F exclusion of bacteriophage T7 occurs at the cell membrane

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Abstract

The F plasmid PifA protein, known to be the cause of F exclusion of bacteriophage T7, is shown to be a membrane-associated protein. No transmembrane domains of PifA were located. In contrast, T7 gp1.2 and gp10, the two phage proteins that trigger phage exclusion, are both soluble cytoplasmic proteins. The Escherichia coli FxsA protein, which, at higher concentrations than found in wild-type cells, protects T7 from exclusion, is shown to interact with PifA. FxsA is a polytopic membrane protein with four transmembrane segments and a long cytoplasmic C-terminal tail. This tail is not important in alleviating F exclusion and can be deleted; in contrast, the fourth transmembrane segment of FxsA is critical in allowing wild-type T7 to grow in the presence of F PifA. These data suggest that the primary event that triggers the exclusion process occurs at the cytoplasmic membrane and that FxsA sequesters PifA so that membrane damage is minimized.

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Introduction

F exclusion of T7 is but one of many instances known where a resident plasmid or prophage interferes with the growth of a (super)infecting phage (for reviews, see Coffey and Ross, 2002; Duckworth et al., 1981; Molineux, 1991; Snyder, 1995; Snyder and Kaufmann, 1994). Exclusion may manifest at many stages of the phage developmental cycle, including adsorption to the host cell, genome penetration of the cell cytoplasm, susceptibility of the phage genome to endogenous restriction nucleases, and intracellular phage gene expression. Exclusion through a block in adsorption was originally called lysogenic conversion, a prophage gene expressed in lysogenic state modifies or makes the cell surface receptor inaccessible for the excluded phage; lysogenic conversion can thus alter the serotype of a bacterium. Aside from the well-known immunity systems expressed by temperate phages, the exclusion of T4 rII mutants by λ rex+
lysogens was one of the first abortive infections to be described. Like T7 and F, and the exclusion of T5 by a resident Col Ib plasmid, the underlying mechanism of rex exclusion remains obscure. The plethora of physiological defects that accompany these classic abortive infections has made it difficult to distinguish the initial events that cause the abortive infection from resulting later events.

Growth of bacteriophage T7 in Escherichia coli strains harboring the F plasmid is aborted midway through the infection cycle. The initial steps of infection occur normally, and early gene products are synthesized just as in a productive infection (Molineux, 1991). However, later events are grossly abnormal. Transcription, particularly over the late region of the T7 genome, is severely reduced (Britton and Haselkorn, 1975; Condit and Steitz, 1975; Young and Menard, 1975; Beck and Molineux, 1991). Inhibition is due both to a decreasing capacity of the abortively infected cell to sustain macromolecular synthesis and to the fact that only about half of the infecting phage particles translocate the late region of the genome into F cells (Garcia and Molineux, 1995). In addition, there is little or no phage DNA replication and the host chromosome is not extensively degraded. ATP and other small molecules leak from abortively infected cells into the surrounding growth medium (Schmitt et al., 1991). Although they do not lyse, the abortively infected cells die.
This abortive infection of T7 in cells harboring the F plasmid is described as F exclusion.

A single F gene, pifA, is responsible for exclusion of T7 (Cram et al., 1984a, 1984b; Morrison and Malamy, 1971; Schmitt and Molineux, 1991); early reports of a pifB gene are erroneous. Expression of pifA is, however, controlled by the operon repressor pifC (Kennedy et al., 1988; Miller and Malamy, 1983, 1984). The pif operon is totally dispensable for normal F plasmid maintenance and conjugation. Plasmids expressing pifA as the only F gene exclude T7 (Cram et al., 1984a, 1984b; Schmitt and Molineux, 1991), and this is the only known function of PifA. No biochemical properties of PifA have been identified and its cellular location is unknown.

Synthesis of either wild-type gp1.2 or gp10 of T7 is required for F exclusion (Molineux et al., 1989). T7 mutants that grow normally in male strains of E. coli necessarily contain a null or missense mutation in gene 1.2 and two missense mutations in gene 10. Genetic analyses suggest that the gene 1.2 mutation and one of the gene 10 mutations both reduce interactions with pifA (Schmitt et al., 1991). The second gene 10 mutation reduces the rate of synthesis of gp10, thereby allowing sufficient progress through the phage developmental cycle so that the exclusion process no longer prevents production of progeny phage (Molineux et al., 1989; Condreay and Molineux, 1989).

Gene 1.2 is not essential for phage growth in wild-type E. coli strains, but is required for growth in optA mutants that overproduce a cellular dGTPase (dgt) (Beauchamp and Richardson, 1988; Saito and Richardson, 1981; Wurgler and Richardson, 1990). Binding of gp1.2 to the dGTPase inhibits its activity, and the enzyme is converted into an rGTP-binding protein (Huber et al., 1988; Nakai and Richardson, 1990). However, it is not clear whether this activity of gp1.2 is pertinent to the process of F exclusion. Mutations affecting the related T3 gene 1.2, which is normally responsible for the ability of wild-type T3 to avoid F exclusion, have been characterized that fail to inhibit dgt but remain active in preventing exclusion, and vice versa (Schmitt et al., 1987). Gene 10 codes for the major capsid protein of T7; assembly of gp10 into proheads is not required to elicit F exclusion (Condreay and Molineux, 1989), and no other activity of the protein has yet been described.

All the physiological defects associated with the abortive infection of T7 in F cells are found when plasmids containing either of T7 gene 1.2 or gene 10 as the only T7 gene are expressed in cells containing pifA as the only F gene (Schmitt, 1989; Schmitt and Molineux, 1991; Schmitt et al., 1991). However, this simplification of the abortive phage infection neither revealed the underlying mechanism of exclusion, nor has it clearly identified the initial event that results in the subsequent complex series of physiological dysfunctions. Furthermore, neither the use of T7 mutants that grow in F cells nor the use of E. coli mutants (containing a wild-type F) that exclude T7 growth to different degrees has yet distinguished the primary cause from subsequent effects in the exclusion pathway. For example, T7 mutants that escape F exclusion still cause nucleotide leakage from infected cells, and E. coli mutants that suppress F exclusion do not completely prevent leakiness (Schmitt et al., 1991; Wang et al., 1999a, 1999b). The loss of membrane integrity that allows small molecules to be lost from the infected cell is therefore only an effect caused by an earlier dysfunction.

