

OspA, a Lipoprotein Antigen of the Obligate Intracellular Bacterial Pathogen *Piscirickettsia salmonis*

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Abstract

No effective recombinant vaccines are currently available for any rickettsial diseases. In this regard the first non-ribosomal DNA sequences from the obligate intracellular pathogen *Piscirickettsia salmonis* are presented. Genomic DNA isolated from Percoll density gradient purified *P. salmonis*, was used to construct an expression library in lambda ZAP II. In the absence of preexisting DNA sequence, rabbit polyclonal antiserum raised against *P. salmonis*, with a bias toward *P. salmonis* surface antigens, was used to identify immunoreactive clones. Catabolite repression of the *lac* promoter was required to obtain a stable clone of a 4,983 bp insert in *Escherichia coli* due to insert toxicity exerted by the accompanying *radA* open reading frame (ORF). DNA sequence analysis of the insert revealed 1 partial and 4 intact predicted ORF's. A 486 bp ORF, *ospA*, encoded a 17 kDa antigenic outer surface protein (OspA) with 62% amino acid sequence homology to the genus common 17 kDa outer membrane lipoprotein of *Rickettsia prowazekii*, previously thought confined to members of the genus *Rickettsia*. Palmitate incorporation demonstrated that OspA is posttranslationally lipidated in *E. coli*, albeit poorly expressed as a lipoprotein even after replacement of the signal sequence with the signal sequence from *lpp* (Braun lipoprotein) or the rickettsial 17 kDa homologue. To enhance expression, *ospA* was optimized for codon usage in *E. coli* by PCR synthesis. Expression of *ospA* was ultimately improved (~13% of total protein) with a truncated variant lacking a signal sequence. High level expression (~42% tot. prot.) was attained as an N-terminal fusion protein with the fusion product recovered as inclusion bodies in *E. coli* BL21. Expression of OspA in *P. salmonis* was confirmed by immunoblot analysis using polyclonal antibodies generated against a synthetic peptide of OspA (110-129) and a strong antibody response against OspA was detected in convalescent sera from coho salmon (*Oncorhynchus kisutch*).

Introduction

A renaissance in the identification of obligate intracellular pathogens as aetiological agents of many poorly understood diseases and emerging pathogens has been made possible by the advent of recombinant DNA technology (Anderson, 1997, Azad and Beard, 1998, Azad, *et al.*, 1997, Davis, *et al.*, 1998, Fryer and Mauel, 1997, Stenos, *et al.*, 1998). Inherent difficulties accompany investigations of obligate intracellular bacteria; *Rickettsiales* have poorly defined genetic systems (Mallavia, 1991), and grow slowly in cell culture making it difficult to produce cells *en masse* and to separate them from host cell material. Historically, characterization of rickettsial pathogenesis and the discrimination of rickettsial antigens has largely relied upon antibody inactivation studies of specific rickettsial surface proteins (Li and Walker, 1998, Messick and Rikihisa, 1994, Seong, *et al.*, 1997). Recently, a few major rickettsial antigens have been identified and characterized further upon sub-cloning into *Escherichia coli* (Anderson, 1990, Anderson, *et al.*, 1987, Carl, *et al.*, 1990, Ching, *et al.*, 1992, Ching, *et al.*, 1996, Hahn and Chang, 1996, Musoke, *et al.*, 1996).

In spite of their gaining importance there are as yet no effective, commercial vaccines against human rickettsial diseases (Sumner, *et al.*, 1995); although whole cell vaccines do elicit protective responses in animal models, they are only partially effective in humans (Sumner, *et al.*, 1995). Current vaccine strategies using recombinant rickettsial protein immunogens have been shown to successfully elicit protective immune responses against bacterial challenge (McDonald, *et al.*, 1987, Sumner, *et al.*, 1995).

Piscirickettsia salmonis, is the aetiological agent of salmonid rickettsial septicaemia (SRS) (Branson and Nieto Diaz-Munoz, 1991, Lannan and Fryer, 1993), an economically significant disease that heavily impacts the rearing of cold water salmonids. *P. salmonis* is an unusually recalcitrant, psychrotrophic, gram-negative bacterium which grows very slowly taking 15-20 days to develop a full cytopathic effect (CPE) with a cell yield ~10% of other rickettsial strains. *P. salmonis* is the first rickettsia-like bacterium to be isolated from an aquatic poikilotherm (Fryer, *et al.*, 1990) and since initial isolation in Chile in 1989, *P. salmonis* has also been identified in Norway (House, *et al.*, 1999), Scotland, and on the Atlantic and Pacific coasts of Canada (Brocklebank, *et al.*, 1993, Jones, *et al.*, 1998) thus appearing to be emerging. No effective vaccines are currently available against *P. salmonis*, leaving heavy antibiotic regimes as the only management alternative. While *P. salmonis* adheres to the historical definition of rickettsiae as being an obligate intracellular bacterium, recent 16S rRNA analysis clearly indicates that *P. salmonis* is phylogenetically unrelated to the majority of *Rickettsiales* (Fryer, *et al.*, 1992, Mauel, *et al.*, 1999).

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Previous studies of *P. salmonis* focused on its growth, purification, and preliminary identification of its surface antigens as possible vaccine candidates. Here we were able to purify enough *P. salmonis* genomic DNA to construct an expression library and to screen it with the intent of cloning and identifying immunoreactive surface antigens as potential recombinant vaccine candidates. We report the first non-ribosomal DNA sequences from *P. salmonis* and the identification, characterization, and optimized expression of a 17 kDa lipoprotein antigen, OspA, with distinct homology to a 17 kDa surface lipoprotein of the genus *Rickettsia*.

Results

Identification of Immunoreactive *P. salmonis* Lambda ZAP II Clones

Screening of the *P. salmonis* expression library with high titre anti-*P. salmonis* rabbit serum identified 18 strongly immunoreactive plaques. These plaques were picked and were rescreened until pure. Initial attempts to excise the pBluescript clones from the lambda phagemids using helper phage were unsuccessful. Endonuclease digests conducted on genomic lambda DNA from all 18 clones showed that 16 of the clones had 7 kb inserts, while the other two clones had inserts sizes of 6 and 5.5 kb. Restriction fragment length analysis with frequently cutting endonucleases suggested that 16 of the clones were identical and all 18 clones contained a common region of DNA (data not shown).

Successful *in vivo* excision of the pBluescript clones was achieved only when the *lac* promoter was repressed using a 1% glucose supplement in all media. Only the 5.5 kb lambda clone yielded a stable insert-containing pBluescript clone, pB12, encoding a 5 kb insert (Figure 1), but was very slow growing even under glucose repression.

To confirm that the insert obtained in clone pB12, was of *P. salmonis* origin and was the same insert encoded by

the lambda phagemid clones, DNA dot blot analysis was used. Genomic DNA from all 18 lambda clones, *P. salmonis*, CHSE-214, and pBluescript plasmid DNA was analyzed by DNA dot blotting using insert DNA from pB12 as the probe. Hybridization revealed that the pB12 insert was of *P. salmonis* origin. The pB12 insert also hybridized with all 18 lambda clone genomic DNA samples indicating that all the inserts encoded an overlapping fragment of *P. salmonis* DNA.

Sequence Analysis of Clone pB12

DNA sequencing of *Exo III/S1* nuclease deletion clones of the pB12 insert revealed that the insert was 4,983 bp long and had a total A+T content of 59% (Figure 1). Coding predictions using Borodovsky's Markov method (Borodovsky, *et al.*, 1995) identified 4 intact open reading frames (ORF) and 1 partial ORF creating a gene fusion in frame with *lacZ* (Figure 1). The predicted ORF's were subjected to BLAST2 (Altschul, *et al.*, 1997) and FASTA3 (Pearson, 1998) analysis to determine if any similar sequences were known.

The 499 bp partial '*alr*' ORF (Figure 1) had a 54% A+T content and was predicted to encode a 176 amino acid (a.a.) protein fused to the N-terminal fragment of LacZ. The predicted molecular weight of the LacZ-'Alr fusion is 22.2 kDa. The predicted a.a. sequence of the '*alr*' ORF shares 49% a.a. identity and 65% a.a. similarity with the 167 a.a. C-terminal region of alanine racemase from *Pseudomonas aeruginosa* (GenBank AAD47082).

A 732 bp ORF (*bax*; Figure 1) with a 61% A+T content was predicted to encode a 243 a.a., 27.6 kDa protein. Both FASTA3 and BLAST2 only identified low scoring a.a. homology (33% identical, 49% similar) between the central 187 a.a. region of the *bax* ORF and a 274 a.a. predicted ORF, BAX, in *E. coli* K12 (GenBank AAB18547).

