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Characterization of the gene encoding the protective paracrystalline-surface-layer protein of *Rickettsia prowazekii*: Presence of a truncated identical homolog in *Rickettsia typhi*

(protein processing/Rickettsiales/bacterial gene/DNA sequence)

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ABSTRACT **The DNA sequence of the gene encoding the protective surface protein antigen (SPA) of *Rickettsia prowazekii* has been determined. The open reading frame of 4836 nucleotides with promoter and ribosome-binding site is present on a 10.1-kilobase *Eco*RI fragment. The encoded carboxyl terminus of the 169-kDa protein contains a potential transmembrane region and hydrophilic regions with many lysine and arginine residues potentially accessible to proteolytic cleavage. Because the rickettsia-derived SPA has an estimated molecular mass of only 120 kDa and does not contain several predicted large carboxyl-region CNBr fragments, the SPA product appears to be processed by the rickettsiae. Eight other CNBr fragments were identical in sequence to those predicted from the encoded gene. A complementary 8.7-kilobase *Eco*RI fragment of *Rickettsia typhi* DNA was cloned. This fragment lacked a 1433-base-pair region that included the promoter, ribosome-binding site, and the initial 1162 base pairs of the open reading frame encoding the *R. prowazekii* SPA but had a 3674-base-pair region identical with the remainder of the *R. prowazekii* SPA gene sequence.**

Many bacteria possess paracrystalline surface layers (S-layers) as the outermost component of their cell envelopes (1–3). The S-layers of many bacteria are composed of protein or glycoprotein subunits arranged on the cell surface in a regularly repeating hexagonal, tetragonal, or linear pattern. The functions of these S-layers are poorly understood. However, an S-layer may function as a protective barrier against environmental hazards, as a molecular sieve and ion trap, as a promoter for cell adhesion and surface recognition, or as a mold for determining cell shape and envelope rigidity. The S-layer proteins of several pathogenic bacteria appear to be important virulence determinants (3, 4).

Rickettsia prowazekii also contains a tetragonally arranged S-layer (5, 6). S-layers have been isolated and characterized as the species-specific surface protein antigens (SPAs) of *R. prowazekii* and *Rickettsia typhi* (7). The evidence that supports the classification of the SPAs as S-layer proteins has been extensively reviewed (8). The SPAs of both *R. prowazekii* and *R. typhi* have apparent molecular masses of 120 kDa. SPAs are readily released by shaking the typhus rickettsiae in hypotonic solution and are partially purified by filtration and by pelleting contaminants with ultracentrifugation (9). Extraction of the SPA in hypotonic solution results in the loss of the repetitive subunits from the rickettsial outer membrane and in a smooth outer-membrane appearance (8). Although this ease of extraction is relatively unusual for an S-layer because strong chaotropic ions or detergents are often used for their release (10), the large amount and high degree of purity of this high-molecular-mass rickettsial protein is exactly that expected for an S-layer because these layers form a monomolecular layer around the entire microorganism (11, 12). The SPAs of the typhus rickettsiae also contain a high proportion of acidic and hydrophobic amino acids and are low in sulfur-containing amino acids, as are other described S-layer proteins (1–3, 8). In addition, SPAs of the typhus rickettsiae, like other S-layer proteins, are rich in β-sheet structure but have little α-helical structure (8).

The SPAs of the typhus rickettsiae are highly immunogenic in humans and animals (13). In addition, the SPAs have been shown to stimulate different classes of human lymphocytes, including lymphokine-activated killers (14), suppressor T cells (15), and γ-interferon-producing T helper cells (16) that participate in the immunological defense against these organisms. More important however, purified preparations of SPA from the typhus rickettsiae have been shown to be effective protective antigens in an animal model. Guinea pigs immunized with different doses of the SPA appear to be protected against challenge with live organisms as compared with nonimmunized controls (13).

