Membrane Association of Active Plasmid Partitioning Protein A in *Escherichia coli*

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QsopA and SopA, proteins essential for stable maintenance of low copy number plasmids and encoded on plasmid QpH1 of *Coxiella burnetii* and the F plasmid of *Escherichia coli*, respectively, are shown to be membrane associated using three independent approaches: isolation of hybrid protein A-PhoA proteins that display PhoA (bacterial alkaline phosphatase) activity indicating a periplasmic location, biochemical fractionation by flotation gradient centrifugation, and subcellular localization by immunoelectron microscopy. These data provide insight into the mechanism by which partitioning protein A spatially direct plasmids into daughter cells at bacterial division.

Thirty-five years ago, Jacob et al. (1) first proposed a DNA segregation model. In this model, replicons (chromosomes or plasmids) bind to equatorial partition sites in the septum of the dividing cell. Envelope growth moves the dividing partition sites apart so that the replicons stably partition into the two daughter cells. Unfortunately, there has not been any solid evidence to support this model. Recently, a number of new models for replicon segregation have been proposed based on genetic and biochemical studies of chromosome and plasmid partitioning systems, although, once again, none of them has been supported experimentally (2, 3). The common feature of these models is that, at the moment of cell division, the DNA molecules (two for chromosomes or at least two for plasmids) are not free in the cytoplasm, but are restrained to some structural site of the dividing cell. The possibility of an attachment between DNA molecules and the bacterial membrane has been suggested by some experimental data (4, 5), but putative candidates (6) responsible for the assumed restraint have not been identified.

The active partitioning systems of low copy number plasmids such as F and P1 (7, 8) of *Escherichia coli* and QpH1 of *Coxiella burnetii* (9, 10) have been studied as models for replicon segregation (6). In these cases, specific genetic loci (*par* or *sop*) are known to be essential for stable plasmid partitioning into daughter cells. Because of the inability to transform *C. burnetii* and the lack of a workable genetic system, it has not been demonstrated that the *sopA* region (*par* equivalent) on QpH1 is required for plasmid partition in this organism. Nevertheless, a common feature for these partition regions is that they can independently stabilize different replicons in *E. coli*.

For example, the partition regions of plasmids R1 (11) and QpH1 (9) could stabilize *sop*-negative mini-F plasmids; the *sop* region of the F plasmid could stabilize an *oriC* plasmid (12), which replicates using the replication origin of the *E. coli* chromosome. A partition (*par*) region usually carries a cis-acting DNA site and a partition operon encoding two trans-acting proteins, protein A and protein B, such as SopA and SopB of F plasmid, ParA and ParB of prophages P1 and P7, and QsopA and QsopB of QpH1. The identified activities for protein B include binding to the centromere-like cis-acting DNA site to form nucleoprotein complexes (13, 14), as well as enhancing the functions of the corresponding protein A (for reviews, see Refs. 7 and 8). Two biochemical activities have also been identified for partitioning protein A. First, this protein has been shown, by DNA footprinting assays, to bind to the promoter region of the protein A gene, such as the *sopA* gene of the F plasmid (15), and the *parA* genes of P1 (16) and P7 (17). The active DNA binding form of P1, ParA, appeared to be a homodimer (18). These DNA binding activities of protein A are generally believed to be responsible for autoregulation of expression from the partition operons (7, 8). Second, the SopA protein of the F plasmid (19) and the ParA protein of P1 (16, 18) both displayed ATPase activity, and this activity could be enhanced by interaction with the corresponding protein B (7, 8). Based on amino acid sequence analysis, all the active partitioning A proteins identified so far including the QsopA protein of QpH1 (10) should contain the ATPase domains (20). Despite the characterization of these biochemical activities, the exact roles of protein A and protein B in the partitioning process remain a mystery. Because the *par* regions function independently of the replication origins (9, 21) and the plasmids F (22) and P1 (23) are maintained perfectly well in chromosome segregation-defective *E. coli* cells (24), it is reasonable to assume that partitioning proteins are involved in the association between plasmid molecules and the host envelope.

Our previous TnphoA mutagenesis studies (9) on *sopAB*, the partition region of QpH1, revealed an interesting phenomenon; three QsopA-PhoA fusions displayed positive PhoA (bacterial alkaline phosphatase) activity in *E. coli*, indicating that QsopA is either membrane-bound or exported, as it is generally accepted that PhoA is only active if localized to the periplasm (25). In view of the presumed function of QsopA as a partitioning protein A, it seemed unlikely that this protein would be exported. Therefore, we decided to pursue the possibility that QsopA and its analog (9, 10) SopA of the F plasmid are membrane-bound. Our studies indicate that both QsopA and SopA are associated with the cytoplasmic membrane in *E. coli* cells.

**EXPERIMENTAL PROCEDURES**

Bacterial Strains, Bacteriophage, and Growth Conditions—All bacterial strains used were *E. coli* K-12 derivatives. CC118 (26) was used as an alkaline phosphatase-deficient *E. coli* host for TnphoA mutagenesis. Strain DH5α (Life Technologies, Inc.) (27) was the cloning host throughout these studies. HB101 (Life Technologies, Inc.) (27) was used as a bacterial host for membrane association analysis. BL21(DE3) (No-
vagen, Madison, WI) was used for overexpression of the QsopA or SopA proteins. BL21 bears a chromosomal copy of the gene for T7 polymerase by using a lysogen of bacteriophage DE3, a λ derivative that contains the T7 polymerase gene under control of the isopropyl-1-thio-β-D-galactopyranoside-inducible lacUV5 promoter (28). The bacteriophage λ::T4phoA (29) was used for mutagenesis. All cultures were aerobically grown at 37 °C.