A 1368 bp ORF (*radA*; Figure 1) with a 57% A+T content was predicted to encode a 456 a.a., 49.4 kDa protein. A high degree of a.a. homology was found between

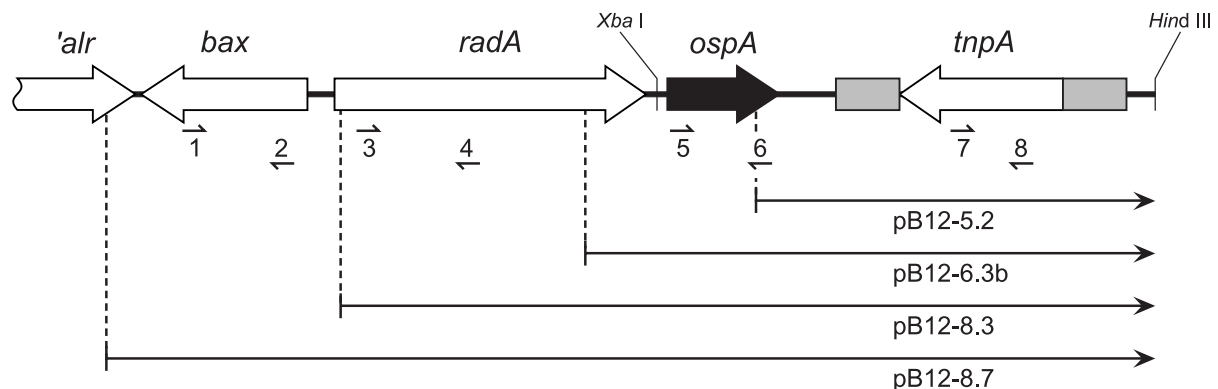


Figure 1. A schematic of the ORF's in the 4,983 bp *P. salmonis* insert of clone pB12. Open arrows depict predicted ORF's and their coding direction. The shaded regions represent a 288 bp direct repeat that flanks the predicted transposase ORF, *tnpA*. The entire pB12 insert has a G+C content of 41%. The regions of clone pB12 encoded by deletion clones pB12-5.2, pB12-6.3b, pB12-8.3, and pB12-8.7 are represented by thin black arrows. The *Xba* I and *Hind* III restriction sites were used to subclone the *ospA* into pBC. Primers 1 and 2 (5'-TGC TCA ACA ACC CTT ATT CTC AG-3' and 5'-TAG GCG TTA TGT GTA TTT AGT GTA TGT ATG-3') were used to generate a 522 bp probe of the *bax* ORF. Primers 3 and 4 (5'-TAA GTG AAG TAA AAG AAG TGC TGC CAA ATC-3' and 5'-CGT GTT TAT TTT TAG TCG GTC ATG TGA-3') were used to generate a 564 bp probe of the *radA* ORF. Primers 5 and 6 (5'-ACA GAG GAT GTT TGC AAG GTA GTA GTC TAA T-3' and 5'-ATG GTC GTT GGC AAG TCA TTT CAA-3') were used to generate a 474 bp probe of the *ospA* ORF. Primers 7 and 8 (5'-AAA AAC GTA ATA AGC CTT CAC AAA TGT CAA C-3' and 5'-AAA GGC CAT TAA CTC TCC CAT ATC ATT CAC-3') were used to generate a 398 bp probe of the *tnpA* ORF.

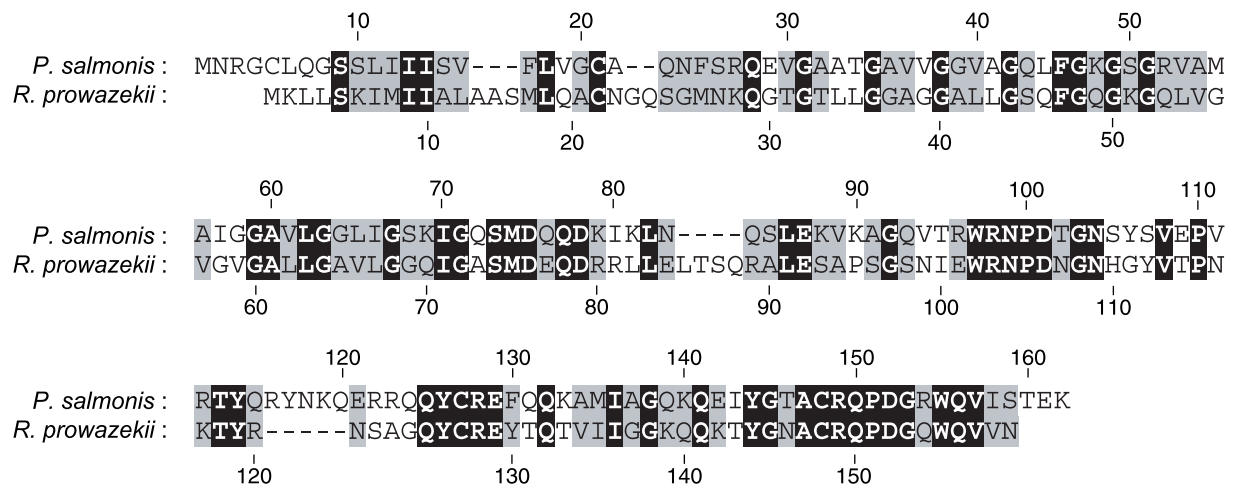


Figure 2. Pairwise sequence alignment of the *P. salmonis* 17 kDa antigen, OspA, and the *R. prowazekii* 17 kDa antigen (SwissProt G112704). The pairwise alignment was generated using the FASTA3 algorithm. The *P. salmonis* 17 kDa antigen shares 41% identity (black background) and 62% similarity (gray background) with the 17 kDa antigen of *R. prowazekii*. Synthetic peptides representing the region from a.a. 110-129 of the *P. salmonis* 17 kDa antigen were used to generate rabbit polyclonal serum.

the entire predicted *P. salmonis* RadA a.a. sequence and the RadA DNA repair enzymes from a variety of bacteria. *P. salmonis* RadA is most homologous to the RadA of *P. aeruginosa* (SwissProt P96963) with 62% a.a. identity and 77% a.a. similarity. *P. salmonis* RadA also has 59% a.a. identity and 75% a.a. similarity to *E. coli* RadA (SwissProt P24554).

A 486 bp ORF (*ospA*; Figure 1), immediately following *radA*, with a 51% A+T content was predicted to encode a 162 a.a., 17.7 kDa protein with the 21-162 a.a. region having sequence similarity to the processed rickettsial 17 kDa genus common antigen (Figure 2). The predicted a.a. sequence of the *ospA* ORF was up to 41% identical and 62% similar to the 17 kDa protein antigens of *R. prowazekii* (SwissProt P16624), *Rickettsia japonica* (SwissProt Q52764), *Rickettsia rickettsii* (SwissProt P05372), and *Rickettsia typhi* (SwissProt P22882).

The final 717 bp ORF (*tnpA*; Figure 1) had a 66% A+T content and was predicted to encode a 239 a.a., 27.7 kDa protein. The *tnpA* ORF is flanked by a perfect 288 bp direct repeat. Amino acid similarity searches returned strong matches between the predicted a.a. sequence of the *tnpA* ORF and a variety of transposases. The closest match