The gene *spaP* encoding the SPA of *R. prowazekii* has been previously cloned (M.E.D., M.C., and G.A.D., unpublished work). We describe here the complete sequence of the 4836 nucleotide (nt) open reading frame (ORF) that encodes a 169-kDa SPA protein as well as its flanking regions.† We provide evidence that the 120-kDa SPA obtained from rickettsiae is derived by truncating the carboxyl end of the encoded gene product. We have also cloned and sequenced a fragment of *R. typhi* DNA that contains an exact 3674-base-pair (bp) copy (*spaPt*) of the 3′ end of *spaP*, but lacks the promoter, ribosome-binding site, and 1162 bp of the 5′ end of the ORF of *spaP*.

MATERIALS AND METHODS

Construction of Recombinant Bacteriophages, Plasmids, and DNA Sequencing. The construction and identification of recombinant λgt11 and recombinant λgtWES bacteriophages containing 3.7-kilobase (kb) and 10.1-kb *R. prowazekii* Breinl-derived DNA fragments, respectively, have been described (M.E.D., M.C., and G.A.D., unpublished work). When plated onto the *Escherichia coli* host strain ED8654 (*supE supF hsdR metB lacY gal trpR*), recombinant bacteriophages containing all or part of the SPA gene were identified by screening plaques with monoclonal antibodies (mAbs) specific for the SPA of *R. prowazekii* (17). Similarly, whole-cell DNA was prepared from purified *R. typhi* (Wilmington strain), digested with a mixture of *Alu* I, *Hae* III, and

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Abbreviations: S-layers, paracrystalline surface layers; PCR, polymerase chain reaction; SPA, surface protein antigen; nt, nucleotide(s); mAb, monoclonal antibody; ORF, open reading frame.

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†The sequence reported in this paper has been deposited in the GenBank data base (accession no. M37647).

Rsa I, and ligated into λgt11 using *Eco*RI linkers (18). The library was titered on *E. coli hsdR* and screened for antigen expression with *R. typhi*-specific mAbs (17). A total *Eco*RI digest of *R. typhi*-derived DNA was ligated into λgtWES (19). Recombinant phage were screened using mAbs as above.

Recombinant bacteriophage DNA was prepared (20), and purified DNA fragments were subcloned into pUC8 and pUC19 plasmids. Recombinant plasmids were sequenced directly from plasmid DNA by the dideoxynucleotide chain-termination method (21) with Sequenase (United States Biochemical) as the polymerase. Sequencing was initiated on both strands with M13 primers (Promega and United States Biochemical). Subsequently, sequencing was continued on both strands by using custom synthetic oligonucleotides as primers.

Restriction Map and Southern Blot Analysis. DNA from the desired plasmids were digested with the following endonucleases: *Bgl* II, *Eco*RI, *Hin*dIII, and *Pst* I, according to the supplier's recommendations (Bethesda Research Laboratories). Plasmid DNA digested with single or double enzymes was then electrophoresed in 0.8% agarose gel.

For Southern blot analysis, DNA was digested with the appropriate restriction enzymes, electrophoresed in 0.8% agarose gels, and transferred to nitrocellulose (Schleicher & Schuell). Hybridizations were carried out largely as described by Southern (22). Probes were derived from recombinant plasmid DNA labeled with deoxyadenosine 5-[γ-[^{35}S] thio]triphosphate by nick-translation (23). Alternatively, probes were generated using polymerase chain reaction (PCR)-derived products labeled with digoxigenin (24), and detection was accomplished by using the stringent conditions recommended by the manufacturer (Boehringer Mannheim).

Preparation and Sequencing of CNBr Fragments from SPA. Cleavage of purified SPA protein at methionine residues with CNBr was done according to the method of Gross and Witkop (25). Fragments were then separated by SDS/PAGE (26), electroblotted onto polyvinyl difluoride paper (Millipore), and stained directly with Coomassie blue. Amino acid sequence analysis was performed on a model 477A sequencer with model 120A automated on-line phenylthiohydantoin analyzer (Applied Biosystems).

