGIIPA -350
<i>S. t.</i>	SISAGLDFFPSVGPQHAYLNSIGRADYVSITDDEALEAFKTLRHEGIIPA -348
<i>P. m.</i>	LESALHALAYALKLIQRHAEKEQLLVNLSGRGDKDIFTVDRLSQKGVSH -400
<i>S. t.</i>	LESSHALAHALKMMHREKPEKEQLLVNLSGRGDKDIFTVDHILKARG--- -395
<i>P. m.</i>	APF -403
<i>S. t.</i>	-EI -397
(b)	
<i>P. m.</i>	MRRFDTLFSQLQKQGAFAVPFVTL -578
<i>K. a.</i>	MERYETLFAQLKNRQEGAFVPFVTL -25

Fig. 3. Comparison of the predicted amino acid sequences of the TrpB and TrpA subunits. (a) Comparison of the deduced amino acid sequences of TrpB from *P. multocida* (top line) and *S. typhimurium* (bottom line). The eight conserved regions found in TrpB proteins are double-underlined. (b) Comparison of the deduced amino acid sequences of TrpA from *P. multocida* (top line) and *K. aerogenes* (*K. pneumoniae*) (bottom line). (:) indicates identical amino acid residues. In both comparisons, alignment was determined using the sequence alignment programs of PC-Genie.

3a) and *Vibrio parahaemolyticus*. Comparison of the *P. multocida* tryptophan synthase deduced amino acid sequence with other TrpB sequences showed significant identity (ranging from 50 to 77%) as well as similarity (64 to 84%). This is not surprising, since within the tryptophan biosynthetic operon, TrpB sequences have the highest similarity. To confirm the identity of the gene product we complemented an *E. coli trpB* mutant (strain W3110 *tnaA2 trpB9578*), restoring it to tryptophan prototrophy (data not shown).

The *P. multocida* tryptophan synthase β subunit shares several features in common with other tryptophan synthases. Every bacterial tryptophan synthase examined to

date contains eight conserved regions (Crawford, 1989). These regions are also found in the *P. multocida* TrpB (Fig. 3). The amino acid residues important for catalysis have been assigned from the results of site-directed mutagenesis from other bacteria (Miles *et al.*, 1989; Nagata *et al.*, 1989; Yutani *et al.*, 1987) and the three-dimensional structure (Hyde *et al.*, 1988) of the *S. typhimurium* tryptophan synthase. Pre-eminent among these residues is Lys-88 (numbers for the *P. multocida* subunit), which forms a Schiff base with the coenzyme pyridoxal phosphate. Gly-234 to Ala-239 form a pocket for the phosphate group of the coenzyme. Ser-379 and Gly-380 bind other parts of the coenzyme molecule. Glu-110 and His-116 are involved in binding the L-serine substrate.

The G + C content (mol%) of the coding regions of *P. multocida trpB* is 46.3%, with 28.6% A, 22.8% C, 23.5% G and 24.9% T. This is comparable to that of the whole *P. multocida* genome (36–43 mol% G + C), but is higher than that of the *P. multocida* toxin gene (34.4 mol%); this may reflect different evolutionary origins between normal housekeeping genes and virulence factors in this organism. Despite the high level of similarity between the *S. typhimurium* and *P. multocida* genes, codon usage is biased between the two organisms, particularly with codons for leucine, isoleucine, and glutamine.

In addition to *trpB*, the 1.8 kbp fragment of pOP43-2G contains the first 77 nucleotides of the 5'-end of the gene (nt 1753–1830) encoding the α -subunit of the tryptophan synthase (TrpA) of *P. multocida* (Fig. 2). The deduced amino acid sequence has 68% identity (92% similarity) with TrpA of *Klebsiella aerogenes* (*K. pneumoniae*) (Fig. 3b). The start codon for *trpA* is found 14 nucleotides upstream within the 3'-end of *trpB* (Fig. 2). There does not appear to be any other gene of the tryptophan operon within 555 nucleotides upstream of *trpB*. In *E. coli* there is an 11 nucleotide spacer between the *trpC* stop codon and the *trpB* start codon (Yanofsky *et al.*, 1981), while in *Lactococcus lactis* there is a 585 nucleotide spacer between the *trpF* and *trpB* genes (Bardowski *et al.*, 1992). Both genes contain putative ribosome-binding sites (RBS) with sequences of AAGGA (Fig. 2). Comparison of the putative RBS of the *P. multocida trpB* and *trpA* genes revealed perfect similarity with only the RBS of the gene encoding the β -lactamase from *P. haemolytica* (Livrelli *et al.*, 1991).

The identification of the 43 kDa protein examined here as a tryptophan synthase offers a potential explanation of why we were unable to detect this protein when *P. multocida* was grown in rich medium. When *E. coli* is grown in rich medium containing high levels of tryptophan, the tryptophan synthase is repressed; however, when tryptophan is lowered to a specific level, activity is derepressed. The growth of *P. multocida* in a defined medium in which the level of tryptophan can be regulated may provide further information on the regulation of the synthesis of this protein.