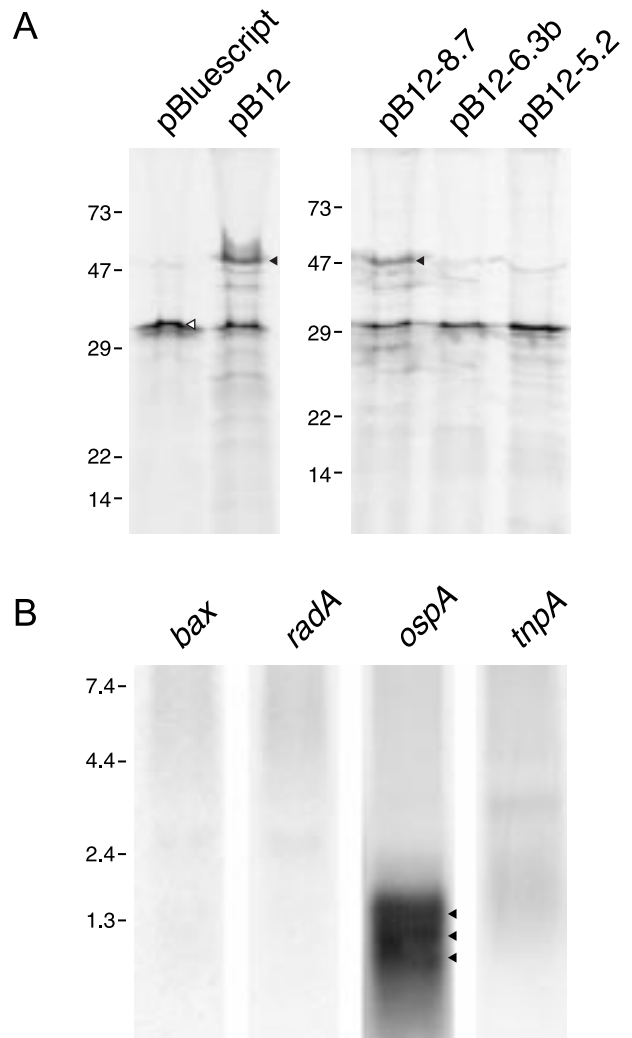


Figure 3. A) *in vitro* transcription and translation of pB12 using *E. coli* S30 extracts. Products of *in vitro* transcription and translation were [^{14}C]leucine labeled and analyzed by 12% SDS-PAGE. A 49 kDa protein (black arrow) was produced by pB12 and pB12-8.7 plasmids but not by deletion clones pB12-6.3b and pB12-5.2 which lack the *radA* ORF. Expression of a 31 kDa product corresponding to β -lactamase (white arrow) was present in all samples. Molecular weights are on the left in kDa. B) Northern blot analysis of total RNA from clones pB12 and pB12-6.3b. 10 μg of total RNA was loaded per lane and analyzed by 1.2% formaldehyde agarose gel electrophoresis. Clone pB12 total RNA was hybridized with probes specific to the *bax* and *radA* ORF's. Clone pB12-6.3b total RNA was allowed to hybridize with *ospA* and *tnpA* probes. The *ospA* probe hybridized with 3 transcripts (1.4, 1.2, and 0.9 kb) in the pB12-6.3b sample. No transcripts specific to the *bax*, *radA*, and *tnpA* ORF's were detected. None of the probes hybridized with *E. coli* DH5 α total RNA (results not shown). Molecular weight standards shown are in kb.

was a transposase encoded in a *Porphyromonas gingivalis* insertion element, IS195 (GenBank U83995), with 47% a.a. identity and 65% a.a. similarity (Lewis and Macrina, 1998).

In vitro Transcription and Translation of Clone pB12

Deletion clones of pB12 were used for *in vitro* transcription and translation experiments. Deletion clones were chosen on the basis of whether or not they encoded particular predicted ORF's (Figure 1). Clone pB12-8.7 was chosen because it only lacked the majority of the *lacZ'*-*alr* construct (Figure 1). Clone pB12-6.3b lacked the majority of the *radA* ORF and pB12-5.2 lacked the *ospA* ORF as well (Figure 1). Five proteins with masses of 17, 22, 28, 28 and 49 kDa were predicted to be expressed from the predicted ORF's of clone pB12. A protein in the 49 kDa range corresponding to RadA was expressed by clones pB12 and pB12-8.7 at levels similar to β -lactamase (31 kDa) in the pBluescript control (Figure 3A). Expression of the 49 kDa product was abolished by elimination of the *radA* ORF in clones pB12-6.3b and pB12-5.2 (Figure 3A). No detectable expression of proteins corresponding to the 3 other ORF's was observed.

Northern Blot Analysis of Clone pB12

Total RNA from clone pB12 was probed for expression of *bax* and *radA* transcripts. Total RNA from clone pB12-6.3b was selected for analysis of *ospA* and *tnpA* expression because it did not encode the *bax* and *radA* ORF's and it grew far better than pB12. Each probe was hybridized with total RNA from *E. coli* and no hybridization was observed with any probe (results not shown). Transcriptional products for the *bax*, *radA*, and *tnpA* ORF's were not detected in clone pB12 and pB12-6.3b (Figure 3B). However, *ospA* transcripts were detected in pB12-6.3b (Figure 3B). The *ospA* probe hybridized with a broad band around 1.2 kb in size (Figure 3B). This band appears to be comprised of three unresolving bands approximately 1.5, 1.2, and 0.9 kb in size.

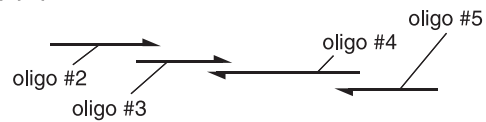
PCR Synthesis of a Codon Optimized *ospA* gene, 17E2

In an effort to obtain high level *E. coli* expression of the *P. salmonis ospA* ORF, a synthetic version of *ospA* (17E2), was constructed by PCR (Figure 4A). Analysis of the codon usage of *ospA* found that 20% of the codons were rare *E. coli* codons (Henaut and Danchin, 1996). When the 17E2 gene was synthesized the Asn-2 codon was removed and codon usage of the entire gene was optimized for high level expression in *E. coli*.

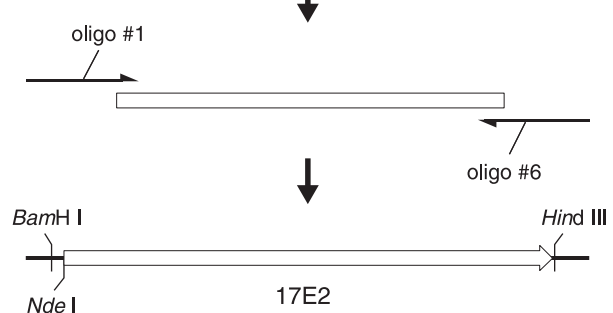
Four putative pET-17E2 clones, identified by *Nde* I / *Hind* III excision of an appropriately sized insert, were DNA sequenced. Only one of the four 17E2 clones was confirmed to have its reading frame intact. The reading frames of the other three clones were destroyed by single base pair deletions at various positions throughout the gene. The chosen pET-17E2 clone contained two base pair substitutions that created transitions of Ala-54→Cys-54, and Arg-149→Pro-149. These base pair substitutions were attributed to the fidelity of *Taq* DNA polymerase during PCR and were considered acceptable because they did not alter the antigenic profile of OspA.

A

PCR 1-1

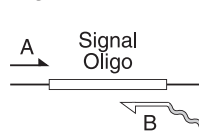


PCR 1-2

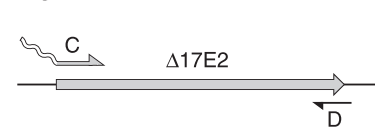


B

PCR 2-1



PCR 2-2



Nde I

Sac I

PCR 2-3

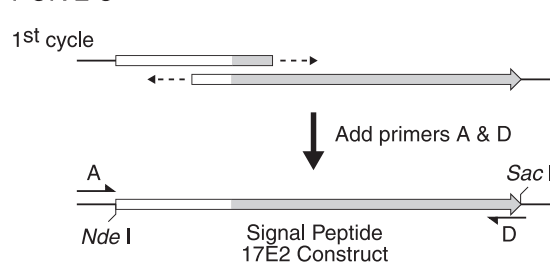


Figure 4. A) A schematic illustration of the PCR strategy used when constructing an *E. coli* codon optimized, synthetic version of the *ospA* gene, 17E2. Assembly of the gene was conducted using two sequential PCR reactions, in which the product of the reaction 1-1 served as the template for reaction 1-2. B) A schematic illustration of the PCR strategy used to add heterologous signal sequences to the truncated version of optimized *ospA*, Δ 17E2. In the two initial PCR reactions, 2-1 and 2-2, homologous regions of the synthetic oligonucleotide signal sequence (signal oligo) and Δ 17E2 are added to each other using primers B and C, respectively. The products of these two reactions are used as primers to each other in the initial round of amplification in PCR reaction 2-3. During the denaturation step following the first cycle of reaction 2-3, primers A and D are added to allow amplification of the full length product in the remaining 29 cycles.