Media and Chemicals—Luria-Bertani (LB) broth (30) (Difco) contained (per liter): 10 g of Bacto tryptone, 10 g of NaCl, and 5 g of Bacto yeast extract. LB plates contained, in addition, 15 g/liter Bacto agar (Difco). M9 minimal medium (30) contained (per liter): 6.0 g of Na₂HPO₄, 3.0 g of KH₂PO₄, 0.5 g of NaCl, 1.0 g of NH₄Cl, and 10 ml of 0.1 M CaCl₂, and was supplemented with 4 ml/g each of cysteine hydrochloride, methionine and 1 mM thiamine-HCl. The chromosomal substrate XP (5-bromo-4-chloro-3-indolyl phosphate p-toluidine salt) was dissolved in dimethylformamide and added to plate media at 40 μg/ml. Antibiotics were added to media at the following concentrations (μg/ml): ampicillin, 100; chloramphenicol, 30; tetracycline, 10; kanamycin (U. S. Biochemical Corp.), 34; and gentamicin (U. S. Biochemical Corp.), 19.

Membrane Association of Partitioning Proteins

Plasmids and their genotypes or characteristics

| Plasmid         | Genotype or characteristic | Reference |
|-----------------|----------------------------|-----------|
| pZL30           | Ap<sup>+</sup>, sopA<sup>+</sup>, high copy number (9) |
| pZL30::20       | Ap<sup>+</sup>, kn<sup>+</sup>, qsoPA::TaphoA, a pZL30 derivative (9) |
| pZL30::206      | Ap<sup>+</sup>, kn<sup>+</sup>, qsoPA::TaphoA, a pZL30 derivative (9) |
| pZL30::8        | Ap<sup>+</sup>, kn<sup>+</sup>, qsoPA::TaphoA, a pZL30 derivative (9) |
| pZL30::49       | Ap<sup>+</sup>, kn<sup>+</sup>, qsoPA::TaphoA, a pZL30 derivative (9) |
| pZL30::210      | Ap<sup>+</sup>, kn<sup>+</sup>, qsoPA::TaphoA, a pZL30 derivative (9) |
| pUC19           | Ap<sup>+</sup>, qsoPA<sup>+</sup>, a cloning vector of high copy number (31) |
| pC1.9           | Ap<sup>+</sup>, qsoPA<sup>+</sup>, a pUC19 derivative (9) |
| pC12            | Ap<sup>+</sup>, kn<sup>+</sup>, qsoPA::TaphoA, a pc1.9 derivative (9) |
| pC35            | Ap<sup>+</sup>, kn<sup>+</sup>, qsoPA::TaphoA, a pc1.9 derivative (9) |
| pC88            | Ap<sup>+</sup>, kn<sup>+</sup>, qsoPA::TaphoA, a pc1.9 derivative (9) |
| pC32            | Ap<sup>+</sup>, kn<sup>+</sup>, qsoPA::TaphoA, a pc1.9 derivative (9) |
| pC20            | Ap<sup>+</sup>, kn<sup>+</sup>, qsoPA::TaphoA, a pc1.9 derivative (9) |
| pC4F            | Ap<sup>+</sup>, qsoAB<sup>+</sup>, a pUC19 derivative (9) |
| pFSOPA6         | Ap<sup>+</sup>, sopA<sup>+</sup>, a pUC19 derivative (9) |
| pF73            | Ap<sup>+</sup>, kn<sup>+</sup>, sopA::TaphoA, a pFSOPA6 derivative (9) |
| pM3-8           | Ap<sup>+</sup>, kn<sup>+</sup>, sopA::TaphoA, a pFSOPA6 derivative (9) |
| pM3-7           | Ap<sup>+</sup>, kn<sup>+</sup>, sopA::TaphoA, a pFSOPA6 derivative (9) |
| pFA             | Ap<sup>+</sup>, sopA<sup>+</sup>, a pUC19 derivative (9) |
| pWSK30          | Ap<sup>+</sup>, a cloning vector of low copy number (33) |
| pQSOPAB         | Ap<sup>+</sup>, qsoAB<sup>+</sup>, a pWSK30 derivative (9) |
| pETX9           | Ap<sup>+</sup>, sopA<sup>+</sup>, a mini-F plasmid (34) |
| pLysS           | Cm<sup>+</sup>, Lys<sup>+</sup> (35) |
| pET3a           | Ap<sup>+</sup>, T7 promoter, an overexpression vector (35) |
| pET3qSOPA       | Ap<sup>+</sup>, a pET3a derivative carrying a truncated sopA (35) |
| pETαSOPA        | Ap<sup>+</sup>, a pET3a derivative carrying a truncated sopA (35) |

1 The abbreviations used are: PBS, phosphate-buffered saline; PCR, polymerase chain reaction; kb, kilobase pair(s); PMSF, phenylmethylsulfonyl fluoride; PAGE, polyacrylamide gel electrophoresis; FGC, flotation gradient centrifugation; GPD, gross particle density; IEM, immunoelectron microscopy.
cloned into the NdeI-BamHI site of pET3a, resulting in a plasmid designated pETASOPA. Plasmid pC4F (9) was a pUC19 derivative carrying fragment C (entire qsopA) of QsOP. pFAB was constructed by ligation of a 3.3-kb EcoRI-StuI fragment (entire sopA) of the F plasmid into pUC19. All the above plasmids except pLyS3 carried an ampicillin resistance gene (ApR). Plasmids pZL30–49, and pZL30–210 were TnphoA mutants of the qsopA gene carried on plasmid pZL30. Plasmids pC12, pC35, pC38, and pC20 were TnphoA mutants of the qsopA gene carried on plasmid pC19; pF73, pM3–6, and pM3–7 were TnphoA mutants of the sopA gene carried on plasmid pFSOPA6. All these plasmids carried both ApR and kanamycin resistance genes (KanR).

