

Length and Location  
of the Dipeptide Permease in E.coli

Submitted in Partial Fulfillment of the  
Requirements for Graduation with Honors to the  
Department of Biology at Carroll College,  
Helena, Montana

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February 1, 1990

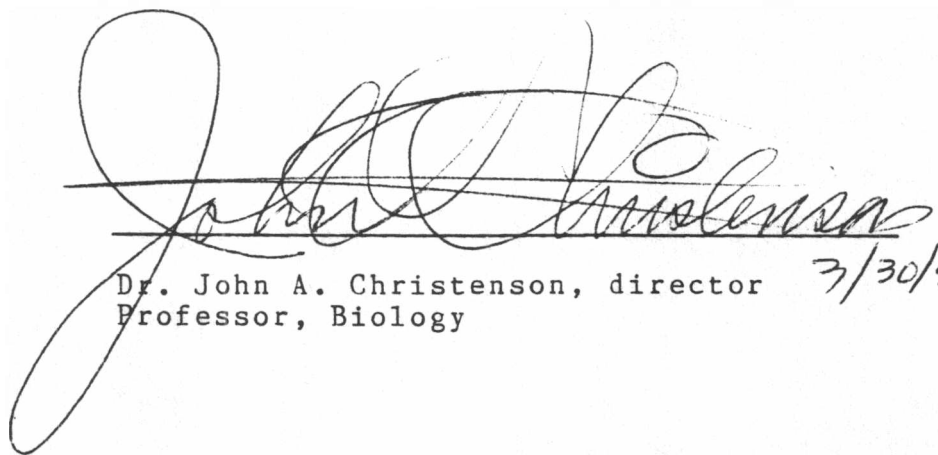
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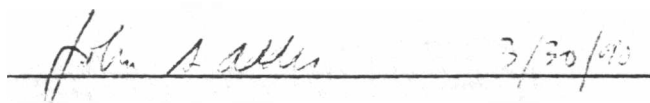
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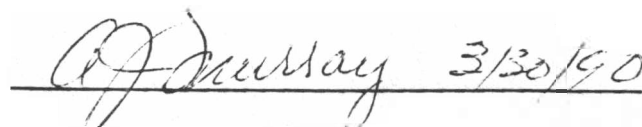
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## ABSTRACT

The gene dpp codes for the dipeptide permease, a transport system in E.coli about which little is known. An attempt was made to determine the length and location of dpp and its protein products in several isolates of the bacterium in order to determine its mechanism of action. A mini-Tn10, which is a transposon, was inserted within the DNA in  $\lambda$  dpp. The location of a mini-Tn10 was found in the 1.7 kb fragment in isolate 1105 and helps determine the extent of dpp. The other isolates, 1108, 1107, and 1114 gave no clear indication where the mini-Tn10 was located. A survival curve of S159 ( $\lambda$  ind-) helped elucidate the dpp protein products by showing which U.V. dose stopped bacterial protein synthesis while allowing substantial phage-encoded protein synthesis.

## INTRODUCTION AND LITERATURE REVIEW

### Escherichia coli (E.coli)

E.coli is a gram-negative bacterium found in the large intestine of vertebrate hosts and in open water systems from fecal contamination.

E.coli is used widely in microbiological research for several reasons. E.coli is easily available not highly virulent, and grows readily on defined media. This wide use led to some of the major triumphs in biology such as the elucidation of most biosynthetic pathways, the refinement of the concept of the gene, the solution of the genetic code, the discovery of molecular mechanisms of gene regulation and the molecular portrayal of viral morphogenesis (Friefelder, 1987).

More is known about E.coli than about any other form of cellular life. Examples of some of this knowledge are; about 1/3 of the gene products of E.coli have been studied in some biological detail, and their genes have been identified; approximately 10% of the E.coli genome has been sequenced; and map positions have been determined for approximately half of its genes (Schaechter and Neidhardt, 1987).