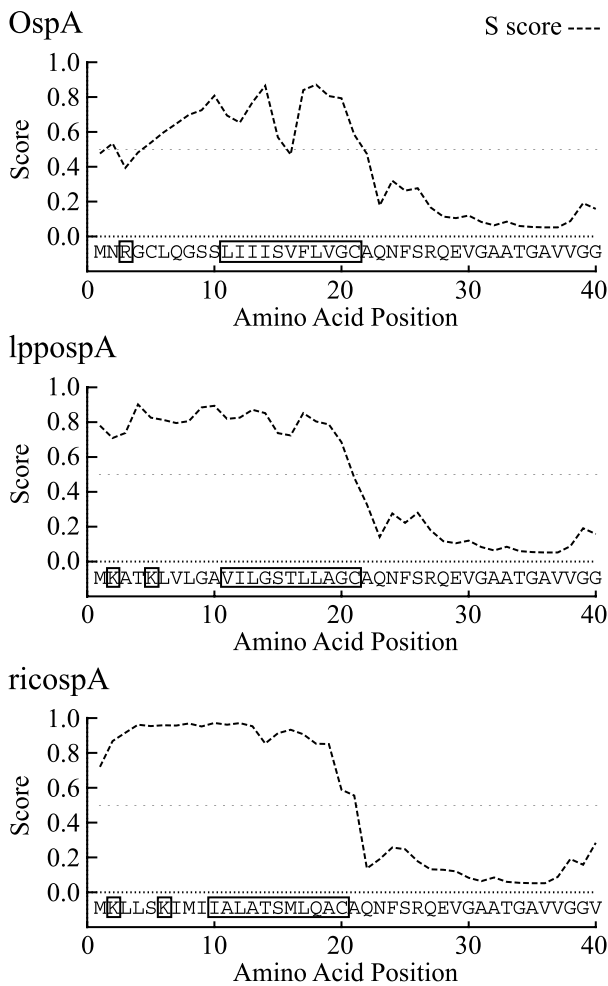


Figure 5. SignalP analysis of OspA and chimeric signal peptide constructs. The signal peptide score (S-score) recognizes signal peptide versus non-signal-peptide positions and should be high at all positions before the cleavage site (Nielsen, *et al.*, 1997). Residues surrounded by boxes represent the PROSITE consensus regions for prokaryotic lipid attachment. Note how replacement of the OspA signal peptide with the signal peptides of Braun's lipoprotein (lppospA) and *R. rickettsii* 17 kDa antigen dramatically improve the signal peptide prediction of OspA.

Removal and Replacement of the OspA Signal Sequence

PROSITE ProfileScan analysis of OspA identified a consensus pattern for prokaryotic membrane lipoprotein lipid attachment (PS00013) with Cys-21 (Figure 5) as the predicted acylation site in the 11-21 a.a. region (Hofmann, *et al.*, 1999).

SignalP calculated a 0.464 probability that OspA has a signal peptide (Figure 5). The signal sequence of 17E2 was removed by PCR to create a truncated version of *ospA*, pET- Δ 17E2. The Δ 17E2 construct lacked the N-terminal 20 a.a. of wild type (w.t.) OspA (Figure 2), and Cys-20 was replaced by a Met start codon. The signal sequence of 17E2 was also replaced by with the type II signal sequences of Braun's lipoprotein (*lpp*) and the *R. rickettsii* 17 kDa antigen (Anderson and Tzianabos, 1989, Inouye, *et al.*, 1977). The codon usage of the signal sequences was

optimized for high level *E. coli* expression and were added to the Δ 17E2 construct by PCR (Figure 4B). Replacement of the OspA signal peptide region with the signal peptides of Braun's lipoprotein and the *R. rickettsii* 17 kDa antigen improved calculated probability of having a signal peptide to 0.991 and 0.998, respectively (Figure 5).

Expression of *ospA* Under the Control of the T7 Promoter

The *Xba*I/*Hind*III fragment of clone pB12-6.3b (Figure 1) was cloned into pBC KS placing *ospA* under the control of the T7 promoter. Only low levels of expression of a 17 kDa product was obtained upon induction in *E. coli* XL1-Blue (Figure 6A).

Comparative Expression of OspA Constructs

Induced expression levels of OspA from codon optimized pET-17E2 in *E. coli* BL21 were not higher than those obtained with w.t. *ospA* from pBC-17kDa (Figure 6A). Replacement of the OspA signal peptide with signal peptides from Braun's lipoprotein and the *R. rickettsii* 17 kDa antigen signal peptides did not result in readily detectable expression levels of OspA (Figure 6A). However, removal of the OspA signal peptide allowed substantially

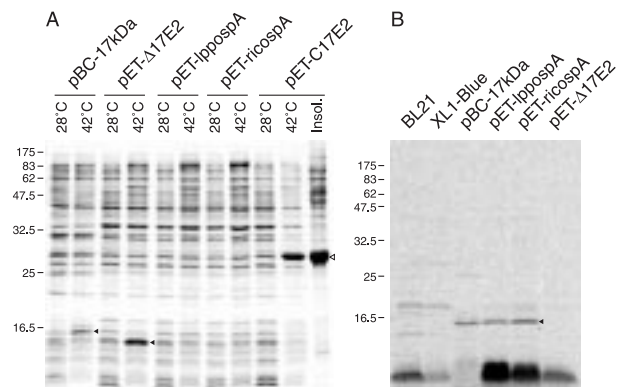


Figure 6. A) Comparative *E. coli* production of OspA protein constructs. Whole cell lysates of *E. coli* OspA clones were analyzed by SDS-PAGE (15% polyacrylamide). Samples of all OspA constructs at time 0 (28°C) and after 10 h induction at 42°C were stained with GelCode. For clarity, only pBC-17kDa is included as representative of OspA production levels obtained from both pBC-17kDa and pET-17E2. *E. coli* XL1-Blue and BL21 carrying pET21a were routinely induced in parallel with OspA constructs and no products that correlated with the m.w. of OspA products were observed at either time 0 or following induction (results not shown). Induced expression of OspA products from constructs pBC-17kDa and pET- Δ 17E2 were visible around 16 kDa, and at 28 kDa in the pET-C17E2 sample (black arrows). The 28 kDa OspA fusion protein was localized to the insoluble (Insol.) fraction of induced pET-C17E2. Quantification of samples revealed that truncated OspA (pET- Δ 17E2) was expressed as 13% of total cellular protein and the OspA fusion was expressed as 34% tot. prot. representing 42% of the insoluble protein fraction. Molecular weights are on the left in kDa. B) [¹⁴C]Palmitate incorporation analysis of OspA constructs by SDS-PAGE (15% polyacrylamide). *E. coli* XL1-Blue and BL21 were induced under the same conditions as the OspA constructs to serve as negative controls for background palmitate incorporation. pET- Δ 17E2 which encoded a truncated form of *ospA* that lacked a signal sequence was included as a negative control for OspA palmitate incorporation. Note the 16 kDa palmitate labeled product expressed in only by induced cultures of pBC-17kDa, pET-lppospA, and pET-ricospA (black arrow). Removal of the OspA signal peptide in pET- Δ 17E2 abolished incorporation of palmitate. Molecular weights are on the left in kDa.

increased expression of 'OspA (13% tot. prot.) relative to OspA expression from pBC-17kDa and pET-17E2 (Figure 6A).

Posttranslational Modification of OspA

[¹⁴C]palmitate incorporation was used to determine whether OspA is processed as a lipoprotein in *E. coli*. Actively growing cultures of pBC-17kDa, pET-lppospA, pET-ricospA, and pET-Δ17E2 were induced and labeled with [¹⁴C]palmitate for 3 hr. Whole cell lysates of the clones were analyzed by 15% SDS-PAGE (Figure 6B). Wild type OspA and the chimeric ricospA and lppospA constructs were all labeled with comparable levels of [¹⁴C]palmitate (Figure 6B). Removal of the signal peptide from OspA abolished [¹⁴C]palmitate incorporation (Figure 6B).

Coupling of OspA with an N-Terminal Fusion Partner

17E2 was cloned in frame with a 91 a.a. N-terminal fusion partner protein in an effort to prevent recognition of the signal peptide and subsequent lipid modification in *E. coli*. Very high expression of the OspA fusion construct (34% tot. prot.), C17E2, was obtained in *E. coli* BL21. The OspA fusion was recovered as inclusion bodies comprising 42% tot. prot. of the insoluble cellular fraction (Figure 6A).

Immunoblot Analysis of OspA

Expression of the immunoreactive product responsible for the initial identification of the lambda clone was not detected in clone pB12. The full length clone pB12 grew very poorly even when the *lac* promoter was catabolite repressed. Clone pB12-6.3b, which lacked the *bax* and *radA* ORF's (Figure 1), grew well under standard conditions and expressed a weakly immunoreactive 17 kDa product (results not shown).

Repeated efforts to generate high titre rabbit polyclonal antibodies against OspA that reacted strongly with *E. coli* produced OspA failed. OspA expressed in *E. coli* was never observed to react strongly with several rabbit antiserum samples. To confirm expression of OspA in *P. salmonis*, the Jameson-Wolf method was used to identify a region of OspA (110-129 a.a.) with a high antigenic index (Jameson and Wolf, 1988). Rabbit antibodies were raised against a 20 a.a. synthetic peptide of this region. Although rabbit antibodies generated against the synthetic peptide reacted with several *E. coli* proteins, the serum did react with a 17 kDa product in induced samples of pET-17E2 (Figure 7) which was not present in *E. coli* BL21 carrying pET21a alone (results not shown). Specificity of anti-peptide serum for OspA was further demonstrated upon strong recognition of a 17 kDa *P. salmonis* antigen (Figure 7).