An E. coli mutant that allows T7 to plate at normal efficiency in the presence of F was isolated by selecting cells that survived co-expression of gene 10A and pifA or gene 1.2 and pifA (Wang et al., 1999a). The mutant contains an up-mutation in the promoter for fxsA that causes 25-fold overexpression of fxsA. The resulting increased synthesis of FxsA alleviates the inhibition of T7 growth by F. Fluorescence microscopy of cells harboring fusions of fxsA to gfp showed that FxsA-GFP was localized to the cell envelope and the protein fractionated with the cytoplasmic membrane (Wang et al., 1999b). The cellular function of FxsA is not known and fxsA null mutants have no obvious phenotype. To gain a better understanding of F exclusion and in particular of how FxsA suppresses the abortive infection of T7, we have determined the cellular locations of the proteins involved. PifA is associated with the cytoplasmic membrane, whereas gp1.2 and gp10 are both cytoplasmic proteins. We show that PifA interacts with FxsA, suggesting that the mechanism of protection by FxsA is to sequester PifA from T7 gp1.2 and gp10. We have also determined the membrane topology of FxsA, showing that it contains four transmembrane segments and that the fourth segment, but not the C-terminal cytoplasmic tail, is essential for suppressing F exclusion.

**Results**

PifA is associated with the cytoplasmic membrane

Overexpression of fxsA in cells containing F alleviates exclusion of T7 (Wang et al., 1999a, 1999b). In those studies, FxsA was shown to be a membrane-associated protein, and it was therefore of interest to determine the cellular location of the protein that causes exclusion. Cells expressing pifA from its natural location on F were fractionated into cytoplasmic and membrane fractions. PifA protein was visualized using anti-PifA antibody and was found exclusively in the membrane fraction (Fig. 1A). Treatment of this membrane fraction with 0.5% Sarkosyl resulted in the complete solubilization of PifA (Fig. 1B). As Sarkosyl selectively solubilizes cytoplasmic membrane proteins (Filip et al., 1973), this result strongly suggests that PifA is tightly bound to the cytoplasmic membrane. Washing with 1 M NaCl does not cause PifA to dissociate from the membrane (Fig. 1C), indicating that the protein is not simply associated with the membrane through ionic interactions.
Membrane proteins were further separated into inner and outer membrane fractions through a 25–55% sucrose step gradient (Osborn et al., 1972). Two distinct peaks of protein were found (Fig. 1D); as expected OmpA, an outer membrane marker, was detected in the heavy fractions, whereas NADH oxidase, a cytoplasmic membrane protein, was found in light fractions. The presence of PifA coincided with the inner membrane fractions, showing directly that PifA is associated with the inner membrane.

Analysis of the predicted amino acid sequence of PifA reveals a Walker A box or P-loop, a flexible loop commonly associated with ATP/GTP-binding proteins (Koonin, 1993). A site-specific mutation altering the conserved lysine of the P-loop motif to alanine, yielding PifA-K59A, completely abolishes activity with respect to exclusion of T7 (Wang, 1997). Cell extracts containing this mutant PifA were also fractionated into membrane and soluble fractions, PifA-K59A remained membrane-associated (Fig. 1E). Two N-terminal deletions of PifA, neither of which interfered with T7 growth, were also examined by this procedure. Removal of the N-terminal 180 amino acids of PifA caused no change in its membrane localization, whereas removal of the N-terminal 462 amino acids (from a total of 717) resulted in a distribution of PifA with about 60% in the membrane and 40% in the soluble fractions. Thus, amino acids 181–462 of PifA contain a membrane-localization sequence with a second determinant in the C-terminal third of the protein.

We then attempted a protease accessibility assay of PifA in spheroplasts and inverted membrane vesicles prepared from F cells. However, insufficient PifA is made from the single-copy F plasmid for reproducible detection of the protein in subcellular fractions. We therefore performed these studies using cells expressing a multi-copy plasmid-borne N-terminal-tagged variant of pifA, his-pifA. Plasmids expressing his-pifA are as effective as comparable pifA plasmids in causing exclusion of T7 (data not shown). After proteinase K treatment of spheroplasts, anti-PifA antibody detected four distinct species (Fig. 2). The predominant protein has an apparent mass that corresponds to intact his-PifA (83 kDa), the minor bands have apparent mass values of approximately 75, 50, and 45 kDa. The 83- and 45-kDa species have the same electrophoretic mobility as proteins detected by anti-his antibody (data not shown), the two other bands have presumably lost their N-termini. It is unclear how these fragments of
PifA were generated; it is likely that they are artifacts of pifA overexpression. Protease treatment of inverted membrane vesicles destroyed almost all protein cross-reacting with anti-PifA antibodies, the small amounts of residual material are likely to be due to vesicles that did not invert. Overall, these data suggest that PifA is a cytoplasmic, membrane-bound protein, but it may exist in more than one conformation when present at artificially high concentrations.

In an attempt to determine whether PifA is a transmembrane protein containing periplasmic domains, we selected Tn<sup>phoA</sup> insertions into different pifA plasmids, and in several experiments examined more than 5000 independent colonies. Only 10 colonies were blue on XP indicator plates, indicative of a periplasmic location of a PhoA fusion protein, but none contained a pifA-phoA fusion. Eight of the 10 colonies contained a plasmid where Tn<sup>phoA</sup> had inserted—at more than one site—into bla, the gene for ampicillin resistance. Because plasmid maintenance and cell growth required β-lactamase, and therefore that Bla–PhoA fusion proteins maintain catalytically active, we believe that this screen for pifA-phoA fusions was quite rigorous. However, we failed to find blue colonies in a separate experiment where the target plasmid for Tn<sup>phoA</sup> transposition contained both pifA and fxsA. Fusions of PhoA to the short periplasmic domains of FxsA (see Fig. 5, below) would yield blue colonies. This negative result left open the possibility that PifA might also contain a short periplasmic domain(s). We constructed four pifA-phoA fusions in vitro using convenient restriction sites corresponding to pifA codons 121, 175, 275, and 520 (full-length PifA contains 717 amino acids). Using synthetic oligonucleotides, an additional eight fusions were constructed within a 50 amino acid region (residues 332 through 381) of PifA that, from the protease accessibility of PifA in spheroplasts, was thought most likely to contain the putative transmembrane segment of PifA. No fusion yielded blue colonies on indicator plates, suggesting that fusions of PhoA to a periplasmic domain of PifA are lethal to E. coli or are rapidly degraded or that PifA is not a transmembrane protein and thus has no periplasmic domains. Although no firm conclusions about a periplasmic domain(s) of PifA can be drawn from this work, we remain certain that PifA is a membrane-associated protein.