PCR. DNA was amplified using the PCR, as described (27). Genomic DNA derived from *R. typhi* and *R. prowazekii*, as well as recombinant plasmids containing inserts derived from these organisms, served as templates. Oligonucleotide primers were selected from those used for sequence determinations. Amplified DNA was detected by agarose gel electrophoresis and ethidium bromide staining.

RESULTS

Recombinant plasmids pMD306 and pMDL7 were created by subcloning 3.7-kb and 10.1-kb inserts (originally derived from *R. prowazekii* Breinl), from recombinant λgt11 and λgtWES bacteriophages, respectively (M.E.D., M.C., and G.A.D., unpublished work). These phages had been detected by screening libraries with SPA-specific mAbs (17). Recombinant phage λgt11-RTW9 contains a 986-bp insert derived from *R. typhi* that is recognized by mAbs specific for the SPA. pMAC1 was created by subcloning this 986-bp insert into pUC19. pMD306 was used as a probe to identify a homologous 8.7-kb *Eco*RI fragment in *R. typhi* genomic DNA. A recombinant λgtWES clone containing this 8.7-kb fragment was identified with SPA-specific mAbs. pMAC2 is a recombinant pUC19 plasmid that contains this 8.7-kb *Eco*RI fragment. The restriction maps of all four of these plasmid inserts are shown in Fig. 1. This data suggested that the pMD306 insert is identical with a region contained within pMDL7 and that the pMAC1 insert is identical with a region contained within pMAC2. Maps for plasmids pMDL7 (derived from *R. prowazekii*) and pMAC2 (derived from *R. typhi*) were identical except for a 1.4-kb fragment of DNA present in pMDL7 but not in pMAC2.

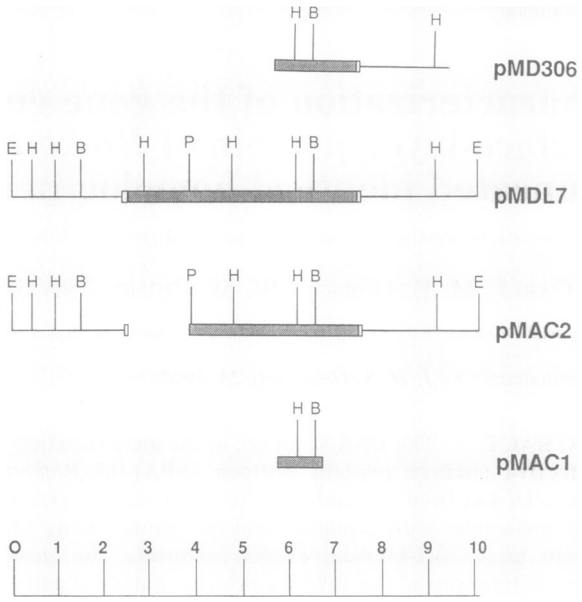

FIG. 1. Restriction maps of the rickettsial inserts of four recombinant pUC plasmids. The inserts from pMD306 and pMDL7 are derived from *R. prowazekii*, and the inserts from pMAC1 and pMAC2 are derived from *R. typhi*. Thickened lines represent sequenced regions; a shaded area represents a continuous ORF. Restriction endonucleases used include *Bgl* II (B), *Eco*RI (E), *Hin*dIII (H), and *Pst* I (P). Scale is in kb.

The complete nucleotide sequences of plasmids pMD306 and pMAC1 were determined for both strands of DNA. The entire sequences for both of these plasmids were contained within pMDL7 and pMAC2. Further sequencing of both strands of pMDL7 revealed an ORF of 4836 nt between the ATG triplet at positions 394–396 and the TAA stop codon at positions 5230–5232 (Fig. 2). Presumptive ribosome-binding site and −10 and −35 regions are underlined. A large inverted repeat forming a stem loop structure consisting of a 14-bp stem and an 11-bp loop is present downstream of the translational stop codons (nt 5271–5309) and might function as a transcriptional termination signal. Amino-terminal amino acid sequences of eight CNBr fragments of purified *R. prowazekii* SPA were found within the ORF and are underlined in Fig. 2. The DNA sequence predicts the existence of an additional seven CNBr fragments at the carboxyl end of the SPA with molecular masses of 0.87–8.14 kDa. However, despite the fact that all other major CNBr fragments throughout the rest of the molecule were identified, no fragments corresponding to these seven predicted carboxyl terminal fragments could be found. A 5.5-kDa CNBr fragment beginning at amino acid 1255 (corresponding to nt 4216–4218) was closest to the putative SPA carboxyl terminus.

