USE OF TN*PHOA* TO STUDY MEMBRANE PROTEIN TOPOLOGY IN *SALMONELLA TYPHIMURIUM*

BY

Michele Beaudet

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THIS IS TO CERTIFY THAT THE SENIOR THESIS PREPARED UNDER MY SUPERVISION BY Michele Beaudet

ENTITLED Use of Tn*phoA* to Study Membrane Protein Topology in *Salmonella typhimurium*

IS APPROVED.

Stanley Maloy
In charge of Thesis

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J. Konisky
Head, Department of Microbiology

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ABSTRACT

In order to determine the structure and function of proline permease, I optimized the techniques for use of Tn*phoA* in *Salmonella typhimurium*. Tn*phoA* is a derivative of Tn5 which has been used in *Escherichia coli* to construct gene fusions which yield information about membrane topology of the protein of interest. Techniques for isolating chromosomal and plasmid Tn*phoA* insertions were developed and several insertions in the *putP* gene were isolated.

INTRODUCTION

Integral membrane proteins carry out many critical functions in living cells including: active and passive transport, energy transduction, and chemical signal reception and transduction across the membrane. Transport, in particular, is an important and often rate-limiting step in a metabolic pathway. Yet, little is known about how integral membrane permeases interact with their substrates and facilitate their translocation across the membrane. Our lack of knowledge on the molecular structure of permeases stems primarily from the difficulty in crystallizing membrane proteins. Crystals are needed for X-ray diffraction analysis to determine the 3-dimensional structure of proteins. However, recently it has become possible to study the structure of membrane proteins using genetics. Ion/solute symport systems are particularly well-suited to genetic analysis.

The major proline permease in *S. typhimurium*, encoded by the *putP* gene (Ratzkin and Roth 1978), is an integral membrane protein that transports proline across the cytoplasmic membrane by sodium symport (Cairney *et al.* 1984). Proline permease is a good model system for studying ion/solute symport because it is well characterized biochemically and physiologically, and genetic manipulation of *putP* is easily accomplished by a variety of *in vitro* and *in vivo* techniques. We are interested in analyzing the structure and function of proline permease at the molecular level in order to use it as a model for

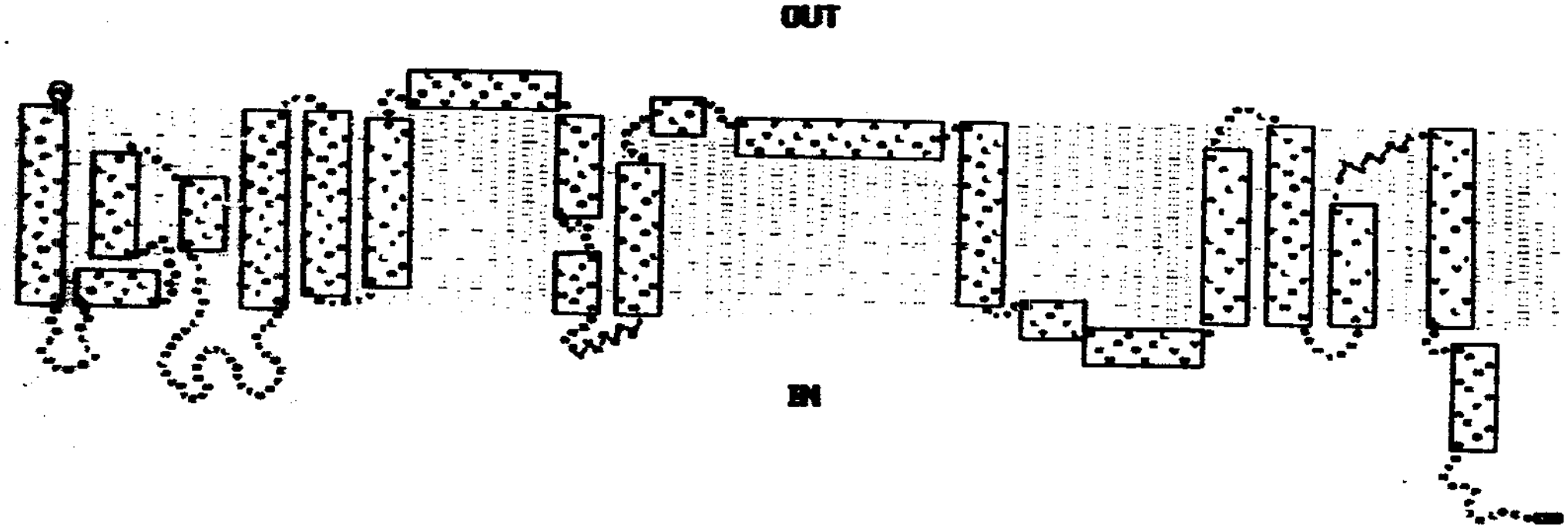
understanding the molecular mechanism of ion-solute transport systems.

To this end, we have undertaken the dissection of the active site of proline permease through analysis of point mutations in *putP*. Substrate specificity mutations in the *putP* gene were isolated in order to define the substrate binding site of proline permease. The selection was based on resistance to the toxic proline analogs, azetidine-2-carboxylic acid (AZT) and 3,4-dehydroproline (DHP) (Dila and Maloy 1986). Most analog resistance mutations have a null phenotype; proline permease fails to transport proline or either of the analogs. Occasionally, rare missense mutants are isolated which transport one or more of the substrates, but not all of them. By an analogous method, rare missense mutants were isolated that appear to alter the cation specificity of proline permease (Myers and Maloy 1988). By determining which amino acids have been altered in these mutants, we will be able to identify important residues at or near the active site.

In order to understand the active site mutations in the context of the three dimensional structure of proline permease, we need to determine its topology within the membrane. Proline permease is estimated to have a molecular weight of about 54,000 daltons and is very hydrophobic, made up of approximately 70% non-polar amino acids (Nakaoa *et al.* 1987). In computer analysis of protein sequences, a stretch of 18-20 hydrophobic amino acids is often indicative of a membrane spanning domain. The hydropathy profile of proline permease (Figure 1) is similar to other integral membrane proteins and predicts 12 membrane-

Figure 1. Cartoon of *E. coli* proline permease secondary structure as determined from computer predictions of hydrophobicity, acrophilicity, amphiphilicity, and hydrophobic moment. (Computer program SEQANAL by H. Robbins and T. Crofts, University of Illinois. Structural prediction and cartoon by Rik Myers, unpublished data).