## Chemotaxis

Study of the mechanisms of motility and taxis in E. coli provide indications of how these systems function in complex eukaryotic cells. Motility and taxis play an important role in the survival of E.coli. Many helical flagellar filaments, each driven by a rotary motor at the cell surface, allow E.coli cells to swim (Macnab, 1987).

Four inner-membrane proteins termed methyl-accepting chemotaxis proteins (MCPs) are responsible for many of the chemotactic responses exhibited by motile E.coli. These proteins function in the sensory transduction systems of bacteria. These four proteins span the cytoplasmic membrane and are therefore able to interact in their periplasmic domain either with specific substrates or the substrate-loaded primary chemoreceptors (periplasmic binding-proteins: GBP, DBP, RBP, MBP). E.coli can sense changes in levels of chemoeffectors due to an adaptation mechanism that continuously allows the signal transducers to be adjusted so that no sensory signals are generated in a homogenous environment. After a stimulus is received, the adaptation process reestablishes the pattern of swimming to the state before the stimulus occurred. Change in the chemoeffector concentration causes signals initiated in the receptor domain of the MCP to be transferred across the inner membrane to the internal signaling domain of the MCP that indirectly control the flagellar motor. Four

cytoplasmic proteins are involved in the transmission of sensory signals from the signal transducer to the flagellar motors (Dahl, 1989; Fig. 1).

The four transducer proteins are Tsr (taxis to serine and away from some repellents), Tar (taxis to aspartate and away from some repellents), Trg (taxis to ribose and galactose), and the Tap (taxis-associated protein). Tsr is the receptor for the attractant L-serine and related amino acids and is responsible for chemotaxis away from such repellents as leucine, indole, and weak acids. It is also involved in thermotaxis. Tar is the receptor for the L-aspartate and related amino and dicarboxylic acids. Tar also interacts with the periplasmic maltose-binding protein to mediate taxis toward the attractant maltose and away from the repellents  $\text{Co}^{2+}$  and  $\text{Ni}^{2+}$ . Trg interacts with the periplasmic ribose and galactose binding proteins to accomplish chemotaxis toward robose and galactose. Tap mediates taxis toward dipeptides by interacting with the periplasmic dipeptide-binding protein. The genes encoding these proteins are designated tsr, tar, trg, and tap. They have been sequenced (Dahl, 1989; Fig. 1).

The four cytoplasmic proteins involved in the transmission of sensory signals from the signal transducer to the flagellar motors are CheA, CheB, CheY and CheW. CheA, CheB, and CheY are regulated by phosphorylation (Macnab, 1987). CheA integrates sensory data from the chemorecep-

tors and transmits it to CheY and CheB. It is thought that CheA phosphorylates CheY and CheB and converts them to the active form. The phosphorylated form of CheY interacts with the switch components of the flagellar motor causing (CW) clockwise rotation. CheZ probably inhibits CheY perhaps by removal of the phosphate from CheY, causing the flagella to rotate counterclockwise (CCW). CheW seems to link the transducers to CheA (Dahl, 1989; Macnab, 1987).

For signals processed through the MCPs (tsr, trg, tar, tap) adaptation is dependent on methylation catalyzed by the CheR methyl transferase. CheB, in the phosphorylated form, functions in the sensory adaptation by catalyzing the demethylation of the four signal transducers. Adaptation to a positive stimulus occurs via increased methylation of specific glutamyl residues in the cytoplasmic domain of the signal transducer proteins caused by decreased CheB activity; suppressing tumbling. Adaptation to a repellent stimulus occurs via more rapid removal of the methyl group generating methanol and enhancing tumbling (Macnab, 1987).

#### Dipeptide permease

Substances enter the E.coli cell by active transport systems. One of these transport systems is the dipeptide permease (dpp), about which little is known. In E.coli

peptides serve as a sole source of carbon or nitrogen and can supplement any auxotrophic requirements for amino acids (Macnab, 1987).

The peptide chemoreceptor is a periplasmic protein, the dipeptide binding protein (DBP), which is coded for by one of the dpp genes. Transport of dipeptides and chemotaxis due to dipeptides are a separate phenomenon, with DBP as the only common element (Manson, 1989).