**T7 gp10A and gp1.2 are both cytoplasmic proteins**

Indirect evidence has suggested that PifA interacts directly with both T7 gp1.2 and gp10A (Molineux, 1991; Wang et al., 1999a, 1999b). Because PifA is membrane-associated, it was therefore of interest to determine the cellular location of the two T7 proteins. To address this question, cell extracts were fractionated by SDS-PAGE and probed with anti-gp10A and anti-gp1.2 serum. To minimize aggregation or inclusion body formation of gp10A due to its low solubility (Cerritelli and Studier, 1996), gp10A was expressed from a low copy number plasmid without induction. T7 gp1.2 is known to be a soluble protein (Myers et al., 1987). Cells containing either T7 gp10A or gp1.2 were lysed and extracts separated into membrane and soluble fractions. Under conditions where FxsA was exclusively found in the membrane fraction, both gp10A and gp1.2 were found only in the soluble fraction (Fig. 3). Overexpression of T7 gene 10A in cells resulted in 15–20% of gp10A fractionating with the membrane (data not shown). This association was weak and could be abolished by the addition of 0.1 M NaCl to the cell extract before fractionation. Moderate concentrations of NaCl are optimal for gp10A solubility (Cerritelli and Studier, 1996), and the apparent mislocalization to the membrane is likely an artifact of high intracellular concentrations. We conclude that both T7 gp1.2 and gp10 are normally cytoplasmic proteins, although during F exclusion of T7, they likely interact with the membrane-associated protein PifA (X. C. and I. J. M., unpublished data).

**The N- and C-termini of FxsA are both cytoplasmic**

In a previous study, FxsA was shown to be a cytoplasmic membrane protein that, when present at elevated levels, allowed T7 to avoid F exclusion (Wang et al., 1999a, 1999b). Most computer algorithms that predict membrane
protein topology suggest that FxsA has three transmembrane domains with the N-terminus of the protein in the cytoplasm and the hydrophilic C-terminal tail in the periplasm. We began to determine the actual topology by constructing the plasmid pHisFxsA, which directs synthesis of an N-terminal His-tagged variant of FxsA. This plasmid allows T7 to grow in strains containing F (see Table 1, below); the his-tag does not therefore interfere with FxsA activity. Consequently, it is unlikely that the positively charged his-tag significantly alters the membrane topology of the native protein.

Cells containing pHisFxsA were converted into either spheroplasts or inverted membrane vesicles, and the susceptibility of the N- and C-termini of his-FxsA to protease digestion was then ascertained. The N-terminus of his-FxsA is not accessible to proteinase K in intact spheroplasts but is rapidly degraded in inverted membrane vesicles (Fig. 4A). Thus, in agreement with computer predictions of FxsA membrane topology, the N-terminus of his-FxsA is cytoplasmic. An antibody raised against the C-terminal 37 residues of FxsA was used to determine the cellular location of the C-terminus of his-FxsA. Like the N-terminus, the C-terminus of his-FxsA is resistant to protease in spheroplasts but is protease-sensitive in inverted membrane vesicles (Fig. 4B). Thus, the C-terminus of his-FxsA is also cytoplasmic, an experimental result that disagrees with most computer predictions of FxsA membrane topology.

Direct visualization of spheroplast or inverted membrane vesicle preparations by microscopy showed few if any intact cells remained. In spheroplasts, the outer membrane protein OmpA was completely degraded by proteinase K whereas the cytoplasmic thioredoxin was resistant (Fig. 4C), indicating that spheroplast formation had preserved the integrity of the cytoplasmic membrane. We had shown previously that FxsA is an integral membrane protein (Wang et al., 1999b); as both the N- and C-termini of his-FxsA are cytoplasmic, there must be an even number of transmembrane domains. Because no fragments of FxsA were detected after proteinase K treatment, we can further conclude that the periplasmic segments must be short. The central periplasmic domain of the λ S holin, a known transmembrane protein, is also resistant to proteinase K after spheroplast formation (Bläsi et al., 1999).

**FxsA contains four transmembrane segments**

To determine both the number and the locations of transmembrane segments in FxsA, a series of fxsA-phoA gene fusions were constructed and assayed for phosphatase activity. Because alkaline phosphatase is only enzymatically active in the oxidizing environment of the periplasm and is inactive when in the reducing environment of the cytoplasm (Boyd et al., 1987a), phosphatase activities of fusion proteins correlate with the cellular location of the domain to which phosphatase is fused. All the fusion proteins migrated on SDS-PAGE gels at their expected sizes (Fig. 5A), and only the K53 fusion protein showed obvious degradation products. Fusion proteins in which the phosphatase moiety is localized to the cytoplasm are frequently unstable (San Millan et al., 1989), and we therefore calculated relative specific activities of phosphatase for the fusion proteins based on their rate of synthesis as determined by pulse-labeling with [35S]methionine. Specific activities of phosphatase are likely to reflect more closely the amount of fusion protein that is potentially available for export.

### Table 1

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Number of N-terminal residues in truncated FxsA</th>
<th>Relative plating efficiency</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>I511/F lac</td>
</tr>
<tr>
<td></td>
<td></td>
<td>30 °C</td>
</tr>
<tr>
<td>pHisFxsA</td>
<td>158 (wild type)</td>
<td>0.37</td>
</tr>
<tr>
<td>pHisFxsA121</td>
<td>121</td>
<td>0.22</td>
</tr>
<tr>
<td>pHisFxsA115</td>
<td>115</td>
<td>0.4</td>
</tr>
<tr>
<td>pHisFxsA111</td>
<td>111</td>
<td>0.2</td>
</tr>
<tr>
<td>pHisFxsA107</td>
<td>107</td>
<td>0.04a</td>
</tr>
<tr>
<td>pHisFxsA102</td>
<td>102</td>
<td>&lt;10⁻⁷</td>
</tr>
<tr>
<td>pTrchisA</td>
<td>b</td>
<td>&lt;10⁻⁷</td>
</tr>
<tr>
<td>pWF185</td>
<td>158 (wild-type)</td>
<td>0.32</td>
</tr>
<tr>
<td>pWSK129</td>
<td>b</td>
<td>&lt;10⁻⁷</td>
</tr>
</tbody>
</table>

a Pinpoint plaques.

b Vector plasmid.

c Wild-type FxsA without his-tag.
Fusions of phosphatase to FxsA residues S18, A24, L84, P88, and L95 all exhibit high levels of enzyme activity, whereas fusions to residues S40, S45, V47, K53, P68, S77, and P122 show much lower activity (Fig. 5B). These data lead to a topological model where FxsA possesses four transmembrane segments. Only two amino acids are predicted to lie between the first and second, and between the third and fourth, transmembrane segments of FxsA. Although these amino acids are predicted to be periplasmic, they are unlikely to be cleaved readily by proteinase K. This idea is consistent with the protease sensitivity experiment shown in Fig. 4. Fusions to residues T32 and P102 exhibit intermediate values of enzyme activity; in the proposed structure of FxsA, these residues fall near the center of a transmembrane segment, oriented from the periplasm toward the cytoplasm. The C-terminal residues of the FxsA portion of these fusion proteins may be unstably inserted into the membrane. Similarly, the higher activity of S40, S45, and V47, relative to the K53 and P68 fusion proteins may reflect the need for the positively charged R48 and K53 to anchor the PhoA portion of the fusion protein in the cytoplasm (Boyd et al., 1987b; San Millan et al., 1989).