Boxes represent helical domains. Membrane-spanning helices are oriented vertically; amphiphilic helices are oriented horizontally at the "lipid/water" interface. The amino acid sequence is indicated with the standard single letter format.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spanning domains (Nakaoa *et al.* 1987). However, there is presently little experimental data in support of the computer predicted structure. Therefore, I used a genetic approach to test and refine the computer generated model experimentally.

Manoil and Beckwith (1986) have developed a technique for determining the topology of membrane proteins using *phoA* gene fusions. Alkaline phosphatase, the *phoA* gene product, must be exported to the periplasm in order to be enzymatically active. Thus, *phoA* gene fusions have high activity only when alkaline phosphatase is directed to the periplasm (see Figure 2). By analyzing *phoA* fusions to the *E. coli* Tsr protein, a chemoreceptor with two known membrane spanning regions, they demonstrated that if the fusion joint was within a periplasmic domain of Tsr, the hybrid protein folded normally and directed export of alkaline phosphatase to the periplasmic space, where it exhibited a high level of activity. In contrast, fusions to regions of Tsr which face the cytoplasm were inactive. Studies with several other membrane proteins agree with these results (Akiyama and Ito 1987; Chun and Parkinson 1988) suggesting that alkaline phosphatase fusions can be used to identify periplasmic domains of membrane proteins.

To facilitate the isolation of random alkaline phosphatase fusions, Manoil and Beckwith constructed Tn*phoA* (see Figure 3), a derivative of transposon Tn5 (Manoil and Beckwith 1985). Tn*phoA* is particularly well-suited to constructing gene fusions because it has a wide host-range and transposes relatively randomly and at a high frequency (Biek 1983).

Figure 2. *phoA* gene fusions only produce PhoA$^+$ hybrid membrane proteins when alkaline phosphatase is directed to the periplasmic space. Two potential classes of *phoA* gene fusions in an integra membrane protein are shown in the cartoon below. Alkaline phosphatase is represented by the curly line.

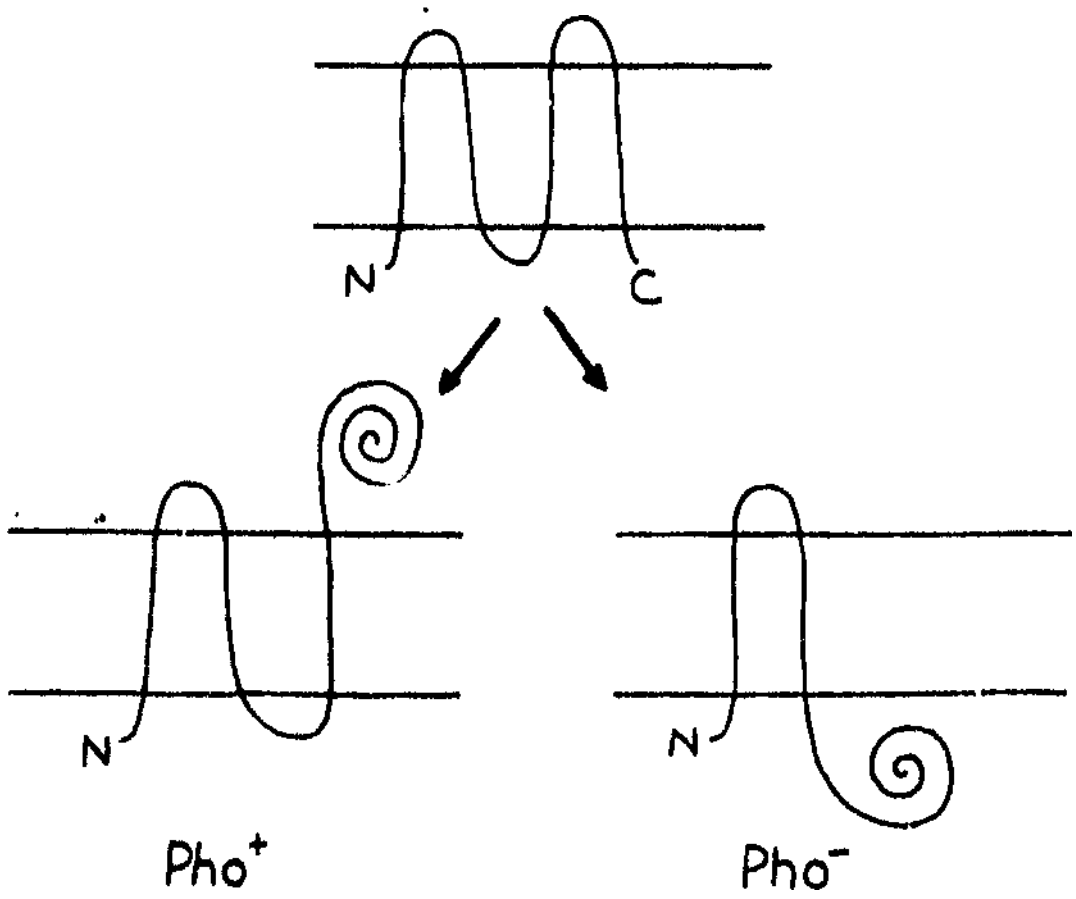
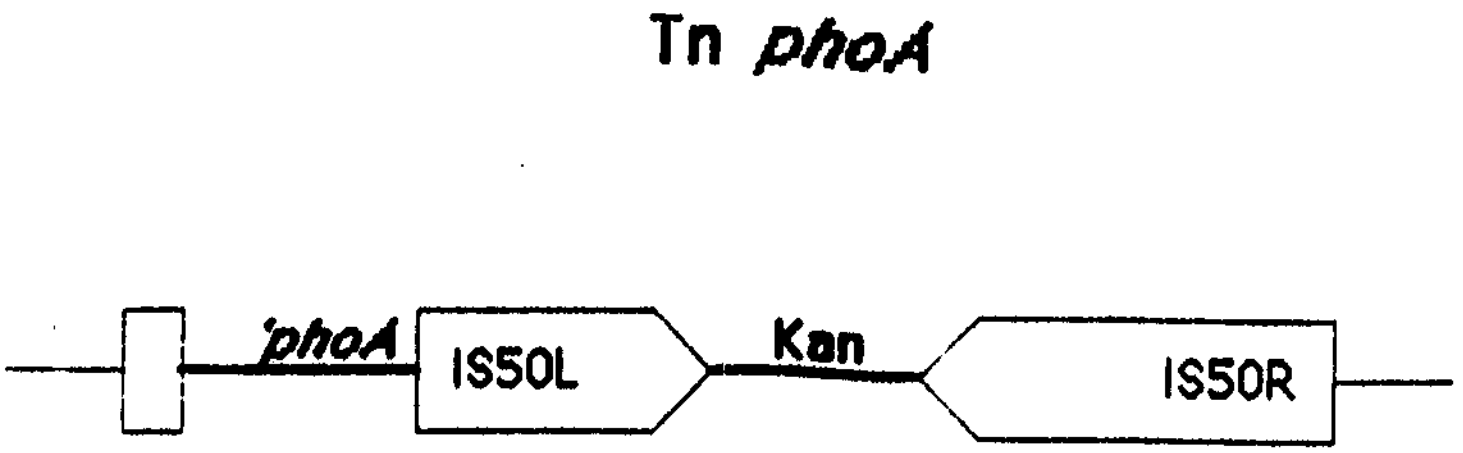