DBP has two functions. Dipeptides are recognized by the DBP protein and conveyed to a poorly characterized uptake system. The peptide substrate probably binds to a cleft in the DBP and the cleft then closes around the substrate. It is not known how the substrate is ultimately transported. One of the models proposes that the substrate is passed through a channel formed by the membrane proteins (Macnab, 1987).

DBP also acts as a chemoreceptor via its interaction with the transducer protein Tap. Tap sends signals which are processed by cytoplasmic components of the chemotaxis apparatus (Che) and transmitted to the flagella (Manson, 1986; Fig. 1).

The structure of the opp (oligopeptide permease) operon is being used as a possible model for the structure of dpp. The first gene in the opp operon encodes the oligopeptide binding protein gene. By analogy, the first gene in the dpp operon is the dipeptide-binding protein

gene (Hogarth and Higgins, 1983; Higgins et al., 1983).

Presently research is being done at Texas A & M (Manson, pers. comm.) to determine the length, location, protein products, and the sequence of the dpp operon. The dpp locus is comprised of genes encoding the proteins of the dipeptide premease, including DBP. The genes may be organized into one or more operons. The dpp locus was cloned in  $\lambda$  SE6 on a 14 kb chromosomal DNA fragment, of which 5,000-8,000bp (5-8 kb) is dpp.

One aid used to locate the dpp was the transposon mini-Tn10. A transposon is a fragment of DNA that contains only a few genes (Freifelder, 1987). The  $\Delta 16 \Delta 17$  mini-Tn10 contains only one functional gene which codes for tetracycline resistance tet<sup>r</sup> (Fig. 2). It is only 3 kb in size (Way et al., 1984).

Cells infected with phage carrying the mini-Tn10 can transduce to tet<sup>r</sup>. Transposons can usually jump from one spot on the bacterial chromosome to another due to the enzyme transposase. Mini-Tn10 does not code for the enzyme transposase, however, so once inserted into a chromosome it is permanent (Freifelder, 1987).

Mini-Tn10 was originally carried on a  $\lambda$  phage. E.coli cells containing a plasmid that marks Tn10 transposase were infected with the phage. Due to the transposase the mini-Tn10 transposed into the E.coli chromosome. By phage PI transduction the mini-Tn10 was recom-

bined into another E.coli cell chromosome. No plasmid was present, thus preventing the mini-Tn10 from transposing further and making it stable (Manson, pers. comm.).

A functional  $\lambda$  CI repressor gene is provided by a lysogenic  $\lambda$  phage in the E.coli bacterial strain S159 (ind-). It codes for the repressor protein CI (ind-). The CI protein in S159 ( $\lambda$  ind-) is non-inducible, which means it cannot be proteolytically cleaved in the presence of the RecA protein. The ind- repressor is only moderately temperature-sensitive and totally resistant to U.V. light induction. The CI repressor controls the  $\lambda$  phage lifecycle. The  $\lambda$  phage is normally lysogenic; meaning it will integrate into the bacterial chromosome (Freifelder, 1987). The CI repressor protein maintains the  $\lambda$  phage in the lysogenic cycle at 30°C but the phage enters the lytic cycle when the CI repressor is destroyed (Manson, pers. comm.). In the lytic cycle a phage converts an infected cell into a phage factory and produces many phage progeny. The phage then burst out of the cell (Freifelder, 1987). When the temperature is raised to 37°C the repressor protein is only moderately stable and the phage will excise from the chromosomes of a relatively few cells (Manson, pers. comm.).

BamHI enzyme is a restriction endonuclease which cuts the DNA internally. BamHI cuts at a specific DNA sequence, in this case GGATCC. The DNA fragment in a  $\lambda$ dpp

that contains the dpp locus can be divided into three smaller fragments by cutting at the four BamHI sites in the phage DNA (Manson, pers. comm.; Fig. 3).