**Membrane domains of FxsA are essential for alleviating F exclusion**

The membrane topology of FxsA that we have determined (Fig. 5B) predicts that the fourth transmembrane domain spans from leucine 95 to histidine 115 and that the hydrophilic C-terminal 43 amino acids are cytoplasmic. To investigate the importance of the C-terminus and the membrane domains of FxsA in protecting T7 from F exclusion,
His-FxsA mutants lacking various lengths of the normal C-terminus were created and their abilities to suppress F exclusion were then examined. His-FxsA truncations synthesizing only the N-terminal 121, 115, or 111 amino acids allow T7 to plate at essentially normal efficiency in cells containing F (Table 1). Thus, the cytoplasmic C-terminal tail of wild-type FxsA is not important in alleviating F exclusion. An even shorter form of FxsA, containing only 107 residues, allows T7 to plate efficiently on cells containing F (Table 1). Thus, the cytoplasmic C-terminal tail of wild-type FxsA is not important in alleviating F exclusion. The idea that this domain is important in exclusion. The activity of T3 gene 1.2 prevents exclusion whereas expression of T7 gene 1.2 actually causes exclusion (Condreay and Molineux, 1989; Garcia and Molineux, 1995; Molineux, 1991; Molineux and Spence, 1984; Schmitt and Molineux, 1991). An activity of T3 gene 1.2 prevents the newly synthesized gp10 capsid protein of the phage from interacting with the F protein PifA. The major capsid protein of all T7 family members that have been tested is capable of triggering exclusion in cells containing the F protein PifA.

The description of T7 as a female-specific phage is usually attributed to Mäkelä et al. (1964), although phages that fail to grow on F+ or Hfr strains of E. coli had been isolated previously (Dettori et al., 1961; Hakura and Hirota, 1961; Hakura et al., 1964; Watanabe and Okada, 1964; Zinder, 1961). However, it was only later that the relatedness between some of these phages and T7 was appreciated. T7 is now the prototype phage of the T7 family, which contains more than 60 representative coliphages plus additional phages that grow on other Gram-negative genera. Most of the coliphages, the salmonellaphage SP6, and the yersiniophage A1122 (also known as phage H) are all excluded in cells containing the F plasmid (Duckworth et al., 1981; Garcia et al., 2003; Molineux, 1991; Scholl et al., 2004; Studier, 1979; Zinder, 1961). However, the Y. enterocolitica phage YeO3-12 and its descendant, the coliphage T3, grow normally in F cells (Pajunen et al., 2001, 2002). The important difference between these last two phages and the remainder of the T7 group is gene 1.2; T3 gene 1.2 prevents exclusion whereas expression of T7 gene 1.2 actually causes exclusion (Condreay and Molineux, 1989; Garcia and Molineux, 1995; Molineux, 1991; Molineux and Spence, 1984; Schmitt and Molineux, 1991). An activity of T3 gene 1.2 prevents the newly synthesized gp10 capsid protein of the phage from interacting with the F protein PifA. The major capsid protein of all T7 family members that have been tested is capable of triggering exclusion in cells containing the F protein PifA.

The pif operon of the F plasmid codes for the autoregulatory repressor PifC and the T7 exclusion protein PifA (Cram et al., 1984a, 1984b; Rotman et al., 1983; Miller and Malamy, 1983, 1984). PifC also represses RP4 conjugal transfer (Miller et al., 1985). Close homologues of PifC have only been found on the large virulence plasmid pLVPK of Klebsiella pneumoniae CG43 and on the Salmonella typhimurium conjugal plasmid R64, both of which also harbor a PifA homologue. R64 (and also R56) has been reported to inhibit the growth of T7 (Hughes and Meynell, 1973). In addition, close homologues of PifA have been found on a transmissible plasmid and in a
pathogenicity island of two uropathogenic *E. coli* strains. However, the only biological function yet known is that of T7 exclusion by F PifA, and no biochemical property of PifA has been determined.

Although we could not identify any transmembrane domains, it is clear that PifA is a membrane-associated protein, and exclusion of T7 is therefore likely to involve membrane functions. In contrast, both T7 gp1.2 and gp10 are soluble proteins and must be recruited to the cytoplasmic membrane to elicit the physiological defects that are associated with exclusion. It is not yet clear whether PifA is directly involved in this recruitment, although complexes between PifA and both T7 gp1.2 and gp10 have been detected (X.C and I.J.M., unpublished observations).

The combination of PifA and either T7 gp1.2 or gp10 is lethal to *E. coli* and must therefore inhibit an essential cell function (Schmitt and Molineux, 1991). *E. coli* FxsA plays a protective role and can prevent exclusion when present at higher than normal concentration (Wang et al., 1999a, 1999b). However, FxsA is a nonessential protein and is not the target of PifA and T7 gp1.2 (or gp10) because *fxsA* null mutants still allow F to exclude T7. FxsA is a membrane protein, and we have shown here that it contains four transmembrane segments with cytoplasmic N- and C-termini.

As the second residue of FxsA is arginine, the initiating methionine should be retained, the cytoplasmic N-terminal segment of FxsA therefore contains only two amino acids with Arg2 serving as the first transmembrane segment anchor. In accordance with the “positive inside rule” (von Heijne, 1989), the second membrane segment may be anchored in the cytoplasm by Arg48 and perhaps Lys53. In higher copy number vectors, where the rate of plasmid protein synthesis was increased 4.5-fold over the experiments shown in Fig. 5, a fusion of Val47 to PhoA gave blue colonies on XP plates and had substantial phosphatase activity (data not shown). The increased rate of protein synthesis is presumably sufficient to allow significant export of the PhoA portion of the fusion protein. Similar observations have been reported for MalF-PhoA (Boyd et al., 1987b) and LacY-PhoA fusions (Calamia and Manoil, 1990). The exact positions of the third and fourth transmembrane segments are less well defined, although it is clear that FxsA has four transmembrane domains. The two periplasmic domains are both predicted to contain just two residues, which is consistent with their being resistant to proteinase K in spheroplasts. Arginines at residues 117 and 120 must help maintain the C-terminal domain in the cytoplasm, but whether Lys111 is within the membrane, as indicated in Fig. 5B, or whether it defines the end of the transmembrane segment is unclear. The cytoplasmic C-terminal tail of FxsA thus contains between 43 and 47 amino acids. Remarkably, the last 22 residues contain 16 charged amino acids, more than half that found in the entire protein.

