Analysis of the Topology of an F Plasmid Protein -
Construction of TraQ::PhoA fusions

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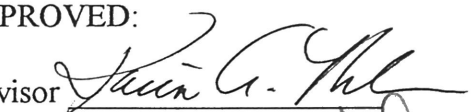
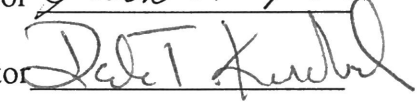
APPROVED:

Fellows Advisor 

Honors Director 

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ABSTRACT

F pilus filaments extend from the surface of bacteria carrying plasmid F and initiate intercellular contacts that allow conjugative DNA transfer to other cells. Secretion of the subunits in this filament depends on a small 94 amino acid inner membreane protein, TraQ. The membrane topology of TraQ was investigated by analysis of *traQ::phoA* fusion products. A plasmid containing both sequences was constructed and fusions were generated by *in vitro* deletion with Bal-31 and religation. Plasmids in transformants were screened for expression of alkaline phosphatase activity and by restriction enzyme analysis; expression of ^{35}S-methionine labeled proteins was also examined. The DNA sequence of plasmids that expressed a *traQ::phoA* fusion was then obtained. The results indicate that the N-terminal region of TraQ spans the bacterial membrane, and the C-terminal portion of the protein extends into the periplasm.

BACKGROUND

Bacterial conjugation is a process in which *E. coli* and other bacteria transfer genetic material from one strain to another. The process was discovered in 1948 by Lederberg and Tatum. It requires direct contact between cells and is dependent on activities expressed by a fertility (F) factor in the donor cell.

The F factor which is required for conjugation is a 100,000 base pair plasmid, carrying the genes necessary for its own replication as well as those involved in conjugation. The plasmid and the copy of its genes that is transferred are replicated during conjugation, so the donor remains F^+ as the recipient changes from F^- to F^+. The ability of the F factor to integrate into the bacterial chromosome also permits transfer of bacterial genes and has been useful in mapping the *E. coli* chromosome.

Conjugation depends on the formation of an appendage called the F pilus, which extends from an F^+ cell and binds to an F^- cell. Pilus subunits then depolymerize, bringing the two cells together. Contact between the cell walls of the two cells appears to be required for DNA transfer, although the mechanism of DNA passage is still unknown. (Joklik et al., 1992)

The F pilus filament consists of repeating units of one protein, the F-pilin subunit, although numerous additional proteins are required for assembly of the subunits into a functional pilus. A 121 amino acid product of the F *traA* gene (propilin) is processed and modified to form F-pilin, a 70 amino acid polypeptide. (Wu et al., 1989)

Prior studies have shown that efficient synthesis of F pilin depends on the product of another F transfer region gene, *traQ* (Wu et al., 1987). If the TraQ protein is absent, propilin remains unprocessed and is rapidly degraded in the cytoplasm. When TraQ is present, propilin is

rapidly translocated and processed, and a pool of stable F-pilin subunits accumulates in the inner membrane of the cell (Maneewannakul et al., 1993).

Recent data suggest that the TraQ product is required for membrane translocation of propilin, and that the 51 a.a. signal sequence is then removed by the host enzyme, Leader peptidase I. Host proteins such as SecA that are required for secretion of other proteins in *E. coli* are not required for pilin synthesis, although the precursor accumulates in cells which are defective in Leader peptidase I (N. Majdalani and K. Ippen-Ihler, unpublished results). Therefore, the current model is that TraQ forms a membrane channel for translocation of the propilin polypeptide into the membrane.