Figure 3. A cartoon showing the essential features of Tn*phoA*. Tn*phoA* is a derivative of Tn5 with the *phoA* gene (minus promoter, translation start, and signal sequence) cloned into IS50L. Sequences derived from Is*50* are shown as open boxes.



When inserted in the proper orientation and reading frame of a gene, Tn*phoA* directs the formation of alkaline phosphatase hybrid proteins. So far this technique has been successfully used to study several membrane proteins in *E. coli*, but we are not aware of any published reports of its application in *Salmonella*. Therefore, one major goal of my project was to determine the optimal conditions for working with Tn*phoA* in *S. typhimurium*. By generating a series of Tn*phoA* insertions throughout *putP*, I attempted to identify the periplasmic domains of proline permease in order to draw a two-dimensional diagram of its membrane topology.

MATERIALS AND METHODS

Bacterial strains. All strains were derived from *S. typhimurium* LT2. The genotypes of strains used in this study are shown in Table 1.

Media and growth conditions. Nutrient broth (8 g/l) with NaCl (5 g/l) was used as the rich medium (NB). Rich medium was supplemented with kanamycin sulfate at a final concentration of 50 µg/ml (Km), tetracycline HCl at 25 µg/ml (Tc), and ampicillin at 35 µg/ml (Ap). The chromogenic substrate 5-bromo-4-chloro-3-indolyl phosphate (XP), potassium salt, was dissolved in dH_2O and added to a final concentration of 40 µg/ml for the detection of alkaline phosphatase activity on plates (Brickman and Beckwith 1975). When using the p-toluidine salt of XP, it was dissolved first in N,N-dimethyl formamide (Brickman and Beckwith

Table 1. *S. typhimurium* strains used in this study.

Strain[1]	Genotype	Source
LT2	Wild Type	Lab collection
TR3400	del(*put-515*)	Ratzkin and Roth
TR4921	del(*put-534*)	Ratzkin and Roth
TR4942	del(*put-555*)	Ratzkin and Roth
TR4944	del(*put-557*)	Ratzkin and Roth
TR4946	del(*put-559*)	Ratzkin and Roth
TR4950	del(*put-563*)	Ratzkin and Roth
TR4959	del(*put-572*)	Ratzkin and Roth
TR4981	del(*put-594*)	Ratzkin and Roth
TR4999	del(*put-679*)	Ratzkin and Roth
TR5023	del(*put-715*)	Ratzkin and Roth
TT11096	LT2 / F' 42 *lacI3 zaf*::Tn*phoA*	J. Roth
TT13206	*phoN51*::Tn*10dTc* (AZT^r)	J. Roth
TT13208	*phoP53*::Tn*10dTc* (AZT^r)	J. Roth
MS308	*phoN51*::Tn*10dTc* (AZT^s)	This study
MS509	*phoP53*::Tn*10dTc* (AZT^s)	This study

MS654	del(*put-516*)	Lab collection
MS1748	*pyrD121* del(*put-521*)	Lab collection
MS1766	*pyrD121* del(*put-521*) / pPC5	Lab collection
MS1878	*putP1204*::Tn*phoA* *putA1020*::Mud*A* *phoN51*::Tn*10dTc*	This study
MS1879	*putP1205*::Tn*phoA* *phoN51*::Tn*10dTc*	This study
MS1880	*putP1206*::Tn*phoA* *phoN51*::Tn*10dTc*	This study
MS1881	*putP1204*::Tn*phoA* *phoN51*::Tn*10dTc*	This study
MS1882	*putA1020*::Mud*A* *phoN51*::Tn*10dTc*	This study

E. coli strains

K12	Wild type	Lab collection
CC118	del(*phoA20*) *araD139* del(*ara-leu*)*7697* del(*lacX74*) *galE galK thi rpsE rpoB* *argE*(am) *recA1*	C. Manoil

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[1]All strains were derived from *Salmonella typhimurium* LT2 unless otherwise noted. Genetic nomenclature is described in Sanderson and Roth (1983).

1975). Five different minimal media were used: a medium with no carbon source and NH_4^+ as the nitrogen source (NCE) (Ratzkin and Roth 1978); a medium lacking both carbon and nitrogen sources (NCN) (Berkowitz *et al.* 1968); lactose + XP agar composed of 0.2% lactose, Tris 121 salts and buffer (Torriani 1966), 40 µg/ml XP, and 15 g/l agar; ß-glycerol phosphate agar composed of 0.25% ß-glycerol phosphate, Tris 121 salts and buffer, 0.3 mM KH_2PO_4, and 15 g/l agar; and MTS (Myers and Maloy 1988) + XP adjusted with Tris base to pH indicated in text. Tris 121 salts and buffer was composed of: 0.12 M Tris, 0.08 M NaCl, 0.02 M KCl, 0.02 M NH_4SO_4, 0.001 M $MgCl_2$, $2x10^{-4}$ M $CaCl_2$, $4x10^{-6}$ M $ZnCl_2$, $2x10^{-6}$ M $FeCl_3$. The pH was adjusted to 7.4 with HCl. When growth on proline as the sole nitrogen source (PSN) was selected, NCN medium was supplemented with 0.2% proline and 0.6% succinate (Hahn and Maloy 1986). To screen analog resistance, azetidine was added to NCE + succinate to a final concentration of 40 µg/ml, or dehydroproline to a final concentration of 12 µg/ml.