The C-terminal tail is, however, not important in alleviating F exclusion of T7. Removal of the last 47 amino acids of FxsA has no effect on the ability of the protein to allow T7 to grow in the presence of F (FxsA111, Table 1). Removing 51 amino acids (FxsA107) still provides a protein with some activity, even though the fourth transmembrane segment of the mutant protein must be incomplete. However, removing 56 amino acids (FxsA102) yields a protein with no detectable ability to prevent F exclusion of T7. These results indicate that the fourth membrane domain of FxsA is required for its activity in preventing F exclusion of T7. The FxsA102-PhoA fusion had an intermediate level of phosphatase activity (Fig. 5), suggesting that only a fraction of the PhoA portion was periplasmic. It is possible that the failure of FxsA102 to alleviate F exclusion of T7 is due to there being insufficient FxsA with its C-terminus in the membrane. Even if this were the case, the fourth transmembrane segment of FxsA must still be considered essential for activity, and we suggest that this segment constitutes part of a binding site for PifA. As PifA is membrane-associated even when FxsA is not overexpressed, it seems likely that the cytoplasmic membrane is the site where T7 exclusion by F is first manifest.

*fxsA* is monocistronic, the gene is transcribed from its own promoter in the opposite orientation to both the upstream *aspA* (aspartase) gene and the downstream *yjeH*, a gene of unknown function (Wang et al., 1999a). The cellular function of FxsA is unknown; although moderate overexpression of *fxsA* is tolerated well by the cell, gross overexpression has a deleterious effect on growth rate (Wang et al., 1999a, 1999b). In contrast, *fxsA* null mutants grow normally; isogenic *fxsA* and *fxsA* null mutants are maintained during competitive growth in both rich and minimal glucose media at 30 and 37 °C (X.C and I.J.M., unpublished data). Although normal *fxsA* expression levels are insufficient to prevent F exclusion of T7, a 25-fold increase, caused by the promoter-up chromosomal mutation *fxsap109*, allows T7 to grow normally in F cells.

T7 exclusion by F is accompanied by a premature inhibition of macromolecular synthesis and a membrane defect that allows intracellular nucleotides and other small molecules to leak from the abortively infected cell. Although leakiness of the membrane has been proposed to be the cause of exclusion, no correlation between leakiness and inhibition of phage growth has been observed. Two chromosomal loci that were found serendipitously to alleviate F exclusion of T7 are *rpsL*, defined here by certain mutations causing a StrR phenotype, and *galU* (Chakrabarti and Gorini, 1975; Remes and Elseviers, 1980). Both mutant strains continue to leak ATP from the cell into the extracellular fluid during T7 growth (Remes and Elseviers, 1980; Schmitt et al., 1991). Furthermore, T7 mutants that grow in the presence of F still leak ATP, albeit at a reduced level (Molineux et al., 1989; Schmitt et al., 1991). Similarly, overexpression of *fxsA* not only allows wild-type T7 to grow in the presence of F but also allows a reduced level of leakiness (Wang et al., 1999b).
Our demonstration that FxsA and PifA interact provides a plausible mechanism for T7 exclusion by F and its alleviation by high concentrations of FxsA. We suggest that in wild-type cells containing F, the membrane-associated protein PifA interacts primarily with an essential E. coli membrane protein and only a small fraction with FxsA. Despite considerable effort, the identity of this essential membrane protein has remained elusive, but only its interaction with PifA is important for exclusion of T7. Although T7 gp1.2 and gp10 are both soluble proteins, some fraction must be recruited to the membrane by either PifA or the E. coli membrane protein whose activity is essential. The primary consequence of the combination of T7 and F proteins binding to the essential E. coli protein is that its activity is inhibited, a secondary event perhaps alters the conformation of the protein. Inhibition of activity ultimately leads to the loss of biosynthetic capacity and active transport in the cell; the hypothesized conformational change in the membrane protein allows intracellular ATP and other small molecules to leak out of the cell. Although they may be related events, there is no obligate coupling between the conformational change in the membrane protein and the inhibition of its activity. FxsA can suppress F exclusion when present at higher than normal intracellular concentrations. In this situation, FxsA simply competes effectively for PifA and thereby sequesters it at sites where it cannot interact with PifA is important for exclusion of T7. Although T7 gp1.2 and gp10 are both soluble proteins, some fraction must be recruited to the membrane by either PifA or the E. coli membrane protein whose activity is essential. The primary consequence of the combination of T7 and F proteins binding to the essential E. coli protein is that its activity is inhibited, a secondary event perhaps alters the conformation of the protein. Inhibition of activity ultimately leads to the loss of biosynthetic capacity and active transport in the cell; the hypothesized conformational change in the membrane protein allows intracellular ATP and other small molecules to leak out of the cell. Although they may be related events, there is no obligate coupling between the conformational change in the membrane protein and the inhibition of its activity. FxsA can suppress F exclusion when present at higher than normal intracellular concentrations. In this situation, FxsA simply competes effectively for PifA and thereby sequesters it at sites where it cannot interfere with the activity of the essential E. coli membrane protein. Consequently, after T7 infection, when gp1.2 and gp10 are synthesized, the phage proteins may interact normally with the essential membrane protein as if they were present in an F− cell, or they may interact with FxsA-bound PifA. In the latter case, the resulting complex must still be unable to inhibit the activity of the essential membrane protein. This idea suggests that FxsA has no mechanistic role in the process of phage exclusion, increased levels of FxsA simply compete effectively for PifA and prevent it from binding an essential membrane protein.

In pure culture, cells harboring F pifA mutant plasmids have not been shown to exhibit growth defects, relative to those harboring F+. Under laboratory conditions, pifA therefore offers no clear advantage to either F or the host bacterium. However, in a natural environment, pifA may be considered to endow a population of cells containing F a level of resistance to the majority of T7-like coliphages which, like T7 itself, are subject to F exclusion. Resistance may be sufficient for maintaining pifA in F and other conjugal plasmids, and also in chromosomal pathogenicity islands that may themselves be plasmid-derived. It is unlikely that this is the primary or sole function of pifA; resistance of a population to T7-like phages would do little more than select for the proliferation of phages like T3, which are insensitive to exclusion. However, our limited knowledge on the population structure of the T7 family of phages suggests that the majority are sensitive to F exclusion. In turn, this line of thought suggests that pifA has another function in the biology of F and related plasmids.