TraQ is a 94 amino acid, 10.9 kDa protein which is located in the inner membrane of the cell and has the amino acid sequence shown in figure 1. Residues 14-32 and 40-60 are hydrophobic, and may span the membrane. Twelve charged amino acids in the C-terminal region suggest this region would extend into the cytoplasm or periplasm. The goal of this research was to determine the membrane topology of TraQ and the orientation and position of various domains of the protein, using a PhoA fusion approach. (Wu et al., 1989)

Figure 1: Amino acid sequence of the *traQ* gene product.

```
MISKRRFSLPRLDITGMWVFSLGVWFHIVARLVYSKPWMAFFLAELIAAILVLFGAYQYLDAWIARVSREEREALEAR
  p+++ p  + - p     p     +   +   p+        -            p       + p+--+-    -

QQAMMEGQQEGGHVSH
pp   - pp-  + p+          p = polar, uncharged a.a.
                          + = positive
                          - = negative
```

The alkaline phosphatase, or *phoA* gene codes for a 471 amino acid polypeptide. As the polypeptide is secreted into the periplasm, a signal peptide consisting of the first 21 amino acids is removed. The active alkaline phosphatase enzyme contains two polypeptide monomers complexed with Zn^{2+} and Mg^{2+}, with disulfide bonds between the four cysteine residues in each polypeptide. Since disulfide bonds do not normally form in the cytoplasm, PhoA is active only in the periplasm. The amino terminus of the mature PhoA protein can be varied without affecting its activity. This allows removal of the first 12 or 13 amino acids and their replacement with large segments of other secreted proteins. Since alkaline phosphatase secretion is dependent on the presence of an amino-terminal signal sequence, the secretion of the modified PhoA depends on whether it is fused to a region of another protein which extends into the periplasm. PhoA activity is in turn dependent on secretion into the periplasm; as a result, PhoA activity is a useful indicator of whether the sequence to which it is fused extends into the periplasm. The alkaline phosphatase activity of a PhoA fusion can be easily determined by growing colonies on media containing the PhoA indicator 5-bromo-4-chloro-3-indolyl phosphate (XP) which turns blue when phosphate is cleaved. (Torriani-Gorini et al., 1987)

MATERIALS/METHODS

Media

Luria Bertoni (LB) broth or LB agar (Miller 1972) containing antibiotics as needed for plasmid maintenance was used. These antibiotics, Ampicillin and/or Kanamycin, were added to a final concentration of 50 ug/ml. Cultures for protein labeling were grown in Jaskusnas Minimal Media (JMM) (Maneewannakul 1992). XP plates for blue/white screening of PhoA activity were made by adding a layer of 3 ml H Top Agar (Miller 1972) containing 10 ul 0.1 M Isopropylthiogalactoside (IPTG) and 10-100 ul 5-bromo-4-chloro-3 indolyl phosphate (XP) over an LB/Ampicillin plate. Several amounts of 4mg/ml XP ranging from 10-100 ul were used, and 50 ul was determined to be most useful for judging the level of PhoA activity expressed by the strain.

Bacterial strains and plasmids

Plasmids used in the construction of *traQ::phoA* fusions and *E. coli* strains used to propagate the fusions are described in table 1 and 2 respectively.

Table 1: plasmids used in fusion construction

Plasmid name	Description/comment	Source
pS13	Carries *phoA-Kan*^{R} cassette	this laboratory
pKI004	pSPORT derivative, carries *traQ* under a T7 promoter	this laboratory

These plasmids were used in the construction of pKI021, a pre-fusion construct containing *traQ* and *phoA*.

Table 2: *E. coli* strains

Strain	Comments/description	Source/reference
CC118	phoA$^-$ strain, used to screen for active fusions.	Manoil and Beckwith 1982
TB1	an *hsdR* derivative of JM83	Focus
XK100	Carries the T7 RNA polymerase gene under *lacUV5* promoter control	this laboratory

DNA purification and bacterial transformation

Several different methods for purifying plasmid DNA were employed in this project. First, the mini preparation (mini-prep) method involved pelleting the cells from a 3 ml overnight culture, resuspending them in .35 ml STET[1], adding 40 ul 10 mg/ml lysozyme and boiling for 1 minute. This mixture was centrifuged for 10 minutes and the pellet removed to produce a cleared lysate. Plasmid DNA was precipitated by adding an equal volume isopropanol, freezing at -70° C for 15 minutes and pelleting for 15 min. The pellet was dried and resuspended in 200 ul sterile glass distilled water (dH_2O). Mini-prep DNA was used mostly for restriction analysis. For some applications, it was necessary to preform a phenol/chloroform extraction to further purify the

[1]STET consists of: 8% Sucrose, 5% Triton X-100, 50 mM EDTA, and 50 mM Tris-Cl.

mini-prep DNA. Second, in purifying pS13 DNA, a Qiagen (Qiagen, Inc., Chatsworth, CA) "maxi-prep" protocol was used according to manufacturer's instructions to obtain large amounts of pure DNA. Third, DNA used in sequencing reactions was purified on a CsCl gradient as described by Maniatis et al..