TnphoA Mutagenesis and Localization of the Transposon Insertions —The methods of TnphoA mutagenesis, isolation of mutants, and localization of the transposon insertions on plasmids have been described (9), except that the target plasmids for mutagenesis were plasmids pZL30, pC19, and pFSOPA6.

Pho Activity Assays —CC118 cells harboring mutant plasmids were grown in LB broth with appropriate antibiotics, to an OD600 (optical density at wavelength 600 nm) of 0.5–0.7, followed by cooling on ice for about 5 min. PhoA activities were measured in CHCl3- and sodium dodecyl sulfate-treated cells according to the published procedure (36).

Affinity Chromatography and N-terminal Sequencing of PhoA Fusion Proteins —CC118 cells harboring p50–20 or pF73 were grown overnight in 50 ml LB broth with the additions of 0.5% SDS, 10 mM Tris-glycine, pH 8.1, 10 ml of 10 mM Tris-HCl, pH 8.1. Phenylnethylsulfonyl fluoride (PMSF) was added to a final concentration of 1 mM. Each sample was sonicated on ice for three times for 1 min each time. After centrifugation at 12,000 × g for 15 min, the supernatant was subjected to affinity chromatography by using immobilized rabbit antibacterial alkaline phosphatase (5 Prime 3 Prime, Inc., Boulder, CO), according to the manufacturer’s instructions. The purified proteins were concentrated using Centricon 30 (Amicon, Beverly, MA) according to the manufacturer’s instructions. Before isolation of the purified fusion proteins by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE), 10% polyacrylamide gel protein gels were prerun with 5 μM reduced glutathione in the running buffer for 40 min. Prior to loading the samples, the cathode buffer was replaced with fresh buffer containing 0.1 mM sodium thioglycollate. After electrophoresis, the separated proteins were blotted onto polyvinylidene fluoride membranes (Westran™, Schleicher & Schuell), visualized by Coomassie Brilliant Blue staining according to the manufacturer’s instructions, and the fusion proteins 30–20 and F73 were cut out for N-terminal sequencing (Laboratory for Biotechnology and Bioanalysis I, Department of Biochemistry and Biophysics, Washington State University).

Protein Labeling and Spheroplast Preparation —HB101 cells harboring an appropriate plasmid were inoculated into the M supplemented minimal medium and grown overnight. Subculturing in fresh medium led to an OD600 of 2.0–3.0. Exponentially growing cultures were labeled for approximately 1 h at a cell density of 1.4 × 108 cells/ml by addition of 56 μCi/ml [35S]methionine (EXPRE-35S, NEEN Life Science Products, Houston, TX). The labeling was stopped by addition of 100 μl of 1 mM cold methionine/ml, and the labeled cultures were cooled on ice. A culture of 7.5 × 108 cells was centrifuged to collect the cells as a pellet. Cells were first converted to spheroplasts to release the periplasmic contents, using a previously described method (37). After conversion to spheroplasts, followed by centrifugation, the supernatant was collected as the periplasmic fraction and the pellet as spheroplasts.

Flotation Gradient Centrifugation (FGC) Analysis —For [35S]methionine-labeled bacteria harboring low copy number plasmids (pQSOAPAB and pX99), spheroplasts were osmotically lysed by resuspending in 0.38 ml of lysis buffer (0.1 M Tris-HCl, pH 8.2, 5 mM EDTA) for 10 min. The periplasmic fraction and spheroplasts were then subjected to three or four cycles of freezing and thawing to completely lyse all spheroplasts. Viscous DNA in lysates was digested by the addition of 5 μg of DNase I (Life Technologies, Inc.) per ml of sample. The periplasmic fraction and spheroplast lysates were centrifuged for 20 min at 338,000 × g with a TLA 100.2 rotor in a Beckman TL100 centrifuge. The supernatants (containing both the periplasmic and cytoplasmic proteins) and pellets (containing the membranes) were combined. A pellet containing membranes of approximately 5 × 1010 cells was subjected to FGC (38). From each FGC, 13 g fractions were obtained in each of each. Approximately one third was used for direct protein visualization on 14% SDS-PAGE gels and the rest was used for immunoprecipitation analysis (see below).

Proteinase K Accessibility of Partitioning Proteins in Intact Spheroplasts —With [35S]methionine-labeled bacteria harboring high copy number plasmids (pC4F and pFAB), the methods to prepare spheroplasts were essentially the same as above. Before collection, spheroplasts were treated with proteinase K (Life Technologies, Inc.) (0.5 mg/ml) for 30 min on ice, and the treatments were stopped by addition of 1 mM PMSF. The treated spheroplasts were then collected by centrifugation, lysed by resuspending in 0.15 ml of lysis buffer followed by two or three freezing/thawing cycles, continued incubation on ice for 20 min and 1 mM PMSF added. A spheroplast lysate prepared from approximately 2.15 × 109 cells was used for immunoprecipitation analysis.

Immunoprecipitation —The immunoprecipitation method was based on the procedure of Randall and Hardy (39). After FGC or proteinase K treatments (see above), samples were centrifuged at 0°C, Eppendorf centrifuge 5415) after precipitation with trichloroacetic acid. The supernatants were discarded, and the residual acid was removed from the tubes by addition of 1 ml of ice-cold acetone to each without suspending the pellets, followed by 2–5 min of centrifugation. Each pellet was washed once with 250 μl of ice-cold acetone and then dissolved in 100 μl of 0.1 M Tris-acetate, pH 7.6, 1 mM PMSF, incubated for 5 min at 98 °C, and briefly centrifuged. Fifteen μl of this 50-μl sample was saved for 14% SDS-PAGE analysis, and the proteins were visualized by Coomassie Brilliant Blue staining.