Convalescent serum from coho salmon, against *P. salmonis*, also strongly recognized OspA and the OspA fusion in induced samples of pET-17E2 and pET-C17E2 (Figure 7). The 12 kDa protein C fusion partner of C17E2 was not recognized by either the rabbit anti-OspA peptide or convalescent coho serum (Figure 7).

Discussion

Rickettsiae are notoriously slow growing bacteria and, like *Coxiella burnetii* and members of the tribe *Ehrlichieae*, *P. salmonis* is even more difficult to grow and to separate

from host cell components because of its location within cytoplasmic vacuoles. Contamination of genomic DNA preparations is a serious concern when constructing genomic libraries of obligate intracellular bacteria. Therefore, with all their imperfections PCR techniques are often preferred over genomic library screening when cloning genes from rickettsiae (Anderson, 1997) but are inadequate for detecting novel genes.

Prior to this study, no genomic sequence data was available from *P. salmonis* with the exception of ribosomal RNA genes (Fryer, *et al.*, 1992, Mauel, *et al.*, 1999). Therefore, to identify antigens with vaccine potential, we constructed a *P. salmonis* genomic DNA expression library and screened it with rabbit anti-*P. salmonis* serum previously shown to predominantly recognize surface associated antigens (Kuzyk, *et al.*, 1996). All 18 immunoreactive clones isolated encoded a common region of the *P. salmonis* genome. DNA sequencing of clone pB12 revealed a 4,983 bp insert with an A+T content of 59% and 4 predicted ORF's and 1 partial ORF (Figure 1).

The *P. salmonis radA* ORF was well expressed by *E. coli* S30 lysates (Figure 3A) and seriously interfered with subcloning of the pB12 insert. *P. salmonis* RadA is apparently toxic to *E. coli* as clones lacking the initial 26 bp of *radA* grew well in *E. coli* DH5α. Even though *radA* was ~1.5 kb downstream of *lacP*, catabolic repression of *lacP* was still required to obtain a stable insert in *E. coli*. *P. salmonis* RadA is closely related to that of *P. aeruginosa* and *E. coli* (≥60% identical). The RadA enzyme family are DNA repair enzymes suspected to be involved in the repair of DNA alkylation (Song and Sargentini, 1996). Even though a *radA* transcript could not be detected (Figure 3B), the presence of an intact *radA* ORF had a dramatic impact upon the growth of *E. coli*. Toxicity is presumably the result of unregulated *radA* expression.

The a.a. sequence of the *ospA* ORF represents a

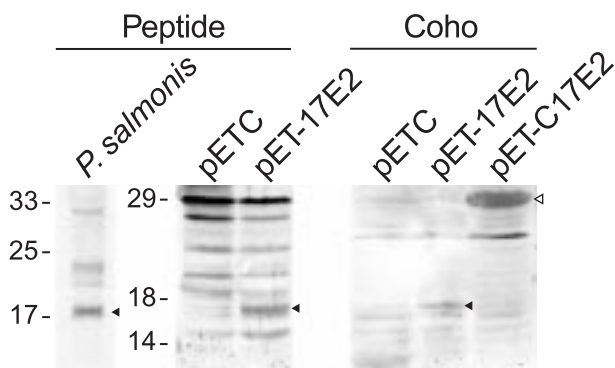


Figure 7. Immunoblot analysis of OspA. Whole cell lysates of *E. coli* clones and *P. salmonis* were analyzed by SDS-PAGE (12% polyacrylamide). Whole cell lysates of *P. salmonis*, pETC and pET-17E2 were reacted with rabbit polyclonal anti-OspA peptide serum. A 17 kDa product was recognized in both the *P. salmonis* and pET-17E2 samples (black arrow). Whole cell lysates of pETC, pET-17E2, and pET-C17E2 were also reacted with convalescent serum from coho salmon. Both OspA (black arrow) and the 28 kDa OspA fusion protein (white arrow) were recognized by the salmonid serum, with the OspA fusion protein being strongly recognized. All molecular weights are on the left in kDa.

putative 17.7 kDa outer surface protein (OspA) that displays homology to the 17 kDa rickettsial genus common antigen, a putative outer membrane lipoprotein (Anderson, *et al.*, 1988, Anderson and Tzianabos, 1989). The rickettsial 17 kDa membrane antigen is one of only three characterized rickettsial surface proteins and is highly conserved amongst *Rickettsia* sp. with greater than 90% amino acid identity (McDonald, *et al.*, 1997). The function of the 17 kDa antigen is unknown and until now has never been identified in any bacteria outside the genus *Rickettsia* (Anderson, 1990).

OspA expression was not detected by *in vitro* transcription and translation although a weakly immunoreactive 17 kDa protein was observed by immunoblot analysis of pB12 deletion clones. In an effort to increase the *E. coli* expression level of OspA for further characterization studies and future protection studies, the *ospA* ORF was cloned under control of the phage T7 promoter (pBC-17kDa). OspA was visibly expressed from pBC-17kDa after anywhere from 3-10 h of induction, but only at a low level (Figure 6A). Therefore, poor expression of the *ospA* gene does not appear to be the result of its promoter being poorly recognized in *E. coli*. It is possible that the ribosome binding site (RBS) of *ospA* is weakly recognized in *E. coli*, or *ospA* mRNA may have secondary structure in the 5' region (Makrides, 1996), or that differential use of minor codons is used as a mechanism to regulate gene expression post-transcriptionally (Saier, 1995).

A PCR strategy to synthesize the *ospA* gene using codons optimized for *E. coli* was formulated (Figure 4A). Previous studies on *E. coli* expression of low m.w. recombinant proteins demonstrated that the position 2 codon can have a significant impact on expression with Arg (CGT) codons being highly favoured (Belagaje, *et al.*, 1997). Therefore, the Asn-2 codon of *ospA* was deleted in the synthetic gene, 17E2, making Arg-3 the second codon in 17E2 (Figure 2). The 17E2 gene was cloned into pET21a under control of the T7 promoter and with an optimal RBS. Degradation makes expression of low m.w. proteins in *E. coli* inherently difficult (Belagaje, *et al.*, 1997), therefore, expression of pET-17E2 and subsequent constructs was studied in *E. coli* BL21, deficient in Lon and OmpT proteases. Levels of induced OspA expression from optimized pET-17E2 were no higher than those previously obtained from pBC-17kDa.

The rickettsial 17 kDa antigen is an outer membrane protein and has a signal peptide that is recognized by signal peptidase II. Removal of type II signal peptides is a three step process involving addition of diacylglycerol to a Cys prior to cleavage of the signal peptide on the N-terminal side of the Cys and addition of a fatty acid to the free amide of the N-terminal Cys (Stanley, *et al.*, 1998, von Heijne, 1990). A signal peptide prediction algorithm, SignalP (Nielsen, *et al.*, 1997), using a neural network trained on known signal peptides marginally identified OspA as a secreted protein (Figure 5) and the 11-21 a.a. region of OspA does contain the consensus PROSITE pattern for prokaryotic membrane lipoprotein lipid attachment (PS00013) with Cys-21 being the predicted acylation site (Figure 5) (Hofmann, *et al.*, 1999).

Presence of a signal peptide alone does not guarantee

transport of a protein across the bacterial inner membrane (Makrides, 1996). Removal of a signal sequence from a *Borrelia burgdorferi* lipoprotein permitted much higher expression in *E. coli* than its lipidated form (Dunn, *et al.*, 1990). To determine the effect the signal peptide of OspA has on its expression and lipidation in *E. coli*, the signal sequence (a.a. 1-20) of 17E2 was removed by PCR. Oligonucleotides of type II signal peptides from Braun's lipoprotein and the *R. rickettsii* 17 kDa antigen that are well recognized by *E. coli* were synthesized and added to Δ 17E2 by PCR (Figure 4B) (Anderson, *et al.*, 1988, Hansson, *et al.*, 1995, Inouye, *et al.*, 1977). To determine if the heterologous signal peptides improved expression or lipidation of OspA, expression of OspA constructs pET- Δ 17E2, pBC-17kDa, pET-lppospA, and pET-ricospA were compared (Figure 6A). OspA expression was significantly improved by removing its putative signal peptide (Figure 6A). The addition of Braun and rickettsial signal peptides did not improve OspA expression and induced products were not detected after 10 h (Figure 6A). But, levels of OspA expression similar to pBC-17kDa had been observed from pET-lppospA and pET-ricospA during shorter periods of induction.