DNA fragments used in construction of pKI021 were gel purified. Plasmid DNA was digested with appropriate restriction enzyme(s) and separated on 1% low melting point (LMP) agarose gels. Bands of interest were excised, the agarose was melted at 75° C for 5 minutes, and the DNA was phenol/chloroform extracted and precipitated with isopropanol. Pellets were resuspended in 20 ul dH_2O and 1 ul was loaded on a diagnostic 1% agarose gel to estimate concentrations. For blunt-ended ligations, a 5:1 insert:vector DNA ratio was used.

Plasmid DNA was introduced into *E. coli* strains using the $CaCl_2$ bacterial transformation procedure described in Maniatis et. al.

Deletions in pKI021

Fusions were generated in pKI021 by Bal-31 deletion and religation. (Fig. 2) First, pKI021 was cleaved with *Hind*III. The *Hind*III digests were done according to manufacturer's instructions, in a total reaction volume of 300 ul including 30 ul 10X buffer (New England Biolabs buffer #2), 9-10 ul *Hind*III enzyme, 30-60 ug pKI021 DNA and dH_2O to a total volume of 300 ul. This reaction mixture was incubated for 3-4 hours at 37° C. After isopropanol precipitation, drying and resuspending in dH_2O, the amount of DNA was calculated using the optical density (O.D.) of the samples at 260 nm.

Deletions of random length from the *Hind*III ends were made using slow Bal-31 exonuclease (Kodak International Biotechnologies, Inc., New Haven, CT).. Approximately 3 ug DNA were added to the Bal-31 digestion mix, along with 10 ul 5X Bal-31 buffer, 10 ul Bal-31 exonuclease, and dH_2O to a final volume of 50 ul. Aliquots were taken at given time points and reactions stopped using 5 ul 0.5 M EDTA and heat inactivation of Bal-31 for 15 minutes at 75° C.

Restriction digests at the unique *Sma*I site, end-filling of any overhangs, and blunt-end ligation resulted in the joining of the 5' end of *phoA* to the 3' end left by the Bal-31 deletion. After the *Sma*I digest, end-filling was used to fill in any overhangs left by the Bal-31 treatment. End-filling reagents included 1.5 ul T4 ligase buffer, 3 units T4 DNA Polymerase, 2 ul 2mM dNTPs, and 0.5 ul dH_2O and were added directly to the *Sma*I reaction mix in each tube. The reaction was incubated at room temperature for 30 minutes. Reagents for the ligation reaction were added to the end-fill reaction mix, including 4.5 ul ligase buffer and 1.5 ul DNA ligase and dH_2O to a final volume of 45 ul. The ligation reactions were incubated overnight in a 15° water bath, and the resulting DNA was used to transform CC118 cells for screening.