The interruption of *aroA* in *P. multocida* results in a highly attenuated mutant (Homchampa *et al.*, 1992). Given the

fact that the enzyme encoded by this gene is involved in the synthesis of aromatic amino acids, one may speculate that interruption of *P. multocida trpB* gene may also result in an attenuated mutant. Little is known about amino acid metabolism in the *Pasteurellaceae* and how it may be involved in virulence. Future work on *trpB* will assess the specific suitability of *trpB* mutants of *P. multocida* as live attenuated vaccines against fowl cholera.

ACKNOWLEDGEMENTS

We thank Dr Charles Yanofsky for providing the *trpB* mutant used in this study and also Drs Gerhart Schurig and Steven Boyle of Virginia Tech for helpful comments related to this work. We also thank Drs Phil Youderian and Scott Minnich at the University of Idaho for helpful suggestions, and Neera Galagedera (Virginia Tech) for production of the *in vivo* antigen preparation. Special thanks are extended to Aline Click at Northern Illinois University for help with preparation of figures.

REFERENCES

- Altschul, S. F., Gish, W., Miller, W., Myers, E. W. & Lipman, D. J. (1990). Basic local alignment search tool. *J Mol Biol* **215**, 403–410.
- Ausubel, F. M., Brent, R., Kingston, R. E., Moore, D. D., Seidman, J. G., Smith, J. A. & Struhl, K. (1989). *Short Protocols in Molecular Biology*. New York: Wiley-Interscience.
- Bardowski, J., Ehrlich, S. D. & Chopin, A. (1992). Tryptophan biosynthesis genes in *Lactococcus lactis* subsp. *lactis*. *J Bacteriol* **174**, 6563–6570.
- Bollag, D. M. & Edelstein, S. J. (1991). *Protein Methods*. New York: Wiley-Liss.
- Crawford, I. P. (1989). Evolution of a biosynthetic pathway: the tryptophan paradigm. *Annu Rev Microbiol* **43**, 567–600.
- Creighton, T. E. & Yanofsky, C. (1966). Association of the α and β_2 subunits of the tryptophan synthetase of *Escherichia coli*. *J Biol Chem* **241**, 980–990.
- Dower, W. F., Miller, F. & Ragsdale, C. W. (1988). High efficiency transformation of *Escherichia coli* by high voltage electroporation. *Nucleic Acids Res* **16**, 6127–6145.
- Hanahan, D. (1983). Studies on transformation of *Escherichia coli* with plasmids. *J Mol Biol* **166**, 557–580.
- Hansen, L. M. & Hirsh, D. C. (1989). Serum resistance is correlated with encapsulation of avian strains of *Pasteurella multocida*. *Vet Microbiol* **21**, 177–184.
- Homchampa, P., Strugnelli, R. A. & Adler, B. (1992). Molecular analysis of the *aroA* gene of *Pasteurella multocida* and vaccine potential of a constructed *aroA* mutant. *Mol Microbiol* **6**, 3585–3593.
- Hu, S. P., Felice, L. J., Sivanandan, V. & Maheswaran, S. K. (1986). Siderophore production by *Pasteurella multocida*. *Infect Immun* **54**, 804–810.
- Hyde, C. C., Ahmed, S. A., Padlan, E. A., Miles, E. W. & Davies, D. R. (1988). Three dimensional structure of the tryptophan synthetase $\alpha\beta_2$ multienzyme complex from *Salmonella typhimurium*. *J Biol Chem* **263**, 17857–17871.
- Laemmli, U. K. (1970). Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature* **227**, 680–685.
- Lax, A. J., Chanter, N., Pullinger, G. D., Higgins, T., Staddon, J. M. & Rozengurt, E. (1990). Sequence analysis of the potent mitogenic toxin of *Pasteurella multocida*. *FEBS Lett* **277**, 59–64.
- Livrelli, V., Peduzzi, J. & Joly, B. (1991). Sequence and molecular characterization of the ROB-1 beta-lactamase gene from *Pasteurella haemolytica*. *Antimicrob Agents Chemother* **35**, 242–251.
- Lu, Y. S., Aguila, H. N., Lai, W. C. & Pakes, S. P. (1991a). Antibodies to outer membrane proteins but not to lipopolysaccharide inhibit pulmonary proliferation of *Pasteurella multocida* in mice. *Infect Immun* **59**, 1470–1475.
- Lu, Y. S., Lai, W. C., Pakes, S. P. & Nie, L. C. (1991b). A monoclonal antibody against a *Pasteurella multocida* outer membrane protein protects rabbits and mice against pasteurellosis. *Infect Immun* **59**, 172–180.
- Manoha, F., Chevalier, G., Wróblewski, H. & Delamarche, C. (1994). Cloning and expression of two *Pasteurella multocida* genes in *Escherichia coli*. *Biochimie* **76**, 9–14.
- Miles, E. W., Kawasaki, H., Ahmed, S. A., Morita, H., Morita, H. & Nagata, S. (1989). The β subunit of tryptophan synthase. *J Biol Chem* **264**, 6280–6287.
- Mock, M., Crasnier, M., Duflot, E., Dumay, V. & Danchin, A. (1991). Structural and functional relationships between *Pasteurella multocida* and enterobacterial adenylate cyclases. *J Bacteriol* **173**, 6265–6269.
- Murray, M. G. & Thompson, W. F. (1980). Rapid isolation of high molecular weight plant DNA. *Nucleic Acids Res* **8**, 4321–4325.
- Nagata, S., Hyde, C. C. & Miles, E. W. (1989). The α subunit of tryptophan synthase. *J Biol Chem* **264**, 6288–6296.
- Petersen, S. K. (1990). The complete nucleotide sequence of the *Pasteurella multocida* toxin gene and evidence for a transcriptional repressor, TxaR. *Mol Microbiol* **4**, 821–830.
- Petersen, S. K. & Foged, N. T. (1989). Cloning and expression of the *Pasteurella multocida* toxin gene, *tox A*, in *Escherichia coli*. *Infect Immun* **57**, 3907–3913.
- Pohl, S. (1981). *Pasteurella multocida*. In *Haemophilus, Pasteurella, and Actinobacillus*, pp. 245–254. Edited by K. Killian, W. Fredericksen & E. L. Biberstein. London: Academic Press.
- Rhoades, K. R. & Rimler, R. B. (1987). Effects of *Pasteurella multocida* endotoxins on turkey poult. *Avian Dis* **31**, 523–526.
- Rhoades, K. R. & Rimler, R. B. (1988). Toxicogenicity and virulence of capsular serogroup D *Pasteurella multocida* strains isolated from turkeys. *J Am Vet Med Assoc* **192**, 1790.
- Rimler, R. B. & Rhoades, K. R. (1981). Lysates of turkey-grown *Pasteurella multocida*: protection against homologous and heterologous serotype challenge exposures. *Am J Vet Res* **42**, 2117–2121.
- Rimler, R. B. & Rhoades, K. R. (1989). *Pasteurella multocida*. In *Pasteurella and Pasteurellosis*, pp. 37–73. Edited by C. Adlam & J. M. Rutter. London: Academic Press.
- Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular Cloning: a Laboratory Manual*, 2nd edn. Cold Spring Harbor, NY: Cold Spring Harbor Laboratory.
- Sanger, F., Nicklen, S. & Coulson, A. R. (1977). DNA sequencing with chain-terminating inhibitors. *Proc Natl Acad Sci USA* **74**, 5463–5467.
- Snipes, K. P., Ghazikhanian, G. Y. & Hirsh, D. C. (1987). Fate of *Pasteurella multocida* in the blood vascular system of turkeys following intravenous inoculation: comparison of an encapsulated, virulent strain with its avirulent, acapsular variant. *Avian Dis* **31**, 254–259.
- Towbin, H., Staehelin, T. & Gordon, J. (1979). Electrophoretic transfer of proteins from polyacrylamide gels to nitrocellulose sheets: procedure and some applications. *Proc Natl Acad Sci USA* **76**, 4350–4354.
- Truscott, W. M. & Hirsh, D. C. (1988). Demonstration of an outer