Genetic techniques. All genetic manipulations used P22 HT105/1 *int*-201, a high frequency generalized transducing phage that cannot form stable lysogens (Schmeiger 1972). Phage lysates were prepared as described (Davis *et al.* 1980), except where indicated. For transductions, phage and cells were mixed directly on selective plates at a multiplicity of infection of approximately 1 PFU per cell, unless otherwise noted in text. When kanamycin resistance was selected, incubation on a non-selective medium was required to allow phenotypic

expression. For phenotypic expression cells and phage were plated on nonselective NB medium, incubated at 37°C for about five hours, then replica printed onto selective medium. Green plates were used in order to isolate phage free transductants (Maloy 1988). Transductants were tested for sensitivity to phage P22 infection by cross-streaking against the P22 clear plaque mutant H-5 (Davis *et al.* 1980). To map *putP*::Tn*phoA* insertions, I carried out transductional crosses with donor phage P22 lysates grown on each of the mutants. The recipients for these crosses were strains carrying deletions of various segments of the *putP* gene (Dila and Maloy 1986).

Alkaline phosphatase assays. Alkaline phosphatase activity was determined by measuring cleavage of p-nitrophenyl phosphate as described previously (Brickman and Beckwith 1975), except that cells were grown to mid-log phase in NCE + succinate medium and were treated with chloroform and sodium dodecyl sulfate before assay (Hahn and Maloy 1986).

RESULTS

Tn*phoA* transposition in *S. typhimurium* by zygotic induction. Strain MS1621 carries an *E. coli* F' harboring a Tn*phoA* insertion. A P22 lysate on this strain will contain transducing particles carrying Tn*phoA* and part of the F'. When used to transduce another strain to Km^r, Tn*phoA* can only be inherited by transposition. There are three reasons for this (Biek 1983). 1) Since the amount of DNA that P22 can package

is less than half the size of the F', an intact F' cannot be moved between strains by transduction. Thus, Km^r cannot be conferred by transducing the Tn*phoA* bearing F'. 2) Tn*phoA* cannot be inherited by homologous recombination between F' DNA and the chromosome since there is not sufficient DNA homology between the F' and the *S. typhimurium* chromosome for recombination. 3) When the transducing fragment enters the recipient, transposition occurs because the recipient strain is free of the Tn5 repressor, allowing transposase to be transiently expressed from Tn*phoA*.

I examined the frequency of transposition using phage grown on MS1621, and LT2 as the recipient at several multiplicities of infection (m.o.i.). At an m.o.i. of 0.8, the frequency of Km^r transductants was $2x10^{-7}$; at an m.o.i. of 2, the frequency of Km^r was $5x10^{-8}$; and at an m.o.i. of 10, the frequency of Km^r was $2.5x10^{-6}$. Since the multiplicity of infection had to be quite high in order to get a reasonable number of Km^r transductants, it seemed possible that fewer than expected phage particles carried Tn*phoA*. Even though the Tn*phoA*-bearing F' was not advertised as being temperature sensitive for replication, this would account for the abnormally low frequency of transposition, since the phage were propagated at 37°C. When a P22 lysate prepared on strain MS1621 grown at 30°C was used to transduce LT2, Km^r colonies were recovered at a frequency of $2x10^{-6}$ at an m.o.i. of 0.4. I therefore prepared all future MS1621 phage stocks by growing the cell culture at 30°C.

Isolation of *putP*::Tn*phoA* insertion mutants. Initially, transductions were performed using LT2 as the recipient and selecting Km^r on NB + Km + XP + EGTA. Cells and phage were incubated at 37°C on NB plates for 5-6 hours and then replica printed onto the selection plates. EGTA was added to the selection plates to chelate Ca^{++} and inhibit further phage infection. The resulting Km^r transductants were screened by replica printing onto three types of plates. 1) PSN plates. A *putP* insertion would render the strain PSN^-, unable to utilize proline as sole nitrogen source. 2) NCE + succinate + AZT plates. A *putP* insertion would confer AZT^r since AZT could no longer be transported into the cell via proline permease. 3) NCE + succinate + DHP. In addition to AZT^r, a *putP* insertion would confer DHP^r since DHP could no longer be transported into the cell via proline permease.

In all, nine *putP* insertions were isolated by this method and the frequency of such insertions among Km^r transductants was approximately 10^{-3}. However, sometimes I screened through several thousand Km^r colonies without finding a single *putP* insertion and became concerned that I may be missing them because the PSN^- phenotype is leaky. The major pathway for proline uptake is by proline permease, but a low amount of proline can also be transported by the *proP* gene product (Menzel and Roth 1980), a glycine-betaine permease (Cairney *et al.* 1985). Thus, even a *putP* knockout mutant can grow slowly on PSN, making PSN^- colonies difficult to spot on a plate with a thousand colonies. Therefore, I decided to concentrate my efforts on working out a simple

plate test for screening PhoA$^+$ transductants.

Use of XP plates. A convenient way to check alkaline phosphatase activity is to screen the color of colonies on a plate containing 5-bromo-4-chloro-3-indolyl phosphate (XP), an indicator dye that is cleaved by alkaline phosphatase to form a blue color. The degree of blueness is a measure of the level of alkaline phosphatase activity (Brickman and Beckwith 1975). An initial problem that I encountered was that *S. typhimurium* LT2 forms blue colonies on XP plates even though it lacks the structural gene for alkaline phosphatase (Schlesinger and Olsen 1968; Kier *et al.* 1977a). One possible explanation was that nonspecific acid phosphatase activity in *S. typhimurium* was high enough to turn colonies blue. Several conditions were tested to find a protocol that would distinguish between PhoA$^+$ and PhoA$^-$ colonies of *S. typhimurium* on XP plates. Since nonspecific acid phosphatase activity increases under phosphate limiting conditions (Kier *et al.* 1977b), I tested the effect of adding 100 mM K_2HPO_4 to five types of plates: NB + XP, E + dextrose + XP, E + succinate + XP, E + succinate + proline + XP, and PSN + XP. Since the presence of nutrient broth has been shown to decrease the specific activity of nonspecific acid phosphatase (Kier *et al.* 1977b), I compared a rich medium to minimal media. Since decreased activity of nonspecific acid phosphatase has been observed under catabolite repressing conditions (Kier *et al.* 1977b), I compared a preferred carbon source (glucose) and a non-catabolite repressing carbon source (succinate). I also tested the effect of induction of the *put*

operon by adding proline to minimal medium plus succinate which would have increased expression of the hybrid protein in a *putP*::Tn*phoA* strain. PSN plates were used because of all the minimal media discussed, it is the only one made with noble agar and therefore contains fewer impurities. Both LT2 and five *putP*::Tn*phoA* derivatives of LT2, produced blue colonies on all five types of plates in the presence or absence of inorganic phosphate.