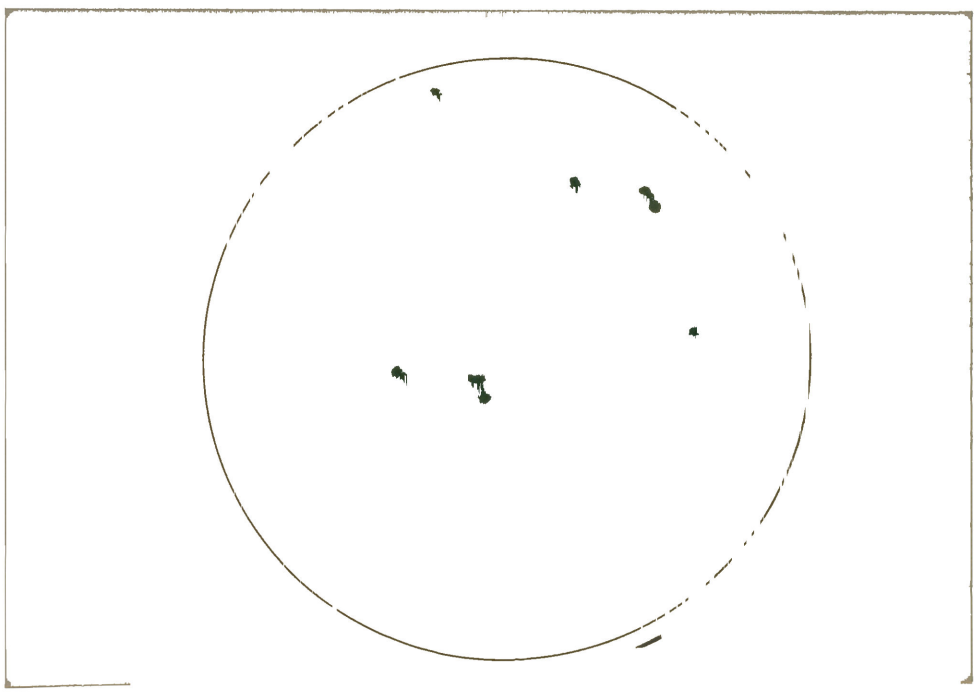
Blue/white screening

Analysis of the prospective fusions by detection of PhoA activity via blue/white selection was done on plates containing XP. Individual colonies were replica plated on LB-Amp (master plate) and LB-Amp-XP (indicator plate) following a grid patter of 50 or 100 squares. A colony expressing a particular fusion peptide was labeled according to the following numbering system:

1. A, B, C or D to indicate the set in which the fusion was generated.
2. A number to indicate the length of the Bal-31 deletion in minutes (e.g. 25, 50, 60).
3. A second number to indicate the location on the grid or the order in which the colony was picked.

For example, a colony might be designated A50.25 to represent a colony whose DNA had undergone a 50 minute deletion in the first deletion reaction and occupied square 25 on the grid.

Fig. 2: Photograph of an XP plate



Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE)

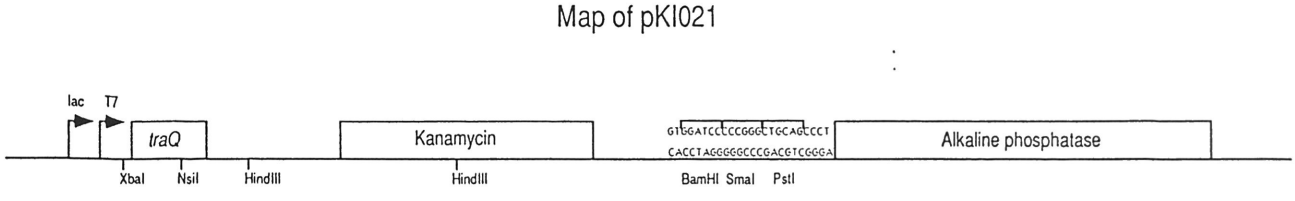
SDS-PAGE and autoradiography were used to analyze ^{35}S-methionine labeled proteins expressed by potential fusions. Nine percent polyacrylamide gels were poured and run using a 20 cm PROTEAN II gel apparatus (Bio-Rad Laboratories, Richmond, CA) and a Laemmli buffer system (Laemmli, 1970). Pellets of labeled cells were resuspended in 200 ul sample buffer (Laemmli, 1970) and boiled for 10 minutes. Five ul of each sample were loaded into separate wells, and the gels were run at 10 Watts for 3-4 hours according to manufacturer's instructions. Gels were stained in Coomassie brilliant blue-45% trichloroacetic acid for 15 min., destained in 7% acetic acid for 20 minutes and dried onto filter paper. Autoradiograms were made by exposing Kodak SB-5 X-ray film to the radioactive gels.