Before overnight incubation on ice with occasional mixing, the Sepharose was centrifuged for ~10 s and washed by suspending in 1 ml of wash solution (0.15 M NaCl, 0.1% SDS, 10 mM Tris acetate, pH 7.6, and 5 mM EDTA) by a 10-s centrifugation. The was wash repeated one more time. The Sepharose pellets were washed at once by suspending in 1 ml of 10 mM Tris acetate, pH 7.6, followed by a 10-s centrifugation. Forty μl of 2× SDS sample buffer was added to each pellet of Sepharose beads and the samples incubated at 100 °C for ~5 min to release the immunoprecipitated proteins from the protein A-Sepharose beads. After a brief centrifugation, 20 μl each was loaded onto 10% SDS-polyacrylamide gel for electrophoresis. Follow-
bacterial lysates were purified as inclusion bodies according to the protocol of Sambrook et al. (40) with some modifications. Every 100 g of thawed bacterial pellet was resuspended in 80 ml of lysis buffer (50 mM Tris-HCl, pH 8.0, 25% sucrose (w/v), 1 mM EDTA, and 1 mg/ml lysozyme) and incubated on ice for 30 min. Into the viscous lysate were added the following chemicals (final concentration): 10 mM MgCl₂, 1 mM MnCl₂, and 10 μg/ml DNase I; and the lysate was shaken at 250 rpm, 37 °C for 30 min or until it was no longer viscous, followed by addition of 200 ml of detergent buffer (0.2 M NaCl, 1% deoxycholic acid (w/v), 1% Nonidet P-40 (v/v), 20 mM Tris-HCl, pH 7.5, and 2 mM EDTA). The suspension was mixed well and the inclusion bodies pelleted by a 10-min centrifugation at 5,000 g. The pellet was washed once with 4 ml of double-distilled H₂O, followed by another 10-min centrifugation. The pellet was then washed by resuspending in 4 ml of 0.5% Triton X-100, 1 mM EDTA, followed by a 10-min centrifugation. The last wash step was repeated until a tighter pellet was obtained. The pellet was resuspended in double-distilled H₂O with 1 mM PMSE.

Approximately 1–2 mg of the purified inclusion bodies were dissolved in SDS sample buffer by incubation in a boiling water bath for 5–10 min and separated from contaminants (<10%) on 10% preparative SDS-polyacrylamide gels by the method of Ausubel et al. (41). Gel slices containing the protein were excised, immersed in 70% ethanol for 30 min, and homogenized in PBS, pH 7.4. The homogenate was used to immunize a New Zealand White rabbit (1–2 mg injection). Anti-QsopA or anti-SopA sera were prepared using the procedures for immunosorbent assay (41), were collected from the immunized rabbits.

**Immunoelectron Microscopy (IEM)**—E. coli HB101 carrying an appropriate plasmid was grown in LB medium to an OD600 of 0.5–0.7, and homogenized in PBS, pH 7.4. The homogenate was used for IEM essentially as described by Maddock and Shapiro (43). Nickel grids mounted with thin sections of the bacterial cells were rocked gently at room temperature for 3 days with anti-SopA in 0.5 × PBS (pH 7.4, 0.3% Tween 20, 1% bovine serum albumin). Anti-SopA labeled colloidal gold particle-conjugated protein A (BioCell Research Laboratory). Examination of grids was carried out using a Hitachi 600 electron microscope at 75 kV.

**Statistical Analysis**—Quantitative analysis of particle distribution in bacteria was carried out by using the NIH 1.52 image program at a magnification of 20,000. With that program, different areas (including an outside-cell area, which was the area surrounded by the curve ~ 25 nm outside of the cytoplasmic membrane) of a given cell were measured and the gold particles in the areas counted (see Table III). All gold particles located within approximately 25 nm of the cytoplasmic membrane were scored as being associated with the membrane. Each microscopic field had a rectangular size of approximately 16 μm², and contained approximately 7–25 cells. Cross particle density (GPD, particle number/μm²) was calculated for cytoplasmic, membrane and outside-cell areas of each cell. Gold particle density of subcellular areas was calculated by subtraction of the subcellular GPD with the field background (outside-cell area) GPD (0.3–0.7/μm²). In total, about 20–25 different fields located on five to eight different grids were examined for each type of bacteria. Significance tests were carried out on the basis of individual cells by using the statistical program SAS.

**RESULTS**

**Isolation of PhoA-positive TnphoA mutants of qsopA and sopA**—From our previous TnphoA mutagenesis of pZL30, a cosmid phC79 (43) derivative containing approximately 80% (30 kb) of the QpH1 DNA, we obtained three qsopA-phoA fusion genes 30–20, 30–8, and 30–49. The encoded fusion proteins displayed PhoA activity (9), indicating that QsopA could be a membrane protein. To confirm that QsopA-PhoA fusions displayed PhoA activity, we did TnphoA mutagenesis of the qsopA locus carried on the plasmids pZL30 and pC1.9. Plasmid pC1.9 carried a 1.9-kb EcoRI-KpnI DNA fragment containing the qsopA locus only (9), so that the PhoA activity of the resulting QsopA-PhoA fusions should not be caused by the presence of other QpH1 DNA. To enhance fusion expression in cases where the promoters of interest display weak promotion or undergo auto-regulation, TnphoA mutagenesis can be carried out on high copy number plasmids (45). We isolated seven more positive qsopA-phoA fusions on these two plasmids (Fig. 1, upper panel). Significantly all the QsopA-PhoA fusions, including those encoded from the qsopA mutants generated from pC1.9, displayed PhoA activity (Table II). These data again suggested that QsopA was bound to the bacterial cytoplasmic membrane or was exported. It was observed that the larger fusions displayed lower PhoA activity. For example, 30–20 retained only the first 12 residues of QsopA but displayed a PhoA activity of 289 units, whereas 30–49 retained the first 159 residues and displayed a PhoA activity of only 10 units. All fusions larger than 30–49 displayed consistently lower PhoA activities (Table II).