The sequence of pMAC2 contained an ORF of 3674 bp identical to pMDL7 from nt 1556–5229 (Fig. 2). A region of DNA just 5′ to this homologous ORF is shown in Fig. 3 (nt 1–87) and is identical to the nucleotide sequence of pMDL7 from nt 36–122. pMAC2, therefore, lacks a 1433-bp segment of DNA (nt 123–1555), present in pMDL7. The DNA sequence of pMAC2 found 3′ to the ORF is also identical to that found in pMDL7 through nt 5322 (Fig. 2) and, therefore, includes an inverted repeat downstream of the translational stop codons. No presumptive ribosome-binding site or −10 and −35 regions could be discerned.

To confirm that this homologous sequence in *spaPt* was not a laboratory artifact, we selected an oligonucleotide primer (primer 1, GGTGTGATAGTATTGTAATC) based on

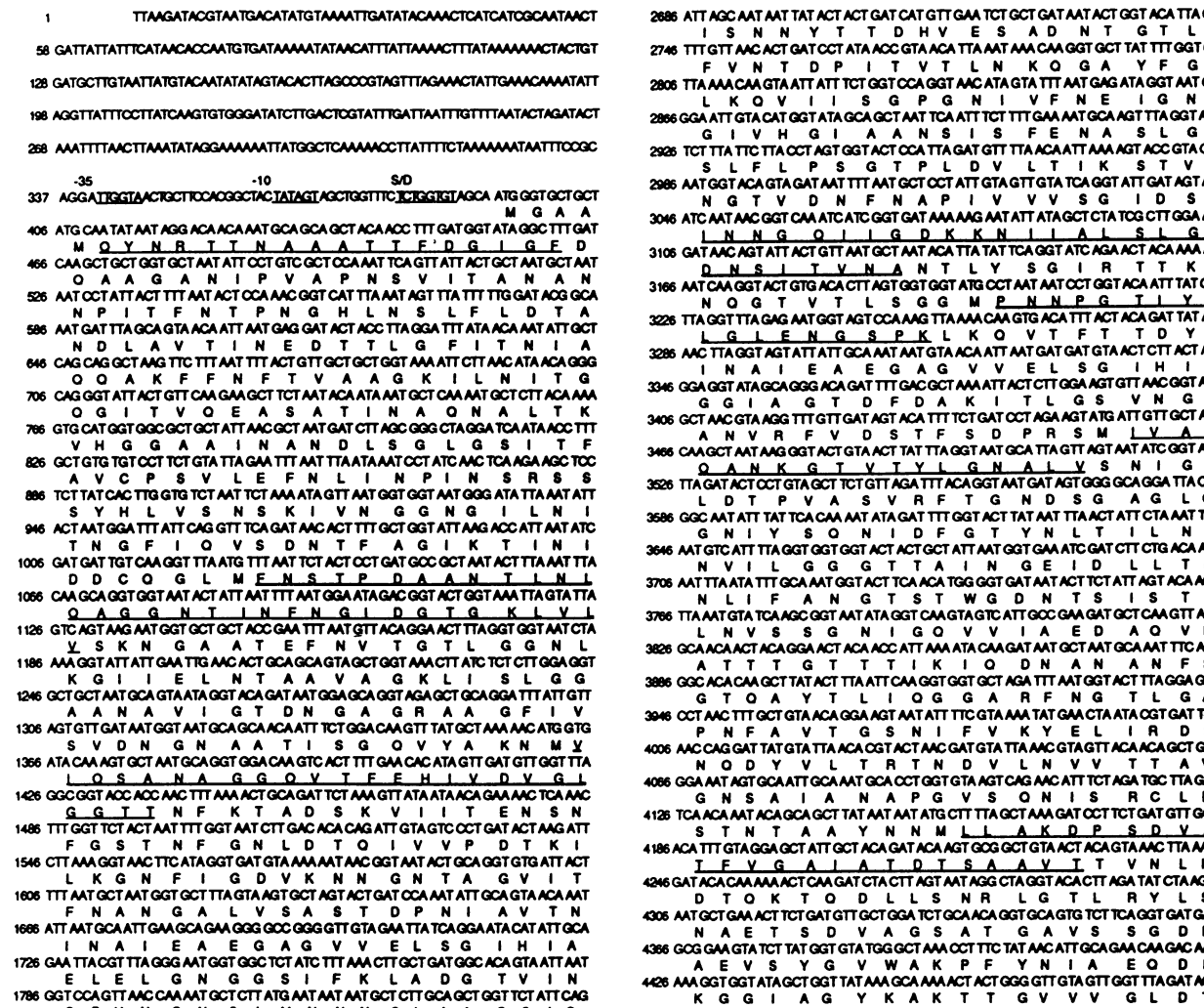

FIG. 2. Nucleotide sequence and deduced amino acid sequence (in one-letter code) of the *spaP* gene. Potential −35 and −10 regions, a potential ribosome-binding site, and a terminal 14-bp inverted repeat are underlined. Deduced amino acid sequences corresponding to sequenced CNBr fragments are also underlined.