RESULTS

Construction of pKI021

In order to obtain a plasmid sequence from which *traQ::phoA* fusions could easily be generated by deletion, I constructed plasmid pKI021. For this construction, an *Eco*RV-*Xba*I DNA fragment corresponding to the *phoA-Kan*^{R} cassette from pS13 was cloned and gel purified. The 5' overhang at the *Xba*I end of the cassette fragment was end-filled in a 10 ul reaction mix containing 1 ul T4 ligase buffer, 3 units T4 DNA Polymerase (NEB), 2 ul cassette DNA, 4 ul dH_2O and incubated at 37° C for 45 minutes. Upon completion of end-filling, I added 0.5 ul T4 ligase buffer, 1 ul T4 ligase, 1 ul pKI004 DNA digested with *Nae*I, and 2.5 ul dH_2O. The ligation mix was incubated overnight at 15° C, and then used to transform *E. coli* strain TB1. Colonies were selected for the acquisition of Kanamycin resistance (*Kan*^{R}). DNA from Kan^R colonies was isolated by mini-prep and digested with *Pst*I and *Eco*RV to determine the orientation of the fragment. One plasmid whose DNA mapped correctly placing Kan^R between *traQ* and *phoA* was designated as pKI021 (See fig. 3).

Fig. 3: Map of pKI021



Deletions in pKI021

Four sets of fusion derivatives were obtained from four separate Bal-31 reactions performed as described in the Methods section. In each case, samples were removed after different deletion periods, and treated with EDTA to stop the reaction. For set A, 12.5 ul aliquots of the reaction mixture were removed and stopped at 25, 50, 100 and 150 minutes. For sets B and C, 7 ul Aliquots were removed at 0, 30, 35, 40, 45, 55 and 60 minutes. Aliquots from the fourth reaction (Set D) were stopped at 45, 50, 55 and 60 min. After the DNA in each sample was digested with *Sma*I, end-filled, and ligated, it was used to transform CC118 cells. Each transformation mixture was then grown on a separate LB plate. Individual colonies were replica plated on indicator plates for blue/white selection.

Blue/White screening

In the case of blue colonies, it was clear that a fusion protein could be expressed, and that the PhoA portion of the protein sequence had been translocated across the inner membrane; it remained to be confirmed that the fusion had occurred in *traQ*, rather than in another open reading frame. In the case of white colonies, there were two possibilities. If the fusion had not placed the *phoA* sequence in frame with the *traQ* reading frame, colonies would be white because no protein would be expressed. However, if the fusion had joined the *traQ* and *phoA* reading frames, but the PhoA portion of the protein expressed had not been translocated across the membrane, the colonies would also be white. To differentiate between active and inactive fusions

in *traQ*, both blue and white colonies were retained for further screening.

In order to eliminate colonies that did not express a *traQ::phoA* fusion protein, two additional types of analyses were performed.

Restriction analysis

Restriction analysis was used to locate fusions of *phoA* which fell within *traQ*. The two sites used were the unique *Nsi*I site located within *traQ* and the *Xba*I site located upstream of *traQ*. If the *Xba*I site was not present, the deletion was assumed to have extended through all of the *traQ* gene, and the derivative was not considered further. If the *Nsi*I site was present, it was clear that at least the first 61 codons of the *traQ* sequence were still present. As indicated below, DNA sequence analysis of DNA from blue colonies obtained in the first digestion reaction showed that many of these expressed proteins derived from fusion to *trbB*, an open reading frame distal to *traQ*. Other results showed that fusions to the *traQ* reading frame that occurred at the *traQ Nsi*I site, or distal to that site also resulted in colonies with a blue color. Therefore, it became most interesting to examine the products of plasmids which retained the *Xba*I site, but had lost the *Nsi*I site.

To determine which restriction sites were present, mini-preparations of DNA were digested with *Nsi*I and examined by agarose gel electrophoresis. Isolates which failed to cut with *Nsi*I were further examined by *Xba*I digestion. The results of this analysis are presented in appendix A.

Since many potential fusions could be screened at one time, this analysis was very helpful for identifying the isolates that would be most interesting to examine further.