Since the nonspecific acid phosphatase has a pH optimum between 5.0 and 5.5 (Weppelman *et al.* 1977), I examined the effect of increasing the pH on the XP phenotype, using MTS + XP plates (MOPS + Tris + succinate + XP) at pH 6.5, 7.0, 7.5, 8.0, 8.5. The same six strains were used and again they all produced blue colonies, regardless of pH. With all of the plates described above, I noticed that the degree of blueness depended on how crowded the colonies were and on how long the plates were incubated. Older growth and larger, less crowded colonies were blue, while smaller, more dense growth was light blue. I then tested the effect of temperature of incubation on blueness and found that growth at 30°C was lighter in color than that grown at 37°C (on NB + XP), possibly due to the effect of decreased growth rate at 30°C.

Next I screened two *E. coli* strains with known *phoA* genotypes to help us decide whether there was something wrong with the media used or whether the problems resulted solely from working in *S.typhimurium*. The strains tested were: *E. coli* K12 (*phoA*$^+$) and CC118 (*phoA*$^-$ derivative of *E. coli* K12). On NB + XP plates, K12 produced pale blue colonies and

CC118 produced white colonies. This indicated that *phoA*+ strains could be differentiated from *phoA*$^-$ strains on NB + XP plates.

Since the NB + XP plates seemed appropriate for screening the PhoA phenotype, I decided to test two derivatives of *S. typhimurium* LT2 lacking nonspecific acid phosphatase activity to see if they would form white colonies on NB + XP. I screened TT13206 (*phoN*::Tn*10dTc*) and TT13208 (*phoP*::Tn*10dTc*). The *phoN* gene is believed to encode the nonspecific acid phosphatase and the *phoP* gene is thought to encode a regulatory protein necessary for the expression of nonspecific acid phosphatase (Kier *et al.* 1979). When streaked for isolated colonies on NB + XP plates, LT2 was light blue, while TT13206 and TT13208 were white. Since the two *S. typhimurium* strains lacking nonspecific acid phosphatase showed no color on the indicator plates, all further work with Tn*phoA* in *S. typhimurium* was performed using derivatives of these strains.

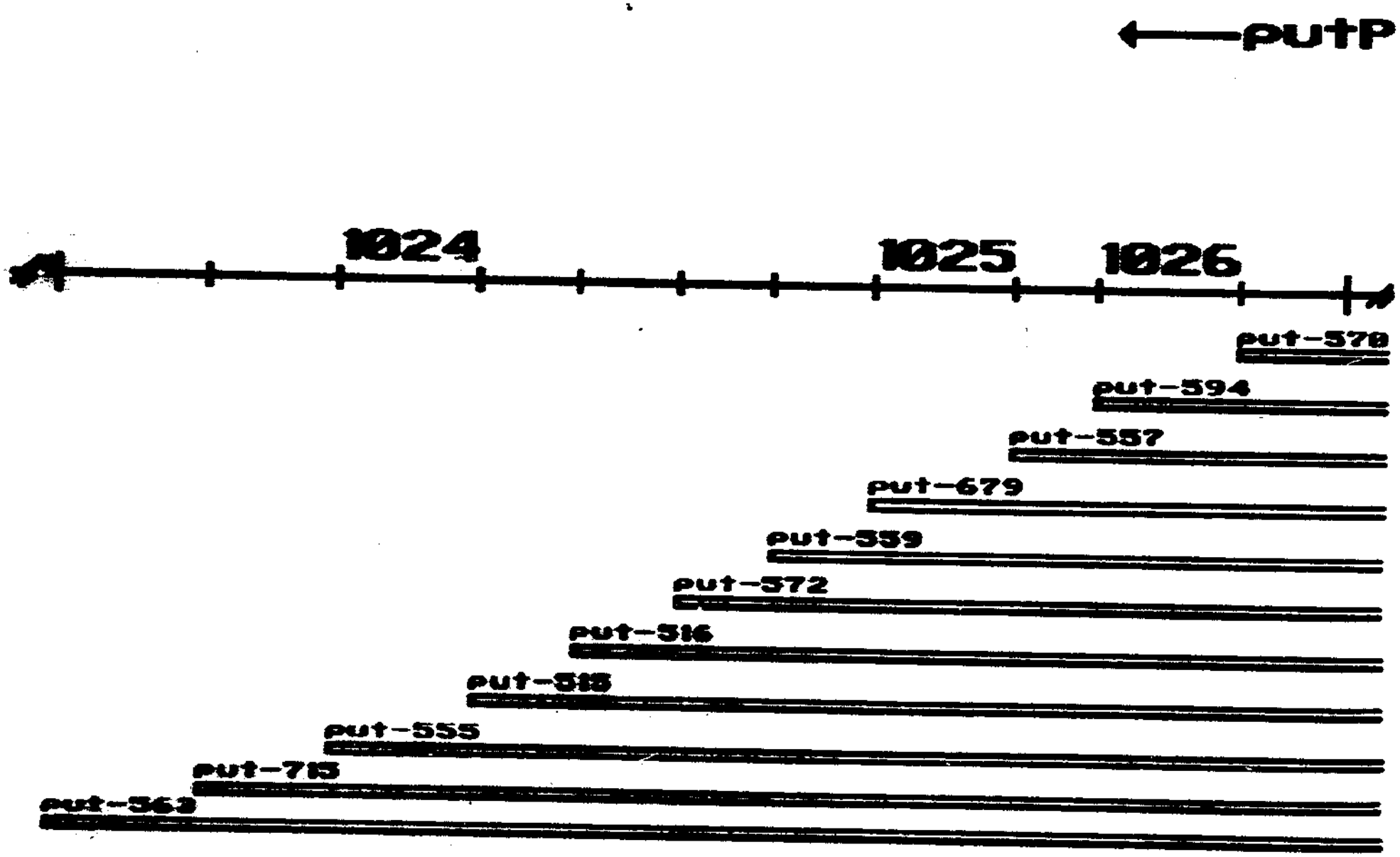
Characterization of *putP*::Tn*phoA* insertions. Since the *phoN* and *phoP* strains produced white colonies on NB + XP plates, I tested the phenotype of my *putP*::Tn*phoA* insertions in these backgrounds. While constructing these strains, I noticed that TT13206 and TT13208 were resistant to azetidine. Since this would have made screening for *putP* mutants difficult, I transduced LT2 to To^r with phage grown on TT13206 and TT13208. I purified 8 To^r colonies for each recipient, and screened their AZT phenotypes. All were AZT^s so I chose one of each and designated the new strains MS308 (*phoN*::Tn*10dTc*) and MS509

(*phoP*::Tn*10dTc*). I used MS308 and MS509 for all further studies. Since the AZT^r phenotype did not cotransduce with *phoN* or *phoP*, I transduced my *putP*::Tn*phoA* insertion strains to To^r with phage grown on TT13206 and TT13208, and screened PSN$^-$, AZT^r, DHP^r, Km^r.