At the amino acid sequence level (10), QsopA was an analog of SopA, the partitioning protein A of the F plasmid. This led to the question whether SopA-PhoA fusions also displayed PhoA activity. To address this question, TnphoA mutagenesis of the sopA gene was carried out. We first subcloned the sopA gene into pUC19 and obtained the target plasmid pFSOPA6. During the construction of pFSOPA6, it was observed that, although both insertion orientations were possible, sopA was never inserted in an orientation under control of the lacZa promoter located on the vector pUC19. From TnphoA mutagenesis of pFSOPA6, three sopA mutants were isolated (Fig. 1, lower panel), and the encoded fusions displayed PhoA activity (Table II), indicating that SopA was also membrane-bound or exported.

Because fusions 30–20 and F73 retained only 12 and 8 residues of QsopA and SopA proteins, respectively, but were exported, it was possible that these fusions were translated from **FIG. 1. PhoA activity-positive TnphoA mutants of qsopA and sopA.** The target cells of TnphoA mutagenesis were E. coli cells harboring plasmids pZL30 or pC1.9 for qsopA and those harboring plasmid pFSOPA6 for sopA (see “Experimental Procedures” for details). Open arrow, open reading frames; vertical bar, positive TnphoA insertions. Labels are mutant names (amino acid residue number before the fusion site). In the case of qopA, mutants for which names begin with “30-” were from pZL30 (30–20, 30–8, and 30–49) and have been reported for fusion generation; Ref. 9, and those with “C” were from pC1.9. 30–210 and C12 were the same qopA mutants but located on two different plasmids. In the case of sopA, the mutants have not been reported previously, and the different initials were assigned because they were obtained by different mutant-screening procedures, with no other particular meaning here.
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TABLE II
Alkaline phosphatase activities of the protein A-PhoA fusion proteins expressed in E. coli

| PhoA fusion | 30–20 | 30–206 | 30–8 | 30–49 | 30–210 | C12 | C35 | C38 | C32 | C20 | F73 | M3–6 | M3–7 |
|-------------|-------|--------|------|-------|--------|-----|-----|-----|-----|-----|-----|------|------|
| PhoA activity (unit) | 289 ± 23 | 140 ± 3 | 376 ± 12 | 10 ± 4 | 10 ± 2 | 90 ± 8 | 13 ± 3 | 9 ± 3 | 18 ± 3 | 22 ± 2 | 460 ± 53 | 27 ± 3 | 16 ± 2 |

Generation of the corresponding protein A-PhoA fusions is presented in Fig. 1. PhoA activities were measured as described under “Experimental Procedures.” Background levels of PhoA activities (<1.7 units) found in PhoA controls have been subtracted from the values shown. Data are presented in mean ± S.D. (n = 9).

An increased quantity of \( \Delta \text{SopA} \) was obtained (data not shown).

QsopA and SopA Proteins Are Associated with the Bacterial Membranes—The above evidence suggested interaction of QsopA and SopA with the bacterial membrane. To determine whether these two proteins are associated with the bacterial membrane, we fractionated E. coli cells carrying the low copy number plasmid pQSQOB (for QsopA) or pXX9 (for SopA), followed by FGC as shown in Fig. 2. Centrifugation of cell lysates clearly resolves membrane-associated proteins from periplasmic or cytoplasmic proteins as indicated by the fact that no detectable EF-Tu was found in the pellet and no detectable OmpA was found in supernatants that contained both periplasmic and cytoplasmic soluble proteins (Fig. 2A). Immuno precipitation studies showed that 49% of the QsopA protein was in the supernatant and 51% in the pellet, and that 56% of the SopA protein was in the supernatant and 44% in the pellet (Fig. 2C, leftmost three lanes). Because protein aggregates might be pelleted with membranes, FGC (Fig. 2, A and B, lanes 1–13) was carried out to demonstrate that the pelleted material was truly membrane-associated. Fig. 2C shows that 75% of the pelleted QsopA floated to the density of membranes, as did 64% of the pelleted SopA. Thus, 38% of the total QsopA and 28% of the total SopA molecules were associated with the bacterial membrane fractions.

In the gradients, the density curve broke at the peak of outer membrane protein OmpA (Fig. 2B). The peaks of QsopA and SopA were located in fraction 3 at a lower density than that of fraction 4, which displayed the OmpA peaks (Fig. 2, compare B with C). Apparently, QsopA peaked in fraction 1 instead of fraction 3 as a result of a higher background in fraction 1. After subtraction of background, it should peak in fraction 3, according to densitometic analysis. Fig. 2C (left) shows that there was a small QsopA peak in gradient fraction 8. In other experiments, this small peak was not present but there was a concomitant increase in percent membrane association of the protein.