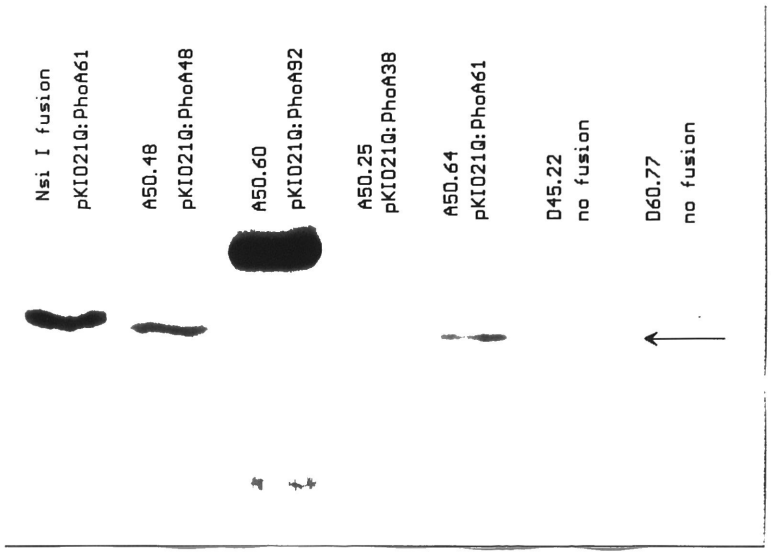
Analysis of gene products

I used a T7 RNA polymerase labeling system (Maneewannakul 1993) to identify the products expressed from selected plasmids. This analysis showed the size of the products expressed from plasmids which had given a blue colony color in the XP screen. It also allowed me to identify *traQ::phoA* fusion plasmids that did express a fusion protein even though the colony color had remained white. For the XP color test, the *traQ* sequence had been expressed by inducing the P_{LAC} promoter on the plasmids with IPTG. However, for this test, I first introduced the plasmids into strain XK100 which carries an IPTG inducible T7 RNA polymerase gene on its chromosome. After addition of IPTG, T7 RNA polymerase is expressed, and can transcribe genes from the P_{T7} promoter that is also present on pKI021. As the T7 RNA polymerase is resistant to rifampicin, but the *E. coli* RNA polymerases are inhibited by this drug, genes that are expressed from the P_{T7} promoter can have their products labeled very specifically in the presence of rifampicin.

Each plasmid to be tested was introduced into strain XK100 by transformation. Subsequently, the XK100 strain carrying the plasmid was grown to OD_{550} ~0.4 in JMM and induced with 10 ul 0.1 M IPTG for 20 minutes to allow T7 RNA polymerase to be expressed. Rifampicin (10 ul, 50

mg/ml) was added and the culture was incubated for an additional 40 min. Ten uCi ^{35}S-methionine were added for 2 minutes to label expressed proteins. Cells were collected by centrifugation, and labeled proteins were analyzed by SDS-PAGE electrophoresis and autoradiography. Fig. 4 shows an example of the results I obtained. It is clear that plasmids pKI021Q::PhoA38, 48, 61, and 92 express fusion proteins in the expected size range. These results are also summarized in appendix A.

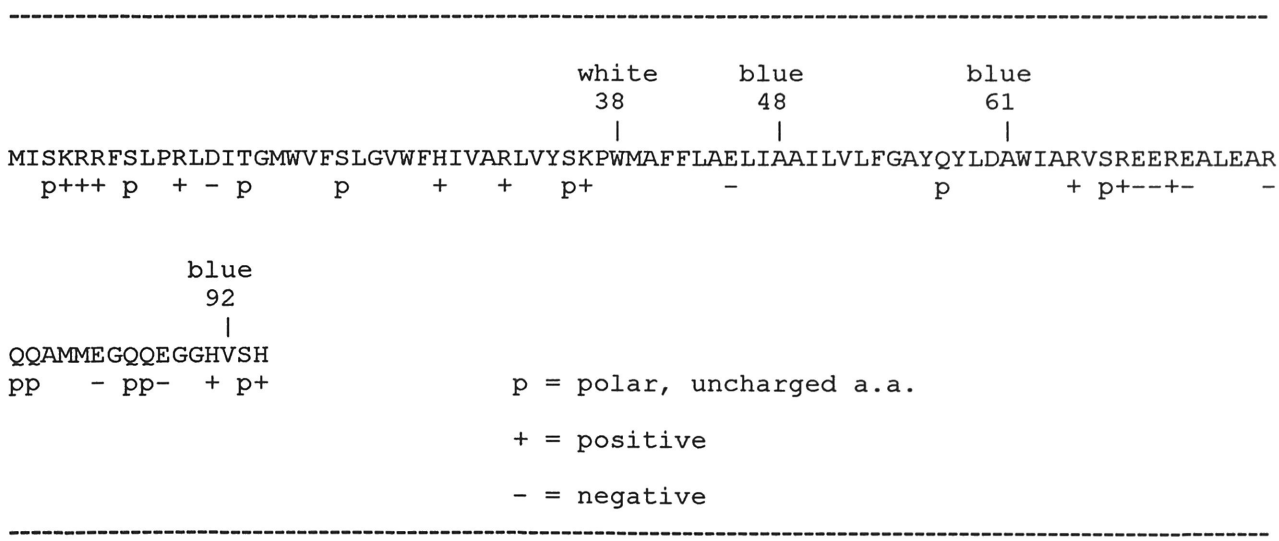
Fig. 4: Autoradiogram of *traQ::phoA* fusions