PifA fractionates with the cytoplasmic membrane, and it is conceivable that the protein thus acts as a sensor for certain environmental changes experienced by the cell. Related, albeit more specific, ideas have been raised for the function of the λ rex system, which, in addition to causing infections by T-even phages that lack the rII genes to be aborted, has been proposed to interact with the stationary phase response of the cell and to inhibit the ClpP family of proteases (Engelberg-Kulka et al., 1998; Slavcev and Hayes, 2003). If PifA acts as a biological sensory system for F, why should the expression of T7 gene 1.2 or gene 10 then abrogate phage development and lead to death of the infected cell? We can speculate that the T7 proteins also have a sensory function that may be distinct from their known activities in inhibiting E. coli dGTPase and in head assembly, perhaps an activity that involves the same cellular proteins or structures that are utilized by PifA. The abortive infection of T7 in F cells may then be a result of both the plasmid and phage genes simultaneously interacting with—and inhibiting as a consequence—the same cellular activities. Transcription in T7-infected cells is terminated earlier than usual when synthesis of both gp1.2 and gp10 is prevented (Beck and Molineux, 1991), possibly suggesting that the two proteins cooperate in sensing the triphosphate pools or overall energy levels in the infected cell. In this hypothetical scenario, PifA would also monitor the same functions that help maintain energy levels in uninfected E. coli cells.

Methods of procedure

Bacterial strains and plasmids

The E. coli strains and plasmids used in this study are listed in Tables 2 and 3. Unless indicated otherwise, bacterial cultures were grown at 37 °C in rich medium (per liter: 10 g Bactotryptone, 5 g yeast extract, 5 g NaCl).

<table>
<thead>
<tr>
<th>Name</th>
<th>Genotype</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>CC118</td>
<td>araD139Δ (ara-leu)7697Δ lacX74Δ phoA20 galE galK thi rpsE rpoB argE (Am) recA1</td>
<td>Manoil and Beckwith, 1985</td>
</tr>
<tr>
<td>CC202</td>
<td>CC118 F42 lacI3 szf-2::TnphoA</td>
<td>Manoil and Beckwith, 1985</td>
</tr>
<tr>
<td>IJ511</td>
<td>ΔlacX74 galK2 galT22 supE44 hisD33</td>
<td>Condreay and Molineux, 1989</td>
</tr>
<tr>
<td>IJ1196</td>
<td>lacX74 galK150 (Am) croDI53 (Am) trp (Am) supF</td>
<td>Wang et al., 1999a</td>
</tr>
<tr>
<td>IJ1199</td>
<td>lacX74 galK150 (Am) cryD45 (Am) trp (Am) supF</td>
<td>Wang et al., 1999a</td>
</tr>
<tr>
<td>IJ1200</td>
<td>IJ1198Δ (trrR-recA)306::Tn10</td>
<td>Wang et al., 1999a</td>
</tr>
<tr>
<td>Top10</td>
<td>mcrAΔ (mcr-hsdRMS-mcrBC) [s80lacZΔ M15] ΔlacX74 deoR recA1 araD139Δ (ara-leu)7697Δ galU galK rpsL endA1 supG</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

Table 2

E. coli K-12 strains
supplemented as necessary with antibiotics to maintain plasmids. All *fxsA-phoA* fusion plasmids were constructed by ligating PCR products of various *fxsA* 5’-proximal coding sequences using oligonucleotides containing a *PstI* or *Smal* site. After digestion, the PCR product was ligated into the polylinker of *pSWFII*, a plasmid containing a *pSC101* replicon, cloning vector Wang and Kushner, SmaI or coding sequences using oligonucleotides containing a *fxsA* by ligating PCR products of various *fxsA-phoA* supplemented as necessary with antibiotics to maintain *pAK25* *pUC9; T7 gene 1.2 under *P* <sup>lac</sup> control Schmitt et al., 1991

<table>
<thead>
<tr>
<th>Name</th>
<th>Replicon, relevant genes</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pAK25</td>
<td>pUC9; T7 gene 1.2 under <em>P</em> &lt;sup&gt;lac&lt;/sup&gt; control</td>
<td>Schmitt et al., 1991</td>
</tr>
<tr>
<td>pGEX-3X</td>
<td>Glutathione-S-transferase fusion vector</td>
<td>Amersham Biosciences</td>
</tr>
<tr>
<td>pGX25</td>
<td>pTrchisB; <em>pifA</em>Δ (1–462) [C-terminal 254 codons]</td>
<td>This work</td>
</tr>
<tr>
<td>pGX26</td>
<td>pTrchisB; <em>pifA</em>Δ (1–180) [C-terminal 536 codons]</td>
<td>This work</td>
</tr>
<tr>
<td>pGX31</td>
<td>pTrchisA; <em>pifA</em>-K94 under <em>P</em> &lt;sup&gt;rec&lt;/sup&gt; control</td>
<td>This work</td>
</tr>
<tr>
<td>pGST1.2</td>
<td>pGEX-3X; <em>gst</em>-T7 1.2</td>
<td>This work</td>
</tr>
<tr>
<td>pGSTFxsa-C</td>
<td>pGEX-3X; <em>fxsA</em> fused to <em>fxsA</em>Δ (1–121) [C-terminal 37 codons]</td>
<td>This work</td>
</tr>
<tr>
<td>pHisFxsa</td>
<td>pTrchisB; <em>his-fxsA</em> under <em>P</em> &lt;sup&gt;rec&lt;/sup&gt; control</td>
<td>This work</td>
</tr>
<tr>
<td>pHisFxsa102</td>
<td>pTrchisA; <em>fxsA</em>Δ (103–158)</td>
<td>This work</td>
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<tr>
<td>pHisFxsa107</td>
<td>pTrchisA; <em>fxsA</em>Δ (108–158)</td>
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<tr>
<td>pHisFxsa111</td>
<td>pTrchisA; <em>fxsA</em>Δ (112–158)</td>
<td>This work</td>
</tr>
<tr>
<td>pHisFxsa115</td>
<td>pTrchisA; <em>fxsA</em>Δ (116–158)</td>
<td>This work</td>
</tr>
<tr>
<td>pHisFxsa121</td>
<td>pTrchisA; <em>fxsA</em>Δ (122–158)</td>
<td>This work</td>
</tr>
<tr>
<td>pQ</td>
<td>pACYC184::lac:: P&lt;sub&gt;T&lt;/sub&gt;&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Lab collection</td>
</tr>
<tr>
<td>pSWFII</td>
<td>pBBR322, signal sequence-less and promoter-less <em>phoA</em></td>
<td>Ehrmann et al., 1990</td>
</tr>
<tr>
<td>pTrchisA,B,C</td>
<td>pBBR322, N-terminal his-tag vector, under <em>P</em> &lt;sup&gt;rec&lt;/sup&gt; control</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pWSK10A</td>
<td>pWSK29, T7 10A under <em>P</em> &lt;sup&gt;lac&lt;/sup&gt; control</td>
<td>This work</td>
</tr>
<tr>
<td>pWF184</td>
<td>pWSK29, <em>fxsA</em>Δ*, <em>fxsA</em>&lt;sup&gt;IP&lt;/sup&gt;109 and <em>P</em> &lt;sup&gt;lac&lt;/sup&gt; control</td>
<td>Wang et al., 1999a</td>
</tr>
<tr>
<td>pWF185</td>
<td>pWSK29, <em>fxsA</em>Δ*, <em>fxsA</em>&lt;sup&gt;IP&lt;/sup&gt;, and <em>P</em> &lt;sup&gt;lac&lt;/sup&gt; control</td>
<td>Wang et al., 1999a</td>
</tr>
</tbody>
</table>