QsopA and SopA Proteins Are Protected from Protease K Digestion of the Spheroplasts—We next asked if the membrane-associated protein A was on the surface of bacterial spheroplasts and accessible to proteinase K. A problem developed when it was discovered that spheroplasts of bacteria harboring the low copy number plasmid expressing the A and B proteins were unstable. Approximately 90% were lysed after treatment with lysozyme. To carry out the proteinase K experiments, it was necessary to prepare stable protein A-containing spheroplasts. This became possible when the partition regions were subcloned into the high copy number vector pUC19. Bacteria harboring these pUC19 derivatives were converted to spheroplasts, and usually more than 90% remained intact. Spheroplasts harboring these pUC19 derivatives were prepared, treated with proteinase K, osmotically lysed, and subjected to FGC. The first six fractions contain membrane proteins and the last six fractions (fractions 8–13) contain cytoplasmic proteins because approximately 100% of the OmpA floated into the first six fractions and 94% of EF-Tu remained in the last six frac-
**FIG. 2.** Membrane association of QsopA (left) and SopA (right). A, separation of membrane-associated and cytoplasmic proteins by cell fractionation followed by FGC. E. coli HB101 harboring plasmids pQSOPAB (for QsopA) or pXX9 (for SopA) was grown at 37 °C in M9 minimal medium, the proteins were labeled by [35S]methionine and the cells were osmotically lysed followed by fractionation into supernatants (periplasm and cytoplasm) and membranes. The membranes were subjected to FGC. Labels: whole cell, total cell proteins as a control; supernatant, periplasmic and cytoplasmic proteins; pellet, membrane-associated proteins; fraction numbers 1–13, top to bottom of the gradient; EF-Tu, the most prominent protein (present in whole cell and supernatant only) as a marker for cytoplasmic proteins; OmpF/C and OmpA, two other markers (visible in whole cell, pellet, and fractions 1–5 only) for membrane proteins (38). Numbers at right are molecular mass standards (in kDa). B, quantitative distribution of OmpA and fraction density throughout the gradients displayed in A. Autoradiographs of the protein gels were analyzed by densitometry for band intensity measurement of OmpA. C, distribution of QsopA and SopA. The remainder (two thirds) of each fraction from A was used for immunoprecipitation of the QsopA or SopA protein with anti-QsopA (immunoabsorbed) or anti-SopA rabbit sera, respectively. Densitometric quantitation of the proteins in each lane (except "whole cell") is shown above the autoradiographs.
FIG. 3. Insensitivity of membrane-associated QsopA (left) and SopA (right) to proteinase K-digestion of intact spheroplasts. A, separation of membrane-associated and cytoplasmic proteins of proteinase K-treated spheroplasts. E. coli HB101 harboring plasmids pC4F (for QsopA) and pFAB (for SopA) was grown at 37 °C in M9 minimal medium, the proteins were labeled by [35S]methionine, and the cells were


\section*{Membrane Association of Partitioning Proteins}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig4}
\caption{Sensitivity of QsoPA (upper) and SopA (lower) in spheroplast lysates to proteinase K digestion. Proteinase K-treated (lane 4) spheroplasts carried pUC19 (lanes 1 and 2) and pC4F (for QsoPA) or pFAB (for SopA) (lanes 3–5). For lane 5, spheroplast lysates were subjected to proteinase K digestion. A spheroplast lysate was immunoprecipitated with preimmune rabbit sera (QsoPA or SopA in lane 1) or anti-QsoPA/SopA rabbit sera (lanes 2–5). Labels: pre-, rabbit preimmune serum; +/−, spheroplasts were treated with proteinase K before (+) but not after (−) lysis.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig5}
\caption{Localization of SopA onto the membrane in bacterial cells. The protein molecules were immunolabeled with gold particles (seen as black spots). The labeling methods have been described under “Experimental Procedures.” A, bacteria harboring pUC19 (SopA-negative cells) showed a low density of random labeling; B, bacteria harboring pFAB (SopA-positive cells) showed that the SopA molecules tended to associate the membrane; C, labeling of a plasmolysed cell demonstrated that the SopA molecules tended to associate with the cytoplasmic membrane.}
\end{figure}

...tions (Fig. 3, A and B). β-NADH oxidase activity is a marker for cytoplasmic membrane fractions, and it was distributed through the flotation gradients as follows: 33% in fractions 1 and 2, 50% in fractions 3 and 4, and 7% in fractions 5 and 6; less than 10% was present in the soluble protein fraction. Thus, excellent separation of membrane-associated proteins from cytoplasmic proteins was obtained. Quantitative distributions of OmpA and EF-Tu in the leftmost three lanes of A are not shown here because we could not detect OmpA in the “supernatant” fraction nor EF-Tu in the “pellet” fraction.

Under these conditions, 13.2% of total QsoPA and 5.2% of total SopA molecules were retained with the bacterial membrane fractions (Fig. 3C). We observed that, with the high copy number plasmids, the overexpression factor was approximately 20–30 for QsoPA and SopA, based on immunoprecipitation analyses following overnight [35S]methionine labeling during bacterial growth in minimal media (labeling started when the bacteria were inoculated); however, this overexpression factor decreased to 2–4 if the bacteria were labeled for only 1.5 h. Importantly, the membrane-associated proteins did not differ in size when compared with those present in the cytoplasmic fractions. It was noticed that there are some protein bands with molecular mass between 41 kDa and 28 kDa in the left panel of Fig. 3C. Because these bands co-existed with QsoPA from the membrane to cytoplasmic fractions, they were not likely to be partial digestion products of QsoPA on the intact spheroplasts in the presence of proteinase K. More likely, they were either partial digestion products of QsoPA caused by lysis of unstable spheroplasts or other proteins that were associated with QsoPA. No evident partial digestion products in membrane fractions were observed for SopA either (right panel of Fig. 3C). Thus, the membrane-associated QsoPA and SopA in spheroplasts were apparently protected from proteinase K digestion.