Next I streaked out the acid phosphatase mutant derivatives of the *putP*::Tn*phoA* strains for isolated colonies on NB + XP. All produced white colonies after 24 hour incubation at 37°C. After about two weeks at 4°C, the colonies of one *putP*::Tn*phoA* strain (MS1881) had become light blue. Alkaline phosphatase assays were performed on the *phoN* derivative of this strain and *E. coli* K12. K12 had 336 units of alkaline phosphatase activity while MS1881 had 4 units of alkaline phosphatase activity. I randomly chose one of the white *putP*::Tn*phoA* *phoN* strains to assay as a representative of the other mutants and it had no alkaline phosphatase activity.

I then mapped the Tn*phoA* insertions in order to determine where they lie in the *putP* gene. For genetic mapping I crossed each Tn*phoA* insertion mutant against a series of nested deletions of *putP* (Figure 4). I mapped three *putP*::Tn*phoA* insertions and each mapped in a different region of the *putP* gene (Figure 4). It is important for my project that Tn*phoA* have little sequence specificity because to get a clear idea of the membrane topology of proline permease we need a random series of Tn*phoA* inserions throughout the *putP* gene. I am in the process of mapping the other six insertions and tentative results indicate that they map throughout the *putP* gene.

Figure 4. Deletion map of the *putP* gene. The positions of three *putP*::Tn*phoA* alleles is shown.


putP
1024
1025
1026
put-578
put-594
put-557
put-679
put-559
put-572
put-516
put-518
put-555
put-715
put-562

Optimized Tn*phoA* chromosomal hop protocol. The following protocol yielded 1,000-2,000 Km^r colonies per plate. A high titer (10^{10}-10^{11} pfu/ml) P22 phage lysate of MS1621 was prepared on an overnight culture of MS1621 grown in NB at 30°C. This phage stock was used to transduce MS1882 (*putA*::MudA *phoN*::Tn*10d*Tc) to Km^r. Expression of the *putP* gene is negatively regulated by the *putA* gene product; insertion mutations in *putA* overexpress the *putP* gene. Therefore, this should enhance the blue color of PhoA+ *putP*::Tn*phoA* insertions. I decided to use the *phoN*::Tn*10dTc* mutation (MS308) for the rest of my work with the nonspecific acid phosphatase minus phenotype because it conferred a cleaner negative phenotype than *phoP*::Tn*10dTc*.

I spread 0.1 ml undiluted phage + 0.1 ml MS1882 (from a saturated culture grown in NB, centrifuged and resuspended in 0.1 volumes of saline) onto an NB plate to allow phenotypic expression of Km^r. The plates were incubated at 30°C, for higher frequency of transposition events (S. Maloy, unpublished observation), until a lawn was visible (about six hours), then replica printed onto NB + Km + XP and incubated at 37°C. I originally added EGTA to the selection plates to inhibit further phage infection but discovered that alkaline phosphatase is inhibited by the presence of EGTA. This is because EGTA chelates divalent cations and alkaline phosphatase requires Zn^{++} and Mg^{++} to be active (Roberts and Chlebowski 1984). Once I stopped adding EGTA to XP plates, I observed a 10x increase in the frequency of blue transductants. After approximately 24 hours incubation, I marked the

position of blue colonies and picked them for further characterization. I then stored the plates at 4°C for 1-2 weeks and rechecked them. Light blue, lower activity fusions became visible after several days in the refrigerator (a phenomenon also observed by Akiyama and Ito 1987). The frequency of blue colonies among Km^r transductants was 10^{-3}.

TnphoA plasmid hops. Of the transductions performed using the optimized protocol, I obtained about 100 blue colonies from a total of 120,000 Km^r colonies but found no *putP*::Tn*phoA* insertions. We reasoned that the frequency of *putP* insertions would be greater if the target size for Tn*phoA* insertion was increased. The high-copy number plasmid, pPC5, carries the *put* operon of *S. typhimurium* (Hahn *et al.* 1988). By increasing the number of copies of *putP* per cell, the frequency of *putP*::Tn*phoA* insertions should also increase. In addition, having the inserts on a plasmid will facilitate restriction mapping to determine the orientation of Tn*phoA*, and determining the DNA sequence of the fusion junctions.

The first step was to perform a large Tn*phoA* "hop" into the plasmid bearing recipient. The resulting transductants were pooled and a P22 lysate was prepared on the pooled cells. When P22 infects a cell, it produces an inhibitor (Abc) of exonuclease V (Poteete *et al.* 1988). In the absence of exonuclease V activity, colE1 plasmids can undergo rolling circle replication to form linear concatemers which can be packaged by P22 (Cohen and Clark 1986). Each transducing particle will

carry a single plasmid species. In this way, potential Tn*phoA* bearing plasmids were moved individually into MS1748, and then screened for antibiotic resistance. Strain MS1748 contains a chromosomal deletion of the *put* operon. This deletion serves two purposes: 1) The presence of a chromosomal *put* operon would have provided extensive homology for recombination with pPC5. 2) The deletion confers a PSN$^-$ phenotype. Isolates of this strain that carry a Tn*phoA* insertion in *putP* will remain PSN$^-$, while all other transductants will become PSN$^+$. Therefore simple screening for PSN$^-$ transductants will identify potential *put*::Tn*phoA* insertion mutants.