The objective of the current study was to localize the four mini-Tn10's within the insert DNA in a  $\lambda$  dpp and to identify dpp gene products with  $^{35}\text{S}$ -methionine labeling. Ligation of the mini-Tn10's will help to determine the extent of dpp, which will in turn help in elucidating the DNA sequence of dpp. Elucidation of the protein products will better help to understand the mechanism of dpp.

## MATERIALS AND METHODS

### Abbreviations

LB	Luria broth
kb	kilobasepairs
MMA	minimal medium A
OD	optical density
TAE	tris acetate buffer
TBE	tris-borate buffer
TE	tris-EDTA
SDS	sodium dodecyl sulfate
TB	tryptone broth
Tris	tris-(hydroxymethyl)-aminomethane
EDTA	ethylene-diamino-tetraacetic acid
SM	$\lambda$ -dil-(composed of NaCl, MgSO <sub>4</sub> -7H <sub>2</sub> O and Tris-Cl)
PEG	polyethylene glycol
HMP	high melting point agarose
tet	tetracycline
DBP	dipeptide binding protein
Tap	taxis to dipeptides
MUV	media for ultraviolet irradiation
$\lambda$ <u>dpp</u>	lambda <u>dpp</u> phage
wild type $\lambda$ <u>dpp</u>	$\lambda$ phage without DNA insert

### Bacterial Strains

All strains used in this work are derivatives of Escherichia coli. These strains are MM1105, MM1107, MM1108, MM1114, MM500, MM634, S159 ( $\lambda$  ind-), and S159(ind-) $\lambda$ pCI<sub>357</sub>. The bacteriophage used was lambda dpp ( $\lambda$  dpp).  $\lambda$  dpp was derived from the lambda phage cloning vector  $\lambda$  SE6 containing a 14 kb insert including the entire dpp locus.

Cells were cultured in Luria broth (LB), tryptone broth (TB), and minimal salts medium. The minimal salts medium was used for <sup>35</sup>S-methionine labeling of protein. The minimal salts medium was supplemented with methionine to a concentration of 10 ug/ml, thiamine to a concentration of 1 ug, glycerol to 1%, and maltose to 0.5% (Maniatis et al, 1982). Methionine was placed in the solution to allow the cells to utilize it while repressing the ability to synthesize their own methionine.

Solid media included 1.5% agar supplemented with LB, TB, LB with tetracycline (LB tet), and minimal medium A (MMA). The MMA agar was supplemented with methionine, threonine, and histidine to a final concentration of 20

ug/ml, prolyl-leucine to a final concentration of 10 ug/ml, 5 mM MgSO<sub>4</sub>, thiamine to a final concentration of 1 ug/ml and glucose to 0.2% (Maniatis et al., 1982).

Production of  $\lambda$  dpp lysates on strains MM1105, MM1107, MM1108, and MM1114

The E.coli strains MM1105, MM1107, MM1108, and MM1114 were each inoculated into 5 ml of LB and grown overnight at 30° C. 0.1 ul of each overnight culture was transferred into 5 ml of LB and placed in a shaking water bath at 37° C for 1 hr or until an O.D. of 0.2 was reached.  $\lambda$  dpp phage were added to a concentration of 10 phage/ml, or 1/10 as many phage as cells. Inoculation continued in a shaking water bath at 37° C for 5 to 8 hr or until lysis occurred. These lysates were renamed 1105, 1107, 1108 and 1114 to correspond to the strain of bacteria that they came from.

0.5 ml of overnight cultures of S159(ind-) and S159 (ind-) $\lambda$ pCi<sub>857</sub> were mixed with 10 ul or 100 ul of each lysate. The mixture was left at room temperature for 20 min and was then spread on tetracycline plates. For each test tube 1/2 of the volume was spread on each of two plates. These plates were incubated overnight at 30° C.

The colonies growing on the tetracycline plates were restreaked on these plates to assure that only tetracycline resistant transductants were selected.