membrane protein with antiphagocytic activity from *Pasteurella multocida* of avian origin. *Infect Immun* **56**, 1538–1544.

Wijewardana, T. G., Wilson, C. F., Gilmour, N. J. & Poxton, I. R. (1990). Production of mouse monoclonal antibodies to *Pasteurella multocida* type A and the immunological properties of a protective anti-lipopolysaccharide antibody. *J Med Microbiol* **33**, 217–222.

Wilson, D. A. & Crawford, I. P. (1965). Purification and properties of the B component of *Escherichia coli* tryptophan synthetase. *J Biol Chem* **240**, 4801–4808.

Yanofsky, C., Platt, T., Crawford, I. P., Nichols, B. P., Christie,

G. E., Horowitz, H., VanCleemput, M. & Wu, A. M. (1981). The complete nucleotide sequence of the tryptophan operon of *Escherichia coli*. *Nucleic Acids Res* **9**, 6647–6668.

Yutani, K., Ogasawara, K., Tsujita, T., Kanemoto, K., Matsumoto, M., Tanaka, S., Miyashita, T., Matsushiro, A., Sugino, Y. & Miles, E. W. (1987). Tryptophan synthase α subunit glutamic acid 49 is essential for activity. *J Biol Chem* **262**, 13429–13433.

.....
Received 18 May 1995; revised 2 September 1995; accepted 20 September 1995.