Palmitate incorporation was conducted to determine if OspA is recognized and processed as a lipoprotein in *E. coli*. Palmitate was incorporated at similar levels into the OspA products from pBC-17kDa, pET-lppospA, and pET-ricospA constructs (Figure 6B). Removal of the signal peptide from OspA abolished incorporation of palmitate (Figure 6B). It appears that the inability to highly express OspA in *E. coli* stems principally from the presence of its signal peptide. Effective expression of secreted proteins in *E. coli* is not a trivial task; protein exporting machinery can become overloaded and cytoplasmic preprotein can be subjected to proteolysis leading to poor expression levels (Makrides, 1996).

Although the improved expression OspA in its truncated form from pET- Δ 17E2 was quite promising, we anticipated that a higher expression level may be obtained by partnering OspA with an N-terminal fusion protein. By adding the fusion partner we aimed to mask the OspA type II signal peptide from secretory machinery and further protect it from proteases. Excellent expression of the 28 kDa fusion protein was obtained from pET-C17E2 in *E. coli* BL21 with the fusion product isolated as inclusion bodies from the insoluble fraction of sonicated cells (Figure 6A). Localization of the OspA fusion to inclusion bodies is not detrimental to our goal of high level expression of OspA because neither biological activity nor proper folding of recombinantly expressed OspA are required for our applications. Putative protective epitopes of OspA are likely to be T cell restricted because of the intracellular nature of *P. salmonis*.

Although the surface localized nature of OspA has not been confirmed, rabbit anti-*P. salmonis* serum originally used to identify the *ospA*-encoding lambda clone was previously shown to predominantly recognize surface associated antigens of *P. salmonis* by immunogold transmission electron microscopy (Kuzyk, *et al.*, 1996). It seems reasonable to assume that OspA is surface associated when its lipoprotein nature and homology to a rickettsial surface lipoprotein are taken into account.

Repeated attempts to generate high specificity rabbit serum against OspA expressed in *E. coli* failed. OspA produced in *E. coli* never strongly reacted with polyclonal rabbit anti-*P. salmonis* serum used to originally identify clone pB12. But, antibodies generated against a 20 a.a. synthetic peptide of OspA did react with a 17 kDa product in *P. salmonis* and weakly recognize the OspA product expressed from pET-17E2 (Figure 7), thereby confirming OspA as an expressed protein in *P. salmonis*. None of the other OspA constructs exhibited better immunoreactivity than w.t. OspA expressed from pBC-17kDa (results not shown).

The ability of whole cell *P. salmonis* to elicit a detectable antibody response in salmon against OspA is of considerable interest. Both OspA and the OspA fusion protein reacted strongly and specifically with convalescent serum from coho salmon (Figure 7). The ability of the natural host of *P. salmonis* to elicit an immune response against OspA during the course of an infection identifies OspA as a potential recombinant vaccine antigen for *P. salmonis*. Further investigation will determine whether recombinantly produced OspA is capable of generating a protective immune response against *P. salmonis* in salmonids.

We have presented the first non-ribosomal DNA sequence data from the AT-rich, obligate intracellular salmonid pathogen, *P. salmonis*, and identified a prospective vaccine candidate antigen. We have confirmed the lipoprotein nature of OspA, a homologue of a rickettsial 17 kDa putative outer membrane lipoprotein. An optimized version of the *ospA* gene was constructed using PCR and the lipoprotein nature of OspA was identified as a limiting factor in its expression. High level *E. coli* expression of immunoreactive OspA targeted to inclusion bodies was achieved by partnering OspA with an N-terminal fusion protein. The OspA fusion was recognized by convalescent salmon sera indicating that during a natural infection in the salmonid host, *P. salmonis* elicits an immune response against OspA. Future work will focus on the ability of OspA to confer immunity against *P. salmonis* challenge in salmon.

Experimental Procedure

Bacterial Strains, Growth, and Isolation

P. salmonis and CHSE-214 samples were obtained from the American Type Culture Collection (ATCC). Type strain *P. salmonis* LF-89 (ATCC VR-1361), herein referred to as *P. salmonis*, was routinely grown on monolayers of a chinook salmon embryo cell line, CHSE-214 (ATCC CRL-1681) as previously described by Kuzyk *et al.* (1996).

E. coli DH5 α , SOLR, XL1-Blue, XL1-Blue MRF' (Stratagene), and BL21 (Amersham Pharmacia) were used for general cloning and lambda phage propagation. *E. coli* was routinely grown using either LB, terrific broth (TB), or MacConkey media. When required, supplements were added to media at the following concentrations: ampicillin (Amp), 100 μ g/ml; chloramphenicol (Cm), 28 μ g/ml; kanamycin (Kan), 28 μ g/ml; maltose, 0.2% (w/v); MgSO₄, 10 mM; X-gal, 40 μ g/ml; IPTG, 1 mM; glucose, 1% (w/v).

All lambda phage manipulations were carried out following protocols and using media described in the documentation provided with the Stratagene Gigapack III Gold packaging extract.

Purification of Genomic DNA and Construction of Gene Library

P. salmonis was purified by density gradient centrifugation as previously described (Kuzyk, *et al.*, 1996) from 12,000 cm² of CHSE-214 cells exhibiting full cytopathic effect 14 days after infection with *P. salmonis*. A single step DNA isolation solution, DNAzol (Life Technologies), was used to obtain genomic DNA from the purified *P. salmonis*. Genomic DNA was further purified by equilibrium centrifugation using a CsCl-ethidium bromide gradient to yield 250 μ g of *P. salmonis* genomic DNA (Sambrook, *et al.*, 1989).

P. salmonis DNA was partially digested using serially diluted *EcoR* I (New England Biolabs). Digests containing an average fragment size of 10 kb were chosen for creation of a *P. salmonis* gene expression library using a Stratagene predigested lambda ZAP II cloning kit.

Immunological Screening of Library

Approximately 10,000 plaques of *P. salmonis* lambda expression library were screened per round with a desired density of 1,000 plaques per 80 mm petri dish. Plaques were lifted in duplicate using 80 mm nitrocellulose discs (BioRad) impregnated with 10 mM IPTG. Screening followed the protocol of Sambrook *et al.* (1989) using anti-*P. salmonis* rabbit serum (Kuzyk, *et al.*, 1996). Immunoreactive plaques were picked and rescreened until pure cultures were obtained. Lambda clones were then amplified and the pBluescript clones excised into SOLR and ABLE K *E. coli* strains using Stratagene ExAssist interference resistant helper phage.

DNA Isolation and Analysis

Plasmid DNA was routinely prepared using a standard alkaline lysis procedure (Sambrook, *et al.*, 1989). Qiagen kits were also used to obtain lambda genomic DNA and plasmid DNA for sequence analysis. DNA preparations and restriction endonuclease digests were analyzed by Tris-acetate agarose gel electrophoresis (Sambrook, *et al.*, 1989).

SDS-PAGE and Immunoblot Analysis

Whole cell lysates were separated by SDS-PAGE using a discontinuous SDS gel system following the protocol of Laemmli as modified by Ames (Ames, 1974, Laemmli, 1970). Protein samples were visualized using GelCode Blue Stain Reagent (Pierce) and quantified with AlphaEase software (Alpha Innotech Corp.).

Western blotting was conducted as previously described (Collinson, *et al.*, 1991). *P. salmonis* antigens were detected by using rabbit anti-*P. salmonis* serum (diluted 1/2,000) and rabbit anti-OspA peptide (dil. 1/100) followed by goat anti-rabbit IgG conjugated to alkaline phosphatase (dil. 1/3,000). Convalescent coho salmon anti-*P. salmonis* serum (dil. 1/30) was also used, followed by an anti-rainbow trout Ig (heavy chain) mAb IPA2C7 (dil. 1/100) (Immuno-Precise Antibodies, Victoria, BC) and goat anti-mouse IgG₁ conjugated to alkaline phosphatase (dil. 1/3,000) (Caltag Laboratories). Salmonids only have a single antibody isotype that is an IgM-like tetramer that lacks a J chain (Press and Lillehaug, 1995). Immunoblots were developed with 5-bromo-4-chloro-3-indolyl phosphate and nitro blue tetrazolium as previously described (Collinson, *et al.*, 1991).

DNA Dot Blot Analysis

DNA samples (0.5 and 1.0 μ g) from CHSE-214 cells, immunoreactive *P. salmonis* lambda clones, and excised pBluescript clones were applied to a nylon membrane, in duplicate (Hybond-N+; Amersham). Blots were denatured in 1.5 M NaCl, 0.5 M NaOH, neutralized in 1.5 M NaCl, 0.5 M Tris (pH 8.0), and rinsed in 0.2 M Tris (pH 7.5), 2 \times SSC (Sambrook, *et al.*, 1989).