**Table 3**

Plasmids

**Cellular localization of proteins**

**PifA**

A 200-ml culture of IJ511/F<sup>lac</sup> at *A<sub>600</sub> = 0.7* was centrifuged and cells were resuspended in 5 ml PBS (pH 7.4) and broken by passage through a French pressure cell. After clarification of the lysate, the membrane fraction was collected by centrifugation at 100 000 × *g* for 2 h at 4 °C and resuspended in 5 ml PBS (pH 7.4). The supernatant was saved as the soluble fraction. For determining Sarkosyl solubility, the membrane suspension was incubated on ice with 0.5% Sarkosyl for 10 min and centrifuged at 100 000 × *g* for 2 h at 4 °C. After dissolving the pellet in 5 ml PBS, both the pellet suspension and the supernatant were dialyzed against PBS (pH 7.4). In some experiments, the original crude membrane preparation was resuspended in PBS containing 1 M NaCl. After shaking at 4 °C for 20 min, the membrane was collected as described above. Samples from each fraction were subjected to SDS-PAGE and examined for the presence of PifA by immunoblotting using anti-PifA antibody.

**T7 gp1.2 and gp10A**

For gp1.2, a 100-ml culture of IJ1199(Q)(pAK25) at *A<sub>600</sub> = 0.4* was induced with 0.1 mM IPTG until *A<sub>600</sub> = 0.7*. For gp10A, IJ1199(Q)(pWSK10A) was grown to *A<sub>600</sub> = 0.7* at 37 °C without induction. Harvested cells were resuspended in 5 ml of 50 mM Tris–HCl pH 8.0 and were disrupted using a French press. After removal of cell debris, cell extracts were centrifuged at 100 000 × *g* at 4 °C for 2 h. The total membrane fraction was suspended in 50 mM Tris–HCl pH 8.0. The presence of gp10A and gp1.2 in the cytoplasmic and membrane fractions was determined by SDS-PAGE and immunoblotting using anti-gp10A or anti-gp1.2 antibody.

**Separation of inner and outer membranes**

Membranes were prepared from strain IJ511/F<sup>lac</sup> using the method of Osborn et al. (1972). A 200-ml culture was grown to a cell density of 5 × 10<sup>8</sup> per ml at 37 °C. Cells were

**Antibodies**

Polyclonal antisera were produced by following the procedure described in “Current Protocols in Molecular Biology” (Struhl et al., 1992). Briefly, purified protein in PBS (pH 7.4) was mixed and emulsified with an equal volume of complete Freund’s adjuvant (CFA, Sigma). A dose corresponding to 0.5 mg protein was injected subcutaneously into a female New Zealand rabbit. Booster immunizations using incomplete Freund’s adjuvant were administered three times every 3 weeks after the primary injection. The following proteins were used as antigens to generate corresponding antisera: gp10A, GST-1.2, His-PifA, and GST-FxsA-C (last 37 amino acids of FxsA fused to the C-terminus of GST). Gp10A was purified following the method of Cerritelli and Studier (1996), other proteins by commercial affinity resins. Antisera were collected 2 weeks after each boost and were stored in aliquots as crude serum at −20 °C. Dr. C. Earhart, University of Texas, kindly provided antibodies to *E. coli* OmpA and thioredoxin. Anti-PhoA antibodies were purchased from Chemicon International.
harvested by centrifugation at 5000 rpm, resuspended in 10 ml of an ice-cold solution of 0.75 M sucrose, 10 mM Tris–HCl (pH 7.8), and incubated with 100 µg/ml lysozyme for 2 min. Twenty milliliters of ice-cold 1.5 mM EDTA (pH 7.5) was then added slowly with gentle mixing over a period of 10 min. Spheroplasts were broken by one passage of the suspension through a French pressure cell at 16 000 psi. After removal of cell debris by centrifugation at 10 000 rpm for 10 min, crude membranes were collected by centrifugation at 150 000 × g for 2 h at 4 °C and were then gently resuspended in 10 ml of 0.25 M sucrose, 3.3 mM Tris–HCl, 1 mM EDTA (pH 7.5). After centrifugation at 150 000 × g for 2 h at 4 °C, the membrane fraction was resuspended in 1 ml 25% (w/w) sucrose, 5 mM EDTA (pH 7.5), and was then loaded on top of a step sucrose gradient (0.5 ml of 55%, 0.7 ml each of 50%, 45%, 40%, 35%, and 30% sucrose (all w/w)). Inner and outer membranes were separated by centrifugation in a SW 55 rotor at 120 000 × g for 14 h at 4 °C; 250-µl fractions were collected from the tube bottom. Protein concentration was determined using the Bradford reagent. For assaying the activity of NADH oxidase, 5 µl of each gradient fraction was incubated for 10 min at 37 °C with 1 ml of 50 mM Tris–HCl (pH 7.5), 0.12 mM NADH, 0.2 mM dithiothreitol. NADH oxidase activity was measured as the decrease in absorbance at 340 nm. Relative amounts of PitA and OmpA in fractions were determined by immunoblotting using anti-OmpA and anti-PitA antibodies after electrophoresis through a 12% SDS-PAGE gel followed by electrophoretic transfer of the proteins to a PVDF membrane.