additional partial sequence of a region 5′ to the 1.4-kb deletion in *R. typhi* (not shown in Fig. 2) and used it in PCR reactions paired with each of two primers 3′ to the deletion (primer 2, CAGTAGTTATATCTAAATCA and primer 3, AGAGCCACCATTCCCTAAAC, reverse complements of bp 2058–2077 and 1733–1752, respectively, Fig. 2). The DNA templates used in these PCR reactions were genomic DNA isolated from *R. typhi* or from *R. prowazekii*. DNA amplified from these two different templates differed by ≈1.4 kb for each of the two primer pairs examined, thus confirming the origin of the DNA inserts in pMDL7 and pMAC2 from *R.*

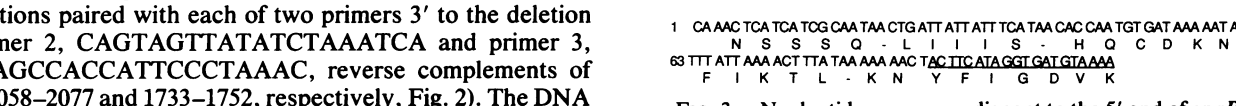

FIG. 3. Nucleotide sequence adjacent to the 5′ end of *spaPt* in *R. typhi*. The underlined nucleotides correspond to nt 1556–1575 in the nucleotide sequence of *spaP* shown in Fig. 2. Amino acids are shown in one-letter code.

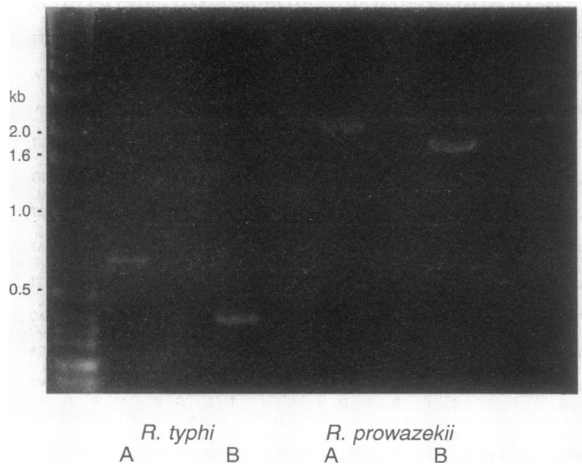

FIG. 4. Polymerase chain reaction-amplified DNA using *R. typhi* or *R. prowazekii* genomic DNA as the template. Primer pair A (primers 1 and 2) was used to amplify a 700-bp fragment from *R. typhi* and a 2.1-kb pair fragment from *R. prowazekii*. Primer pair B (primers 1 and 3) was used to amplify a 400-bp fragment from *R. typhi* and a 1.8 kb-fragment from *R. prowazekii*.