The construction of phage pools was performed as follows: I transduced 0.1 ml saturated culture of MS1766 with 0.1 ml of phage MS1621 diluted 1:25 in T2 buffer, selecting Km^r. Transductants (approximately 100-200 per plate) were pooled by adding 0.3 ml NB + 2x Ap + 2x Km to the selection plates, suspending the cells with a spreader. Cell suspensions were transferred to tubes containing 2 ml NB + 2x Ap + 2x Km + EGTA and incubated on a shaking water bath at 37^{o}C. Cultures were centrifuged and resuspended in 2 ml NB to remove EGTA and high titer P22 phage stocks were prepared as described in Materials and Methods.

Several procedures were tested to find the best method for transducing MS1748 with the phage pools. The protocols and results are described in Table 2. Protocols A and B involved mixing cells and phage at an m.o.i. of 1 and simultaneously selecting Km and Ap resistance, so

TABLE 2. Results of transductions with plasmid pools.

The protocols used are described in the legend.

		# Colonies			
Protocol	Pool	Ap^r	Km^r	Ap^rKm^r	PSN^-
A	1			1	0
	2			0	
B	1			4	0
	3			1	0
C	1		2500	0	
			2500	0	
			2500	0	
			2500	0	
D	5	LAWN		3	0
		LAWN		9	0
		LAWN		18	0
		LAWN		0	0
	11	LAWN		17	0
		LAWN		13	0
		LAWN		11	0
		LAWN		4	0
	12	LAWN		23	0
		LAWN		28	0
		LAWN		10	0
		LAWN		15	0
	13	LAWN		17	0
		LAWN		6	0
		LAWN		8	0
		LAWN		13	0
	14	LAWN		75	0
		LAWN		46	0
		LAWN		90	0
		LAWN		77	0
	15	LAWN		47	0
		LAWN		33	0
		LAWN		41	0
		LAWN		32	0
	16	LAWN		59	0

TABLE 2 continued.

		# Colonies			
Protocol	Pool	Ap^r	Km^r	Ap^rKm^r	PSN^-
		LAWN		79	0
		LAWN		57	0
		LAWN		38	0

A: MS1748 (1 x 10^9 cells) + phage pools were mixed at an moi = 1, and incubated at 30°C for 30 min. Cells with adsorbed P22 were separated from free phage by centrifugation, resuspended in 0.2 ml T2 buffer, and plated on NB. Following phenotypic expression, NB plates were replica printed onto NB + 2x Ap + 2x Km plates and incubated at 37°C. PSN was scored by patching Ap^rKm^r transductants onto PSN plates.

B: MS1748 (5 x 10^9 cells) + phage pools were mixed directly on NB plates at an moi = 1. Following phenotypic expression, NB plates were replica printed onto NB + 2x Ap + 2x Km plates and incubated at 37°C.

C: MS1748 (1 x 10^8 cells) + phage pools were mixed directly on NB plates at an moi = 1. Following phenotypic expression, NB plates were replica printed onto NB + Km and incubated at 37°C. Km^r transductants were replica printed onto NB + 2x Ap plates to score for coinheritence of pPC5.

D: MS1748 (5 x 10^9 cells) + phage pools were mixed directly on NB + 2x Ap plates at an moi = 1, and incubated at 37°C. Ap^r transductants were replica printed onto NB + Km plates to score for coinheritence of Tn*phoA*. PSN was scored by patching Ap^rKm^r transductants onto PSN plates.

that only Tn*phoA* bearing plasmids could gro Only one Km^r Ap^r strain was isolated. We reasoned that the double selection might have been to harsh on the cells, so I next tried selecti for either Km^r or Ap^r and then screening the other phenotype. With the Km selection, about 2,500 transductants were isolated, however none of them carried Ap resistance. It is possible that in some of these isolates, Tn*phoA* had transposed into the β-lactamase gene. With the Ap^r selection, a lawn of Ap^r transductants grew and a few of them also carried Km^r. These were screened and found to be PSN^+, which means the Tn*phoA* insertion was on the plasmid, but not in the *putP* gene. The number of these Ap^r Km^r transductants is shown in Table 2, but it should be noted that within a given phage pool, any isolates obtained may be siblings.

It seemed that none of the phage pools tested so far contained plasmids carrying *putP*::Tn*phoA* insertions. This could have been because the original pools contained only 100-200 Km^r colonies per pool. Given the frequencies observed so far for *putP*::Tn*phoA* insertions, it was likely that none of these colonies carried one. Therefore, new phage lysates were prepared on pools of approximately 900 colonies each. The results of transductions with these pools are shown in Table 2. The number of Ap^r Km^r isolates did increase, however none were PSN^-.

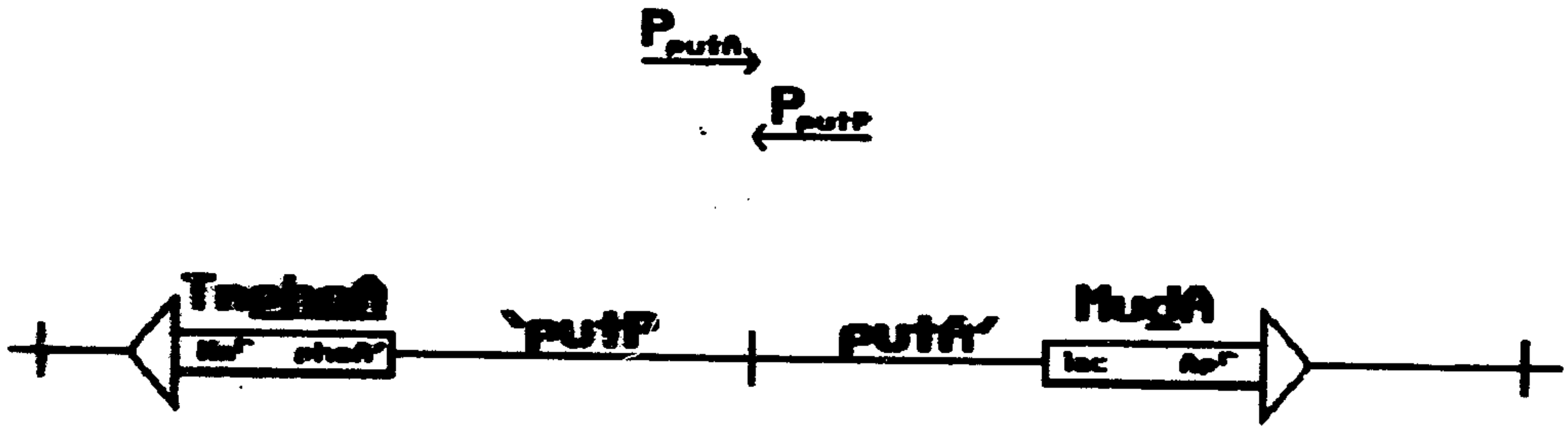
<u>Selecting PhoA+ derivatives of MS1881</u> As previously discussed, chromosomal Tn*phoA* hops into *S. typhimurium* yield about 10^{-3} $PhoA^+$ colonies among Km^r transductants. By performing hundreds of these transductions, several active *putP*::Tn*phoA* insertions could eventually

be isolated. However, it would be useful to find a more efficient alternative to this brute-force method.