**SDS-PAGE and immunoblotting**

Proteins were resolved by SDS-PAGE using 12% (for gp10A, PitA, and FxsA) or 15% (for gp1.2) acrylamide. Proteins were then electrophoretically transferred to a PVDF membrane. After blocking with 5% nonfat milk in TBS (20 mM Tris–HCl, 500 mM NaCl, pH 7.5) for 1 h, the membrane was incubated with primary antibody in TTBS (20 mM Tris–HCl, 500 mM NaCl, 0.05% Tween 20, pH 7.5) for 1 h and then incubated with TTBS containing a secondary antibody conjugated to alkaline phosphatase for 1 h. Primary antibodies were used at the dilution of 1:10 000 for anti-gp10A antibody, 1: 5000 for anti-PitA antibody, 1: 3000 for anti-gp1.2 and FxsA antibodies. Secondary antibodies were diluted in TTBS at 1: 5000 for goat anti-rabbit antibody (Bio Rad) and 1:3000 for goat anti-mouse antibody (Kirkegaard and Perry Lab, Inc). Proteins of interest were visualized using AP color reagents (Bio Rad).

**Proteinase K accessibility**

**Spheroplasts**

One hundred milliliters of IJ1200(pWF185) or IJ11-98(pHisFxsA) was grown at 37 °C to A600 = 0.7 and harvested by centrifugation. Cells were suspended in 10 ml of ice-cold 0.5 M sucrose, 100 mM Tris–HCl, pH 8.0, 5 mM EDTA. After addition of lysozyme to 80 µg/ml, an equal volume of ice-cold H2O was added and the mixture was incubated on ice for 10 min. Spheroplast formation was monitored by microscopy. After adding MgSO4 to 20 mM, spheroplasts were collected by brief centrifugation and were then resuspended in 10 ml of ice-cold 0.25 mM sucrose, 50 mM Tris–HCl (pH 8.0), 10 mM MgSO4 (Cao and Dalbey, 1994).

**Inverted membrane vesicles (IMV)**

One hundred milliliters of IJ1200(pWF185) or IJ1200(pHisFxsA) cells were grown and harvested as described above for spheroplasts. Cells were suspended in 10 ml of 50 mM Tris–HCl, pH 8.0, 0.1 mM NaCl, 20 mM EDTA and were disrupted by a single passage through a French pressure cell at 16 000 psi. Unbroken cells and debris were removed by centrifugation at 5000 × g for 10 min at 4 °C. This procedure results in the conversion of at least 95% of cells into inverted membrane vesicles (Bläsi et al., 1999; Gründling et al., 2000).

**Proteinase K treatment**

One milliliter of a spheroplast or IMV suspension was treated with 1 mg/ml proteinase K at 37 °C for various times; digestion was stopped by the addition of 2 mM PMSF. A separate sample was treated with 2% Triton X-100 before proteinase K treatment. Spheroplasts were collected by centrifugation at 10 000 rpm for 1 min, IMV by centrifugation at 100 000 × g for 2 h. Pellets were dissolved in 2 × SDS-loading buffer and boiled for 5 min before SDS-PAGE analysis. The presence of FxsA was determined by immunoblotting using antibody against either the C-terminus of FxsA or (for His-FxsA) anti-his-tag antibody.

**Pulse-labeling and detection of FxsA-PhoA fusion proteins**

E. coli CC118 cells containing a fxsA-phoA fusion plasmid were grown in M9 minimum medium supplemented with 0.2% glucose, 100 µg/ml arginine and leucine, 20 µg/ml thiamine, 1 mM MgSO4, and 100 µg/ml ampicillin at 37 °C to A600 = 0.3. One milliliter of culture was labeled with 100 µCi [35S]methionine (1175 Ci/mmol; NEN) for 1 min. Immunoprecipitation followed the procedure of Ito (1981). After centrifugation, the cell pellet was dissolved in 20 µl of 1% SDS, 50 mM Tris–HCl (pH8.0), 1 mM EDTA, and boiled for 2 min. Ice-cold buffer (2% Triton X-100, 50 mM Tris–HCl, pH 8.0, 0.15 M NaCl, 0.1 mM EDTA, 660 µl) was then added. A nonspecific precipitate was removed by centrifugation, 2 µl of anti-PhoA antibody was added, and the mixture was incubated at 4 °C for 1 h. Forty microliters of 33% protein A-agarose beads was then added and the mixture was again incubated at 4 °C for 1 h. Beads were collected, washed twice with buffer containing Triton X-100, once with 10 mM Tris–HCl, pH 8.0, and then boiled for 2 min in a reducing SDS-PAGE loading buffer. After
electrophoresis, the fusion proteins were visualized by phosphorimaging and bands were quantitated using Image-Quant software.

**Alkaline phosphatase assays**

Alkaline phosphatase activities were measured by a modified procedure of Brickman and Beckwith (1975). Log-phase CC118 cells containing the test plasmid (1.5 ml of culture) were resuspended in 1 ml 1 M Tris–HCl pH 8.0 and the A_{400} was measured. Cells were permeabilized by the addition of 10 μl CHCl₃, 10 μl 10% SDS, and 50 μl of 80 mg/ml p-nitrophenyl phosphate (Sigma 104 phosphatase substrate). The mixture was incubated at room temperature for 10 min and the reaction terminated by the addition of 100 μl 1 M K₂HPO₄. After removal of cell debris by centrifugation, the A_{420} of the supernatant was measured. Activity is calculated as units = 1000 \cdot \frac{A_{420}(t \times A_{600})}{t \cdot C_{2}}. Background values were obtained from a strain containing the vector plasmid pSWF11 and were subtracted from the activity of each fxsA-phoA fusion.

**TnphoA insertions**

TnphoA insertions into various pifA plasmids were selected using the strain CC202 as described (Manoil and Beckwith, 1985). Plasmid DNA was isolated and transformed into the phoA strain CC118. Transformants that were blue on plates containing 5-bromo-4-chloro-3-indolyl phosphate (i.e., are PhoA^+) were purified, and plasmid DNA was isolated. The site of transposon insertion into the pifA plasmid was determined by DNA sequencing.

**Affinity chromatography of FxsA**

His-PifA (0.5 mg) and His-tagged SP6 RNA polymerase (0.5 mg) in 0.5 ml were each incubated with 0.1 ml Ni-NTA agarose beads in a buffer (50 mM Na₂HPO₄ pH8.0, 25 mM NaCl) containing 10 mM imidazole for 2 h at 4 °C. The resin was then washed with the same buffer to remove unbound protein. Cells from a 5-ml culture of IJ1199 (overexpressing fxsA) were harvested, lysed in the presence of 2% Triton X-100 to ensure that membrane proteins were solubilized, and incubated with 50 μl of PifA resin. After extensive washing with buffer containing 20 mM imidazole, proteins were eluted using buffer containing 250 mM imidazole. The eluate was subjected to SDS-PAGE, electro-transferred to a PVDF membrane, and probed with anti-FxsA antibodies.

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References


Garcia, E., Elliott, J.M., Ramanculov, E., Chain, P.S.G., Chu, M.C., Molin- 