Determination of the DNA sequence:

I made purified DNA preparations of all plasmids that were found to express a fusion protein. These preparations were then sent to the Gene Technologies Laboratory, TAMU, for DNA sequence determination. From the sequence data obtained, I could determine the actual position at which the DNA sequences of *traQ* and *phoA* were joined in each plasmid. As shown in Fig 6, the fusions in plasmids I had identified and purified had occurred after TraQ residues 38, 48, 61, and 93.

Figure 6: Location of protein-expressing fusions in *traQ*.



DISCUSSION

Significance:

It is important to understand the way in which DNA is passed from one cell to another in conjugation because this exchange is responsible for genetic variation. It allows bacterial strains to acquire new genes, including new virulence characteristics such as antibiotic resistance or toxin genes.

Synthesis of pilin subunits, which bring cells in contact with each other, is essential for conjugation among gram negative bacteria. Thus, an understanding of the mechanism of pilus synthesis could lead to development of ways of blocking conjugation. This could prevent the transfer of unwanted genes and characteristics from one bacterial strain to another.

A study of pilin translocation into the membrane is also important because it seems to be a unique process. No other known bacterial protein is translocated across the membrane by the action of a single other protein, as appears to be the case for pilin. Therefore, this could be a unique model system for understanding the way in which protein sequences are transmitted into and through the bacterial membrane.

Conclusions:

Based on these experiments, I can make the following statements about the topology of the TraQ membrane protein. *TraQ::phoA* fusions that included residues 48, 61, and 93 exhibited alkaline phosphatase activity. This indicates that this segment of the protein spans the inner membrane and allows secretion of PhoA sequences to the periplasm. Fusion at residue 38 did not exhibit any alkaline phosphatase activity and therefore retains PhoA sequences in the cytoplasm. Since no fusions upstream of residue 38 have been identified at this time, the topology of the N-terminal region is not yet known. However, the von Heijne (von Heijne, 1989) positive-inside rule suggests that the amino terminal of TraQ is cytoplasmic.

Further research by myself and others to isolate fusions at the N-terminal region should allow us to complete our knowledge of the topology of TraQ.

Appendix A

Results of restriction and protein labeling experiments

Key:

+..... positive result
- ..... negative result
nd .....test not done

Name	Blue color	*Nsi*I digest	*Xba*I digest	Protein present
A25.23	+	+	nd	nd
A25.45	+	+	nd	nd
A25.68	+	+	nd	nd
A25.82	+	+	nd	nd
A25.83	-	+	nd	nd
A25.88	-	+	nd	nd
A50.6	+	+	nd	nd
A50.19	+	+	nd	nd
A50.25	-	-	nd	+
A50.35	-	+	nd	-
A50.38	+	+	nd	nd
A50.43	+	+	nd	nd
A50.48	+	-	nd	+
A50.58	-	+	nd	nd
A50.60	+	-	nd	+
A50.64	+	+	nd	+
A50.70	-	+	nd	nd
A50.81	-	+	nd	-
C45.7	-	+	nd	nd
C45.10	+	+	nd	nd
C45.12	+	+	nd	nd
C55.28	+	+	nd	nd
C55.30	+	+	nd	nd
C55.34	-	+	nd	nd
C55.42	+	+	nd	nd
C55.44	+	+	nd	nd
C60.63	+	+	nd	nd
C60.67	-	+	nd	nd