The proteinase K was active as demonstrated by the degradation of OmpA (Fig. 2A). Without proteinase K treatment, the membrane association percentage was 6.6% for QsoPA and 5.4% for SopA. Proteinase K treatment increased the percentage from 6.6% to 13.2% for QsoPA, probably as a result of...
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The SopA molecules distributed in the bacteria were identified as gold particles by immunoreaction with anti-SopA and, in turn, with gold particle-conjugated protein A. Bacteria harboring plasmid pUC19 was a SopA-negative control, plasmid pFAB as SopA-positive. Membrane association of SopA was determined by comparing gold particle density around the cytoplasmic membranes with that in the cytoplasm. Significance test: the difference between two neighboring numbers is insignificant ($P > 0.80$) for identical symbols, significant ($P < 0.02$) for $\dagger$ and $\ddagger$, or very significant ($P < 0.0001$) for $\ddagger$ versus $\ddagger$, $\dagger$ versus $\dagger$, or $\ddagger$ versus $\dagger$.

| Plasmid | Cells examined | Subcellular area | Gold particles | Gold particle density |
|---------|----------------|------------------|----------------|----------------------|
|         |                | CA               | MA             | ICA                  | % | no./μm² | (Ratio) |
| pUC19   | 308            | 0.417² | 0.1176 | 0.3606 | (163) | 25.2 (41) | 74.8 (122) | 0.41² | 0.35² | (1.2) |
| (SopA⁻) |                | 0.3745³ | 0.1152 | 0.3210 | (445) | 40.0 (178) | 60.0 (267) | 4.21⁺ | 1.75⁺ | (2.4) |
| pFAB    | 309            | 0.417 | 0.1176 | 0.3606 | (163) | 25.2 (41) | 74.8 (122) | 0.41³ | 0.35³ | (1.2) |
| (SopA⁺) |                | 0.3745⁴ | 0.1152 | 0.3210 | (445) | 40.0 (178) | 60.0 (267) | 4.21⁻ | 1.75⁻ | (2.4) |

* CA, area surrounded by cytoplasmic membrane; MA, the area covering approximately 25 nm outside and inside of the cytoplasmic membrane; ICA, cytoplasmic area whose edge is approximately 25 nm inside of the cytoplasmic membrane; MA = OCA – ICA (also see “Experimental Procedures”).

The percentage of gold particles in the cytoplasm or associated with the membrane and the actual number of gold particles counted in parentheses.

‡ Gold particles per subcellular area, including both cytoplasmic and membrane areas. The numbers in ( ) are density ratios (membrane/cytoplasm).

digestion of QsopA released from unstable spheroplasts during treatment.

There are three possible explanations for the inability of proteinase K to degrade the membrane-associated QsopA or SopA. 1) Both proteins were inherently resistant to digestion by proteinase K, 2) the membrane-associated molecules are buried in a membrane, or 3) the molecules are associated with the cytoplasmic face of the membrane. We performed further proteinase K sensitivity experiments to evaluate these possibilities. Spheroplasts containing overexpressed protein A were treated with proteinase K before or after lysis, and the undigested protein A was examined by immunoprecipitation studies. QsopA and SopA were completely digested by proteinase K treatment of lysed spheroplasts (Fig. 4, lanes 5). Treatment of presumed intact spheroplasts with proteinase K resulted in the digestion of less than 10% of QsopA or SopA (Fig. 4, compare lanes 3 with 4), probably a result of digestion of the proteins released by spontaneous lysis of unstable spheroplasts as described above.

Membrane Association of Partitioning Protein A Is Further Verified by Immunoelectron Microscopic Studies—Immunoelectron microscopic localization of QsopA within the bacteria independently supported the above biochemical findings. Immunogold labeling of *E. coli* HB101 cells harboring plasmid pFAB, the pUC19 derivative carrying the sopAB locus to increase the quantity of QsopA and thus the sensitivity of immunoelectron microscopy (IEM) (45), was carried out. The labeling density was quite low, with an average density of approximately 1.5 particles/μm² (Fig. 5, A and B). All the particles located in the membrane areas were generally distributed around the edges of the bacteria, without evident polarization in any fields examined. However, statistical analysis of particle distribution revealed that QsopA was significantly associated with the bacterial membrane (Table III). The specific membrane density (particle density ratio of membrane/cytoplasm) was 2.4 for SopA-positive cells, twice as much as that (1.2) for the negative control. In plasmolyzed cells, the protein was clearly labeled on the cytoplasmic membrane (Fig. 5C). We carried out similar studies for QsopA with immunosorbed anti-QsopA rabbit serum. Despite a high background, the specific membrane density for QsopA-positive cells was 1.8 times as much as for the negative control. Thus, the specific membrane densities appeared to be similar between QsopA and SopA.

**DISCUSSION**

Isolation of PhoA-positive TnphoA mutants of *qsopA* and *sopA* is of considerable interest because neither QsopA nor SopA carries a hydrophobic N terminus. N-terminal sequencing of 30–20 and P73 demonstrates that the fusions retain only 12 or 8 residues of the protein A, and yet they appear to be localized to the membrane. One may argue that the PhoA activity displayed by the fusions was a result of leakage or slow activation of cytoplasmic PhoA. However, recent studies have demonstrated that signal-sequenceless PhoA proteins expressed at a high level were retained in the *E. coli* cytoplasm and were not activated during incubation on ice for 15 min; nor did incubation at 37 °C activate cytoplasmic PhoA (46). In our experiments, the bacterial cultures were held on ice for approximately 5 min before activity measurements (see “Experimental Procedures”). Therefore, it is reasonable to assume that the protein A-PhoA fusions displayed PhoA activity apparently caused by translocation across the bacterial cytoplasmic membrane and not to leakage or cytoplasmic activation.