prowazekii and *R. typhi*, respectively (Fig. 4). Therefore, the observed 1.4-kb deletion seen is present in *R. typhi* and did not occur as a deletional event within the transformed *E. coli* host. Although large enough to encode a protein of 120 kDa, we are certain that *spaPt* does not encode the *R. typhi* SPA because some sequenced CNBr fragments of *R. typhi*-derived SPA are not present in the deduced amino acid sequence of *spaPt* (W.-M.C., unpublished work). Therefore, we hypothesized that there exists another gene (*spaT*) highly homologous to *spaPt* that encodes the *R. typhi* SPA. To prove this, Southern blot analysis of DNA derived from *R. typhi* was done. A digoxigenin-labeled DNA probe (generated using PCR) from the 5′ end of the *spaP* gene (nt 394–1641) hybridized to a single 3.4-kb *Pst* I fragment (presumably containing part of *spaT*), whereas a second digoxigenin-labeled DNA probe (nt 3238–4437) hybridized to the same 3.4-kb *Pst* I fragment and also to a 12-kb fragment (presumably containing part of *spaPt*, Fig. 5). Because the second

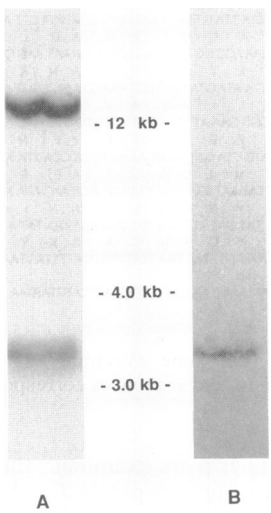

FIG. 5. Genomic DNA purified from *R. typhi* was digested with *Pst* I, electrophoresed in 0.8% agarose gel, and transferred to nitrocellulose before Southern blot analysis. (*A*) Probe used for hybridization was a PCR-generated product, and its nucleotide sequence corresponded to nt 3238–4437 of *spaP* (Fig. 2). (*B*) Probe used for hybridization was generated using the PCR, and its nucleotide sequence corresponded to nt 394–1641 of *spaP* (Fig. 2).

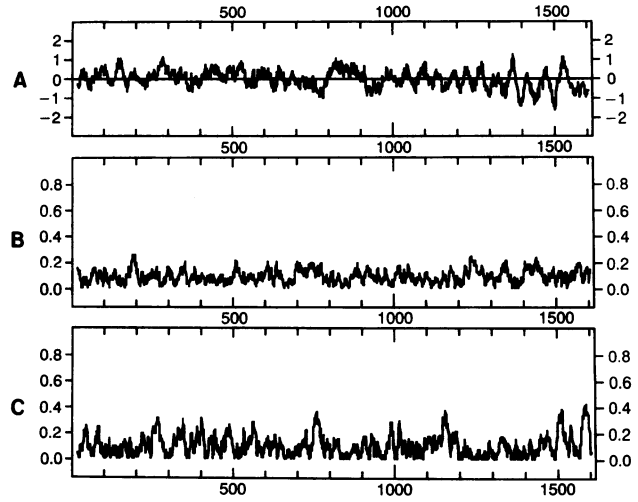

FIG. 6. Theoretical structure analysis of the *R. prowazekii* SPA. Hydropathy (*A*), α-helical structure (*B*), and β-sheet structure (*C*) are based on the deduced amino acid sequence.

probe consisted of DNA the sequence of which was located entirely within a single *Pst* I fragment derived from *R. prowazekii*, this data strongly suggests the presence of two highly homologous genes, only one of which (*spaT*) contains sequence homology to the 5′ end of *spaP*.