The insert DNA from clone pB12 was used as a probe. The pB12 insert was agarose gel purified using Sephaglas (Pharmacia) and labeled with [α -³²P]dATP by nick translation (Amersham Pharmacia). Blots were hybridized with probe under standard conditions at 60°C for 18 hr (Sambrook, *et al.*, 1989). Following stringency washes, blots were visualized using a storage phosphor screen imaging system (Molecular Dynamics).

Construction of Deletion Clones and DNA Sequence Analysis

A directional deletion library of *P. salmonis* clone pB12 was constructed to facilitate sequence analysis. A *Exo* III/S1 nuclease double-stranded nested deletion kit (Amersham Pharmacia) was used to create deletions in the direction of *lacZ* on the pBluescript vector. Restriction endonucleases *EcoR* I and *Sac* I were used to generate opposing overhangs protecting the vector from *Exo* III digestion. Upon ligation using T4 DNA ligase (Life Technologies) and screening, 32 deletion clones were selected that represented the entire insert and differed in size by 100-500 bp.

Double stranded plasmid DNA samples were sequenced using a combination of dye primer (Sequenase; Amersham Pharmacia) and dye termination (Dynamic ET Terminator; Amersham Pharmacia). Sequencing reactions were analyzed using an automated sequencer (ABI 377; Perkin-Elmer). Sequence data were assembled and analyzed using the Lasergene software package (DNASTAR). These sequence data have been submitted to the DDBJ/EMBL/GenBank databases under accession number AF184152.

Antibody Generation

Antibodies were generated in New Zealand white rabbits against 10 and 20 a.a. synthetic peptides based on a.a. 110-129 of the predicted OspA sequence. Peptides were glutaraldehyde conjugated to keyhole limpet hemocyanin (KLH) using a 40:1 molar ratio of peptide:KLH in standard phosphate buffered saline (pH 7.5). Conjugation was carried out for 1 h at

4°C in a 10 ml reaction volume with 500 µg/ml KLH and 1% glutaraldehyde. For the primary immunization, rabbits received 250 µg of conjugated peptide mixed 1:1 with Freund's complete adjuvant. Each rabbit was boosted three times at 2 week intervals with 250 µg of conjugated peptide per boost mixed 1:1 with Freund's incomplete adjuvant. Anti-*E. coli* antibodies were adsorbed out using *E. coli* DH5α lysates following the protocol of Sambrook, *et al.* (1989).

Anti-*P. salmonis* polyclonal rabbit serum was prepared against purified *P. salmonis* as previously described (Kuzyk, *et al.*, 1996). Convalescent serum was collected from coho salmon fry that had been challenged with *P. salmonis* but did not succumb to infection.

In vitro Transcription/Translation

E. coli S30 lysates (Promega) for transcription/translation of plasmid DNA were used to analyze protein expression from *P. salmonis* inserts in clone pB12 and deletion clones pB12-5.2, pB12-6.3b, and pB12-8.7. Each reaction used 5 µg of plasmid DNA and L-[U-¹⁴C]leucine (Amersham Pharmacia) was used as a radiolabel. Protein was acetone precipitated and rehydrated in SDS-PAGE sample buffer for analysis by 12% SDS-PAGE. Gels were vacuum dried at 80°C and visualized using a storage phosphor screen imaging system.

RNA Isolation and Northern Blot Analysis

Qiagen RNeasy columns were used to isolate total RNA from *E. coli* DH5α and clones pB12, and pB12-6.3b all of which were in DH5α. 10 µg of each total RNA sample, including DH5α, were analyzed by 1.2% formaldehyde-agarose gel electrophoresis sample. Gels were vacuum blotted onto nylon membranes (Hybond-N+; Amersham Pharmacia) and air dried and baked for 1 hr at 80°C. PCR generated probes (Figure 1) were labeled with [³²P]dATP by nick translation. Blots were hybridized with probes for 18 hr at 65°C under standard conditions. Following stringency washes, blots were visualized using a storage phosphor screen imaging system.

Coding regions of the four predicted ORF's on the clone pB12 insert were PCR amplified using primers illustrated in Figure 1. All PCR reactions were carried out using *Taq* DNA polymerase (Boehringer Mannheim) and standard PCR conditions. Prior to radiolabeling, probes were agarose gel purified using Sephaglas to ensure removal of template DNA.

Primary Structure Analysis of OspA

SignalP 2.0 was used to analyze potential signal peptide regions of OspA, pET-*lppospA* and pET-*ricospA* chimeric OspA constructs. SignalP uses neural networks trained on proteins with characterized signal peptides to determine if query sequences have signal peptides (Nielsen, *et al.*, 1997). The OspA protein sequence was also queried against the PROSITE protein family database to identify conserved protein domains and sequence patterns (Bateman, *et al.*, 2000, Hofmann, *et al.*, 1999).

Creation of *E. coli* Optimized ospA and Subsequent Signal Peptide Constructs

The products of all completed PCR strategies were initially subcloned into either pBC KS(+) (Stratagene) or pGEM-5Zf(+) (Promega) and electroporated into XL1-Blue. Following confirmation of positive clones by DNA sequencing, any inserts not already in pET21a(+) (Novagen), were sequentially subcloned into pBC KS(+) then pET21a.

pBC-17kDa

The *Xba* I/*Hind* III fragment of deletion clone pB12-6.3b (Figure 1) was subcloned into vector pBC KS(+) to place w.t. *ospA* under T7 polymerase promoter control. Following ligation, pBC-17kDa was electroporated into *E. coli* XL1-Blue MRF'.

pET-17E2

The EditSeq application, of the Lasergene software suite (DNASTAR), was used to reverse translate the OspA amino acid (a.a.) sequence using non-degenerate codons optimal for strong expression in *E. coli*. The synthetic *ospA* gene sequence (herein referred to as 17E2) was then modified to reduce the number of instances identical codons were used by replacing them with equally favoured codons wherever possible. Oligonucleotides of 17E2 were synthesized as six overlapping regions (94-151 bp in length) (Figure 4A) using standard techniques on a PCR-MATE 391 DNA synthesizer (ABI). *Taq* DNA polymerase (Boehringer Mannheim) was used throughout the construction of 17E2.

The central region of 17E2 was created in PCR reaction 1-1 using 0.5 pmol of oligonucleotides #3 (5'-AAA GGC TCT GGT CGT GTG GCG ATG GCC ATC GGC GGT GCG GTT CTG GGC GGT CTG ATT GGC TCT AAA ATC GGT CAG AGC ATG GAC CAG CAG GAT A-3') and #4 (5'-CCT GTT TGT TGT AAC GCT GGT AGG TGC GAA CCG GTT CCA CAG AGT AGC TGT TAC CCG TGT CCG GAT TAC GCC AAC GAG TAA CCT GGC CGG CTT TCA CTT TTT CCA GAG ACT GGT TCA GTT TGA TTT TAT CCT GCT GGT CCA TGC TCT GAC C-3') as templates with 2.5 pmol of

oligonucleotides #2 (5'-TGG GTT GCG CCC AGA ACT TCA GCC GCC AGG AAG TTG GCG CGG CCA CCG GTG CGG TTG TGG GCG GTG TTG CCG GCC AGC TGT TCG GTA AAG GCT CTG GTC GTG TGG CGA TG-3') and #5 (5'-GGT GCC GTA GAT TTC CTG TTT CTG ACC TGC GAT CAT GGC TTT CTG CTG AAA TTC GCG GCA GTA CTG CTG ACC GCG TTC CTG TTT GTT GTA ACG CTG GTA GGT-3') as primers (Figure 4A). PCR reaction 1-1 was carried out for 30 cycles (92°C 30 s, 55°C 30 s, 72°C 30 s). All PCR reactions were 100 µl volumes and contained 200 µM of each dNTP, 10 µl of 10× reaction buffer (100 mM Tris/HCl, 15 mM MgCl₂, 500 mM KCl, pH 8.3) and 2 units of *Taq* DNA polymerase.

Full length 17E2 was constructed in PCR reaction 1-2 using 1 µl of the completed first reaction, diluted 1/100, as template. The 100 µl reaction contained 2.5 pmol of oligonucleotides #1 (5'-CGC CAG GGT TTT CCC AGT CAC GAG GGA TCC GTC TCA TAT CCG TGG TTG CCT GCA GGG CAG CTC TCT GAT CAT TAT CTC TGT TTT CCT GGT GGG TTG CGC CCA GAA CTT CAG CCG CCA G-3') and #6 (5'-CGT CCT CTC GTC CTG GTC CCA GAT AAG CTT AAT TAA TTT TTT CGG TGC TAA TCA CCT GCC AGC GGC CAT CCG GCT GAC GGC ACG CGG TGC CGT AGA TTT CCT GTT TCT GAC-3') as primers (Figure 4A). The first cycle of PCR reaction 1-2 had an annealing temperature of 55°C which was raised to 70°C for the remaining 29 cycles; denaturing and elongation temperatures were the same as for PCR 1-1.