Data from cell fractionation, proteinase K treatment studies, and FGC analyses indicate that active plasmid partitioning protein A (QsopA and SopA) is associated with the cytoplasmic side of the bacterial inner membrane. In fact, the data presented in both Figs. 2 and 3, and in other experiments, show that these proteins are found in fractions of lighter density than those containing OmpA, suggesting an association with the cytoplasmic membrane. Because normal active partitioning may only require protein A of plasmids pTAR (47) and QpH1,² this membrane association potentially represents one of the most important functions described for partitioning proteins (6–8) and provides current partitioning models (1–3, 6, 19, 48, 49) with experimental evidence that protein A mediates attachment of the plasmid molecules to the membrane of the host cell. Because of the low expression of protein A, we were unable to examine the possible polar location of these proteins in the bacteria by IEM.

Cloning of the partition regions into the high copy number plasmid vector pUC19 resulted in a reduced level of percent membrane association of the partitioning protein A (Fig. 3). It is possible that the decreased percent membrane association observed is a result of membrane saturation in the presence of excess partitioning protein A. This possibility is supported by studies in which we found that when expression levels of fusions were increased; the increase in PhoA activity displayed...
by a QsopA-PhoA fusion was less than the β-galactosidase activity displayed by the fusion in which the PhoA portion was replaced with LacZ. This suggests a mediating system located in the cytoplasmic membrane, able to translocate the QsopA-PhoA fusion protein across the membrane for PhoA activation. 

What is not understood is how the presence of high copy number plasmids (pC4F and pFAB) stabilized the spheroplasts. Given that the difference in molar concentration between low and high copy number plasmids was much greater for the plasmid DNA (approximately 100) than that (20–30) for the expressed protein A, this stabilization might be a consequence of higher affinity of the soluble QsopA or SopA molecules for high copy number DNA binding sites located on the plasmids than that for membranes. The difference in molar levels of QsopA and SopA expressed from the low and high copy number plasmids was underestimated by immunoprecipitation followed by autoradiography analyses when the [35S]methionine labeling of the proteins lasted for only a short period of time (1–2 h) (see “Results”). Under these conditions, the labeled molecules represented a minority of the existing molecules because of slower bacterial growth in a minimal medium and autoregulation of protein expression. Although overexpression by a factor of 20–30 might skew the proteinase K treatment data, use of the high copy number plasmids was the only way to avoid autolysis of the spheroplasts. The fact that protein labeling for 1–2 h did not reach a steady state should not affect the data unless FGC and proteinase K digestion had a preference between labeled and unlabeled protein molecules.

IEM data show a significant level of membrane association of SopA, expressed from pFAB (Table III). SopA molecules that were loosely membrane-associated did not dissociate from the membrane because the bacteria were not subjected to cell fractionation steps but rather were fixed in formaldehyde and glutaraldehyde immediately after harvesting. In situ fixation might also reveal any nonspecific interaction between the membrane and the SopA molecules when SopA molecules were present in excess. One argument could be that the significant membrane association observed was a result of formation of inclusion bodies of the partitioning protein because of its expression from a high copy number plasmid. Precipitation of the inclusion bodies on the membrane during centrifugation (20 s, Eppendorf centrifuge 5415) (or harvesting) of the bacteria might partially account for the significant degree of membrane association. However, the expression level of SopA from pFAB was generally very low, as we failed to detect the protein by Western blot analysis. This is most likely caused by autoregulation, which keeps a controlled molar concentration of free SopA molecules in the cytoplasm, suggesting that inclusion bodies are unlikely to form. The observed increase in protein levels with the high copy number plasmid pFAB could result mainly from a high copy number DNA-bound form of the protein molecules. As IEM showed, the membrane-associated gold particles did not tend to be located on the same side of the cells on each thin section, indicating that the membrane association was not a result of a centrifugation-caused deformation of SopA inclusion bodies on the membrane. The membrane association definition utilized (−25 nm within cytoplasmic membrane) might be considered arbitrary, but it should not affect the data presented for at least two reasons. 1) The diameter (20 nm) of gold particles helped to define the distance from the membrane. 2) Enlarging the membrane area coverage would dilute the particle density in the membrane area because the background or cytoplasmic particle density was always lower; reducing the membrane area coverage would also decrease the particle density in the membrane area because the background density could be increased by leaving some of the membrane-associated particles out as outside-cell particles.

Several explanations are possible for the fact that protein A is not quantitatively recovered bound to membranes. First, FGC may underestimate the amount of association existing in vivo. During the course of FGC, some protein A might dissociate from the membrane. The observation of protein A in the gradient at densities greater than that for membrane, but too low to represent the equilibrium position of proteins is likely to represent protein A dissociated during centrifugation. It is also possible, because protein A has ATPase activity, that a nucleotide is required for stable association.

A more interesting possibility is that protein A cycles during its function between a cytosolic and a membrane-bound form, a situation reminiscent of SecA, a protein mediating export of proteins in E. coli (50–55). Protein A is essential for plasmid partitioning, rather than protein translocation, but it shares several features with SecA: 1) lack of a hydrophobic membrane domain (but approximately one third of the protein molecules are recovered bound to the bacterial cytoplasmic membrane and the rest remain free in the cytoplasm), 2) ATPase activity, 3) formation of a homodimer, 4) cooperation with other protein(s), and 5) possible membrane saturation when the protein is overexpressed. SecA functions in a cycle; it binds to a polypeptide, mediates translocation of the polypeptide through the membrane, and then releases into the cytoplasm again (56). It is possible that protein A also functions in a cycle, by binding plasmid molecules to the membrane only when plasmid partitioning occurs at cell division.

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