The ORF of pMDL7 encoded a protein with a calculated molecular mass of 169,874 and with a theoretical pI of 5.81. Codon usage in the *spaP* gene is similar to that described for the *R. prowazekii* citrate synthetase gene (28). The hydrophobicity profile, α-helical structure, and β-sheet structure (29) are plotted in Fig. 6 (DNA Strider, Institut de Recherche Fondamentale). Although most of the protein appears to be hydrophobic, the carboxyl portion of the molecule appears more hydrophilic.

DISCUSSION

We describe the cloning and sequencing of the *spaP* gene. Although *R. prowazekii*-derived SPA has an estimated molecular mass of 120 kDa, the gene described here encodes a protein with a molecular mass of 169.87 kDa. Although widely disparate molecular masses for the SPA have been obtained by different methods and the SPA migrates anomalously by PAGE (8, 30, 31), this discrepancy is rather large. However, seven CNBr fragments predicted at the carboxyl end of the molecule from the deduced amino acid sequence were absent in the rickettsia-derived SPA. It is striking that the carboxyl end of the SPA for *R. prowazekii* contains both a hydrophobic region with α-helical configuration consisting of 24 amino acids (amino acids 1356–1379, corresponding to nt 4519–4590, Fig. 5), which could span the bacterial cell membrane and an adjacent 16 hydrophilic amino acids in a β-turn region (amino acids 1382–1396, corresponding to nt 4597–4641, Fig. 5) could serve, respectively, as a hydrophobic anchor and translocation stop. This hypothesis is consistent with what is presently known about hydrophobic anchor sequences that have been identified in transmembrane proteins (32, 33). Cleavage of the SPA protein near this anchor region would then result in a soluble surface protein with approximately the same molecular mass (130 kDa) and almost the identical amino acid composition as determined for *R. prowazekii*-derived SPA (9). Although the calculated pI of 5.4 for the protein that results from this hypothesized cleavage would differ from the pI of 4.1 already determined for the SPA (34), various modifications of the protein might account for this difference—such as phosphorylation, deamidation, and glycosylation.

We have compared the deduced amino acid sequence of the *spaP* gene for *R. prowazekii* with previously published sequences for the S-layer proteins of several prokaryotes (35–39) and are unable to identify any major regions of homology despite the fact that most S-layer proteins typically have a high proportion of acidic amino acids and usually a small amount of sulfur-containing amino acids (8). This result seems to suggest that similar characteristics of S-layer proteins of various prokaryotes did not arise via common ancestors or via the transfer of genes among bacteria. Signal sequences that are cleaved from mature S-layer proteins have been demonstrated for *Halobacterium*, *Bacillus brevis*, and *Deinococcus* (35–39). No cleavage of the relatively hydrophobic amino terminus was detected in the S-layer of *Caulobacter* (40). The amino terminus of *R. prowazekii* SPA is not very hydrophobic, does not exhibit homology to other signal sequences, and is not cleaved in the mature protein.

In the present study we have also cloned and sequenced *spaPt*, which is identical to the *spaP* gene but lacks the promoter region, the ribosomal-binding site, and 1162 bp of coding sequence located at the 5′ end of the *spaP* gene. Because the SPA of *R. typhi* appears highly conserved among different strains and its gene appears highly homologous to *spaPt* (17), it is not readily apparent why or how *spaPt* is maintained stably in *R. typhi*. Deletion of the 1.4-kb fragment of DNA from an ancestor of *R. typhi* and *R. prowazekii* may be one of many such events that occurred during divergence of *R. typhi* as a species. The fact that the ORF of *spaPt* is identical to that of *spaP* suggests that some selective pressure exists to maintain *spaPt*, perhaps even an unknown functional role for the protein encoded by this truncated gene.

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