So far I have one *putP*::Tn*phoA* insertion, which maps to the ninth deletion interval and has measurable, albeit low, alkaline phosphatase activity. The low activity may be the result of a gene fusion that links Tn*phoA* to a domain of proline permease only partially exposed to the periplasm. Growth of cells on a medium with XP as the sole phosphate source requires expression of high levels of alkaline phosphatase activity. For instance if MS1881 was plated on this medium, a spontaneous deletion within *putP* that created a fusion of the Tn*phoA* to a periplasmic domain of proline permease would result in greater alkaline phosphatase activity. It may also be possible to obtain deletions large enough to create a fusion between the Tn*phoA* and another gene upstream. To localize the deletions to the *putP* gene, a strain (MS1878) was constructed with a Mu*dA* gene fusion in *putA*. The *put* genes are divergently transcribed from a common control region (Figure 5). The *MudA* contains the *lacZ* gene whose expression is driven by the *putA* promoter. By selecting PhoA$^+$ on minimal lactose + XP, the *lacZ* gene must be expressed. Therefore, deletions resulting in PhoA$^+$ cannot extend past the *putA* promoter.

A culture of this new strain, MS1878, was grown to saturation in NB, diluted 1:10 in saline, and 0.1 ml aliquots were plated on lactose +

Figure 5. Strain MS1878 (*putP1204*::Tn*phoA* *putA1020*::Mud*A* *phoN*::*Tn10dTc*) displays a weak PhoA$^+$ phenotype. Selection for growth on Lac + XP medium requires higher level expression of the *phoA* gene fusion. Internal deletions of the *putP* gene can theoretically fuse *phoA* to domains of proline permease that better direct the fusion to the periplasm than the original Tn*phoA* allele. The 5' of the deletion can not extend past the promoter for *putA* since growth on lactose is required.



P putA
P putP
'putP'
putA'
MudA
lac
Ap^r

XP. About 500 blue, PhoA$^+$ mutants were isolated per plate. These isolates seem to have different levels of alkaline phosphatase activity, because different degrees of blueness were observed. These mutations must be mapped to check that they are intragenic, and then sequenced. With so many different isolates, there are probably a whole series of deletions through the first 8 or 9 deletion intervals of *putP*. The logical next step would be to clone the *phoA* gene just downstream of *putP* and perform the same selection. In this way, deletions that hook-up Tn*phoA* to periplasmic regions coded for by sequences within the last two deletion intervals could be isolated.

DISCUSSION

One approach to analyzing the three-dimensional structure of integral membrane proteins is based on the use of gene fusions. These gene fusions encode hybrid proteins whose N-terminus is composed of varying lengths of the protein of interest, and whose carboxy-terminus is composed of an indicator protein. Alkaline phosphatase has been used as an indicator protein because its activity depends of its cellular location. Tn*phoA* serves as a useful tool for creating a series of *phoA* fusions throughout a gene, but has not been used extensively in *S. typhimurium*. In this study, I worked out the techniques for use of Tn*phoA* in *S. typhimurium*, while attempting to characterize the membrane topology of proline permease.

The optimum conditions for chromosomal Tn*phoA* "hops" were determined: 1) A greater number of phage particles carried Tn*phoA* (based on frequency of Km^r) when the donor, MS1621, was grown at 30°C rather than at 37°C. This suggests that the F' may be temperature sensitive for replication. 2) The maximum number of Km^r transductants was seen when an m.o.i. of one was used for "hops". 3) The conditions for use of XP plates were determined. Nonspecific acid phosphatase activity in *S. typhimurium* contributes sufficient background color to obliterate any effect of $PhoA^+$. In order to screen the XP phenotype in *S. typhimurium*, a *phoN*$^-$ or *phoP*$^-$ strain must be used. Also, EGTA must not be added to the indicator plates because it chelates divalent cations required for alkaline phosphatase activity. 4) To screen for the presence of low activity Tn*phoA* fusions on a plate of Km^r colonies, the plate should be stored at 4°C for about a week to identify colonies that have become light blue.

I also worked out the methods for isolating Tn*phoA* insertions into *putP* on a multi-copy plasmid. A transduction was performed which yielded about 1,000 colonies per Km selection plate. These transductants were screened for Tn*phoA* insertion onto the plasmid by preparing a phage lysate of the pooled cells, and moving the plasmids individually into a clean background. The best results were obtained by selecting Ap^r and screening Km^r.

A third method for isolating Tn*phoA* insertions throughout *putP* was devised, which involved selecting for high alkaline phosphatase activity

on a plate containing XP as the sole phosphate source. A culture of cells that contain a low activity fusion was spread on these plates, and hundreds of PhoA$^+$ derivatives were isolated. These isolates need to be mapped to find the location of the fusion and then the DNA sequence of the fusion junction must be determined. Because the frequency of random insertions into *putP* has been low, this technique of selecting PhoA$^+$ with a strain carrying a local Tn*phoA* insertion is promising.

Now that the details for using Tn*phoA* and these techniques have been worked out, it should be possible to rapidly saturate *putP* with Tn*phoA* insertions. Once the DNA sequences of the fusion junctions have been determined, a detailed map of the membrane topology of proline permease will be constructed. The combination of data about the membrane topology, and the location of amino acids at or near the binding site will further our understanding of the molecular mechanism of ion/solute transport.

SUMMARY

A genetic technique for the study of the topology of integral membrane proteins has been devised using Tn*phoA* to construct a random series of fusions within a gene of interest. This study involved the optimization of techniques for use of Tn*phoA* in *S. typhimurium*. Protocols for obtaining chromosomal and plasmid insertions were devised, as well as a method for selecting PhoA$^+$ in a low activity fusion strain. Several chromosomal *putP*::Tn*phoA* insertions were isolated which may be useful for defining the topology of proline permease.

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