Name	Blue color	*Nsi*I digest	*Xba*I digest	Protein present
C60.69	+	+	nd	nd
C60.77	+	+	nd	nd
D45.3	-	+	nd	nd
D45.6	-	+	nd	nd
D45.8	-	-	-	nd
D45.10	-	+	nd	nd
D45.13	-	+	nd	nd
D45.15	-	+	nd	nd
D45.19	-	+	nd	nd
D45.22	-	-	+	-
D45.24	-	+	nd	nd
D50.27	-	+	nd	nd
D50.30	-	+	-	nd
D50.33	-	+	nd	nd
D50.36	-	-	-	nd
D50.39	+	+	nd	nd
D50.41	-	-	-	nd
D50.44	-	+	+	nd
D50.47	-	+	nd	nd
D50.50	-	+	nd	nd
D55.51	-	-	-	nd
D55.54	-	-	-	nd
D55.56	+	+	nd	nd
D55.59	-	+	nd	nd
D55.63	+	+	+	nd
D55.66	-	nd	nd	nd
D55.68	-	-	+	-
D55.71	-	-	+	-

Name	Blue color	*Nsi*I digest	*Xba*I digest	Protein present
D55.75	+	+	+	nd
D60.77	-	-	+	-
D60.80	-	-	-	nd
D60.83	+	-	nd	nd
D60.86	+	+	+	nd
D60.89	-	-	+	-
D60.91	-	-	+	-
D60.93	-	-	+	-
D60.96	-	+	nd	nd
D60.98	+	+	nd	nd
D50.101	nd	-	-	nd
D50.102	nd	-	-	nd
D50.103	nd	-	-	nd
D50.104	nd	+	nd	nd
D50.105	nd	nd	nd	nd
D50.106	nd	-	-	nd
D50.107	nd	-	-	nd
D50.108	nd	-	-	nd
D50.109	nd	+	nd	nd
D50.110	nd	+	nd	nd
D50.111	nd	+	nd	nd
D50.112	nd	+	nd	nd
D55.113	nd	+	nd	nd
D55.114	nd	-	-	nd
D55.116	nd	+	nd	nd
D55.117	nd	+	nd	nd
D55.118	nd	-	-	nd
D55.119	nd	+	nd	nd

Name	Blue color	*Nsi*I digest	*Xba*I digest	Protein present
D55.120	nd	-	-	nd
D55.121	nd	+	nd	nd
D55.122	nd	+	nd	nd
D55.123	nd	+	nd	nd
D55.124	nd	+	nd	nd
D60.125	nd	+	nd	nd
D60.126	nd	+	nd	nd
D60.127	nd	+	nd	nd
D60.128	nd	-	-	nd
D60.129	nd	+	nd	nd
D60.130	nd	+	nd	nd
D60.131	nd	+	nd	nd
D60.132	nd	-	-	nd
D60.133	nd	+	nd	nd
D60.134	nd	+	nd	nd
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D60.136	nd	+	nd	nd
D50.137	nd	+	nd	nd
D50.138	nd	+	nd	nd
D50.139	nd	+	nd	nd
D50.140	nd	+	nd	nd
D50.141	nd	-	-	nd
D50.142	nd	+	nd	nd
D50.143	nd	-	-	nd
D50.144	nd	-	-	nd
D50.145	nd	+	nd	nd
D50.146	nd	+	nd	nd
D50.147	nd	+	nd	nd

Name	Blue color	*Nsi*I digest	*Xba* I digest	Protein present
D50.148	nd	+	nd	nd
D55.149	nd	-	-	nd
D55.150	nd	+	nd	nd
D55.151	nd	-	-	nd
D55.152	nd	+	nd	nd
D55.153	nd	+	nd	nd
D55.154	nd	-	-	nd
D55.155	nd	+	nd	nd
D55.156	nd	-	-	nd
D55.157	nd	+	nd	nd
D55.158	nd	+	nd	nd
D55.159	nd	-	-	nd
D55.160	nd	+	nd	nd
D60.161	nd	+	nd	nd
D60.162	nd	+	nd	nd
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D60.172	nd	-	-	nd

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