The full length product was selectively amplified with primers FOR (5'-CGC CAG GGT TTT CCC AGT CAC GAC-3') and END (5'-TGT GAA GCT TAT TTT TCG GTG CTA ATC ACC TGC CA-3'), both complementary to regions outside the 17E2 coding region, in a third PCR reaction. The synthetic 17E2 product was digested with *Bam*H I and *Hind* III and ligated into vector pBC KS(+). The ligation mixture was electroporated into XL1-Blue and plated on MacConkey agar (Cm) for selection overnight at 37°C. Positive clones were confirmed by DNA sequencing. The optimized 17E2 insert was then subcloned from pBC-17E2 into pET21a using the *Nde* I and *Hind* III restriction sites to construct pET-17E2.

pET-C17E2

The *Bam*H I/*Hind* III fragment of pBC-17E2 was subcloned into vector pETC to place 17E2 in frame with an N-terminal fusion partner, Protein C. The pET-C17E2 ligation mixture was electroporated into *E. coli* BL21. Transformants were selected on MacConkey agar (Amp) and confirmed to be positive upon plasmid purification and excision of the insert with *Bam*H I/*Hind* III digestion.

pET-Δ17E2

A truncated version of 17E2 lacking its predicted signal sequence was created by PCR using a forward primer encoding *Bam*H I and *Nde* I restriction sites (5'-CTT GGA TCC GTC TCA TAT GGC CCA GAA CTT CAG CCG CC-3'). The 38 bp forward primer was complementary to only 19 bp of 17E2 in the region of codons 22-27. The 17E2 Cys-21 codon was replaced with an ATG start codon. The reverse primer, 17E2_{REV} (5'-GCC GAG CTC TTA TTT TTC GGT GCT AAT CAC CTG CC-3'), encoded a *Sac* I restriction site immediately following the 17E2 TAA stop codon. The 50 µl volume PCR reaction contained 100 ng of pET-17E2 as template, 10 pmol of each primer, 200 µM of each dNTP, 5 µl of 10× *Pfu* buffer (200 mM Tris/HCl, 20 mM MgCl₂, 100 mM KCl, 1% Triton X-100) and 1.25 units of *Pfu* DNA polymerase (Stratagene). PCR was carried out for 30 cycles (94°C 60 s, 55°C 60 s, 72°C 60 s). The Δ17E2 PCR product was digested with *Bam*H I and *Nde* I and ligated into pET21a(+) and confirmed by DNA sequencing to create pET-Δ17E2.

pET-*lppospA*

A 114 bp oligonucleotide, LPPSIG (5'-CGC CAG GGT TTT CCC AGT CAC GAC ATA TGA AAG CGA CCA AAC TGG TTC TGG GCG CGG TTA TCC TGG GTT CTA CCC TGC TGG CGG GTT GCG AGC TCG GAC CAG GAC GAG AGG ACG-3'), was synthesized encoding the 21 res. signal sequence of *E. coli* Braun's lipoprotein (Prosite P02937) with optimized *E. coli* codon usage (Inouye, *et al.*, 1977), an *Nde* I site overlapping the ATG start codon, and flanking primer sites for amplification (Figure 4B). A 21 bp region complementary to codons 2-8 of Δ17E2 were added to the 3' terminus of the LPPSIG oligo in reaction 2-1 (Figure 4B) using primers FOR SYNTH (5'-CGC CAG GGT TTT CCC AGT CAC GAC-3') and LPPSP_{REV} (5'-CCT GGC GGC TGA AGT TCT GGG CGC AAC CCG CCA GCA GGG TAG AAC-3'). A 23 bp region complementary to the 3' region of LPPSIG was also added to the 5' region of Δ17E2 in reaction 2-1 (Figure 4B) using primers OSPLPP FOR (5'-GTT CTA CCC TGC TGG CGG GTT GCG CCC AGA ACT TCA GCC GCC AGG-3') and 17E2_{REV}. Reaction conditions were identical to those used during construction of pET-Δ17E2. The first cycle of PCR was allowed to anneal at 55°C to allow all non-homologous regions of primers to be filled in. The subsequent 29 cycles annealed at 65°C (94°C 60 s, 65°C 60 s, 72°C 60 s).

The *lpp* signal sequence was added to Δ17E2 by using the products of reactions 2-1 and 2-2 as templates in reaction 2-3 (Figure 4B). PCR

reaction conditions remained unchanged, and 1 μ l of reactions 2-1 and 2-2 diluted 1/10 were used as template. The first cycle of PCR contained no primers allowing the 44 bp overlap of the two templates to anneal at 55°C and *Pfu* DNA polymerase to synthesize the complementary regions. Following the first cycle, 10 pmol of primers FOR SYNTH and 17E2 REV were added and the remaining 29 cycles annealed at an elevated temperature of 65°C (94°C 60 s, 65°C 60 s, 72°C 60 s).

The product of reaction 2-3 was ligated into pGEM-5Zf(+) using the incorporated *Nde*I and *Sac*I restriction sites. Ligation mixture was electroporated into XL1-Blue and clones were confirmed by DNA sequencing. The confirmed *lppospA* insert was then subcloned into pET21a to create pET-lppospA.

pET-ricospA

A 111 bp oligonucleotide, RICSIG (5'-CGC CAG GGT TTT CCC AGT CAC GAC ATA TGA AAC TGC TGT CTA AAA TCA TGA TCA TCG CTC TGG CTA CCT CTA TGC TGC AGG CTT GCG AGC TCG GAC CAG GAC GAG AGG ACG-3'), was synthesized encoding the 21 res. signal sequence of *R. rickettsii* 17 kDa lipoprotein (GenBank AAA26381) with optimized *E. coli* codon usage, an *Nde*I site overlapping the ATG start codon, and a flanking primer sites (Anderson and Tzianabos, 1989).

Addition of the *R. rickettsii* 17 kDa signal sequence to Δ 17E2 followed the same strategy as used when creating the pET-lppospA construct. Only the primers used to create overlaps between the *R. rickettsii* signal sequence and Δ 17E2 differed. The primer RICOsp (5'-CCT GGC GGC TGA AGT TCT GGG CGC AAG CCT GCA GCA TAG AGG TAG CC-3') was used to add a 21 bp Δ 17E2 overlap to the 3' terminus of the *R. rickettsii* signal sequence. The primer OSPRIC (5'-GGC TAC CTC TAT GCT GCA GGC TTG CGC CCA GAA CTT CAG CCG CCA GG-3') was used to add a 25 bp region complementary to the *R. rickettsii* signal sequence to the 5' terminus of Δ 17E2.

Following subcloning into pGEM-5Zf(+) and DNA sequence confirmation, the 17E2 construct encoding an *R. rickettsii* signal sequence was subcloned into pET21a to create pET-ricospA.

Induction of Expression and [¹⁴C]Palmitate Incorporation

For induction experiments, pGP1-2 encoding T7 polymerase (Tabor and Richardson, 1985) was cotransformed with each pET21a-based construct into *E. coli* BL21. Cotransformants were selected overnight at 30°C on MacConkey media (Kan/Amp). pBC-17kDa was co-transformed with pGP1-2 into XL1-Blue MRF⁺ and selected at 30°C on MacConkey media (Kan/Cm).

For expression studies of the *ospA* constructs, 3 ml TB cultures containing appropriate antibiotics were grown overnight at 30°C and subcultured 1/100 into 5 ml TB broth. Once cultures were actively growing (OD₆₀₀ 0.5) temperature was shifted to 42°C for induction for 3 hr. Inclusion bodies were collected by sonicating induced cultures of pET-C17E2 3 \times 60 s followed by centrifugation at 10,000 \times g.

Toluene was removed from [1-¹⁴C]palmitate (Amersham Pharmacia) under vacuum and the palmitate was dissolved in a 1/5 vol. of isopropanol. The [¹⁴C]palmitate was added to cultures after being shifted to 42°C at a final concentration of 5 μ Ci/ml. Labeling was carried out for 3 h before samples were collected for SDS-PAGE. Samples were analyzed by 15% SDS-PAGE and the gels were vacuum dried at 80°C and visualized using a storage phosphor screen imaging system.

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