A single colony was taken from these tetracycline plates for each of the four strains MM1105, MM1107, MM1108, and MM1114. They were inoculated in 2x 2 ml of LB and grown in shaking water bath at 37°C or 42°C for 5 to 8 hr, until lysis occurs. The elevated temperature partially inactivated the repressor protein, allowing the ind- phage and  $\lambda$  dpp phage to enter the lytic cycle in some of the cells. Chloroform was then added to kill the remaining bacterial cells; The lysates were placed over 0.1 ml of chloroform for 15 min and then centrifuged for 10 min at 3000 rpm. The supernatant containing the phage was removed and stored over chloroform. The supernatant contained both the ind- phage and the  $\lambda$  dpp phage.

A 0.1 ml sample of each of the lysates was then combined with 0.1 ml of MM500 cells in 2.5 ml TB top agar containing 0.5 ml LB and the mixture was poured on LB agar plates. The plates were incubated overnight at 37° C. Plaques originating from a single phage grew up in the lawn of MM500.

For each of the four isolates about 50 plaques were picked with sterile toothpicks and stabbed into tetracycline plates spread with 10 cells of strain S159 ( $\lambda$  ind-) and once into soft agar overlay of strain MM500 on LB plates. The phage that formed plaques on the MM500 and transduced S159 (ind-) to Tet were  $\lambda$  dpp phage that had picked up the mini-Tn10.

Sterile Pasteur pipettes were used to remove three plugs of agar from plaques of isolates that had picked up the mini-Tn10. These plugs were placed in 0.5 ml of SM medium at room temperature, vortexed and left for 1 hr to allow the phage to resuspend. An overnight culture of strain MM634 was grown at 30°C, in TB, and 0.1 ml was added to 5 ml of LB and incubated 1 hr at 37° C. The resuspended phage were added to the cells and the mixture incubated at 37°C in a shaking water bath for 5 to 8 hr or until lysis occurred. At this time, 0.2 ml of chloroform were added to the cells, vortexed, and the mixture left standing for 15 min. They were then centrifuged for 10 min at 300 rpm. The supernatants, which contained the phage, were removed and stored over chloroform. These lysates were then titered using strain MM500 (Tables 1 and 2).

The isolates were spotted on tetracycline plates spread with S159 ( $\Delta$  ind-) to check for the ability to transduce to Tet<sup>r</sup>.

MM1114 was inoculated from a single colony and grown overnight in LB. The cells were then washed twice by centrifugation in 10 mM MgSO<sub>4</sub> and resuspended in 10 mM MgSO<sub>4</sub>. MMA plates containing the dipeptide L-prolyl-L-leucine were spread with the MM1114 strain containing /g+4-lac5 phage. This strain is dpp-, pro-, and leu-. It can obtain proline and leucine from the dipeptide only if

dpp can transport it. The 1105, 1107, 1108 and 1114 were then spotted on the MMA plates spread with MM1114. These were then placed overnight in 30°C. This test was done to see if the phage isolates contained dpp. The plates spotted with the 1105, 1107 and 1108 should show growth if dpp is present while the plates spotted with 1114 should not because the MM1114 contains  $\lambda$  phage already in the same place where the 1114 isolate  $\lambda$  phage would insert.

#### Large Scale lysates

Forty ml of TB containing 10mM MgSO<sub>4</sub> were added to a sterile 125-ml flask. This was inoculated with strain MM634 and grown shaking overnight at 30°C. The next day, 20 ml of fresh overnight cells were mixed with 5x10<sup>8</sup> phage in a 2-liter flask. This was incubated for 5 min at room temperature for phage absorption. One liter of LB containing 10mM MgSO<sub>4</sub> was added. The lysates were shaken vigorously at 37°C for 5 to 8 hr until lysis occurred. NaCl was added to 0.5 M (2.9 g/100ml) and 1 ml of chloroform was added. Shaking was continued for 5 min. Each lysate was then centrifuged at 6000 g for 10 min to remove cell debris. The supernatant was decanted and stored over chloroform in the refrigerator. The lysates were titered on strain MM500. To provide sufficient phage for DNA preparation and for  $\lambda$ -directed protein labeling experiments, the phage titer must be at least 10<sup>-10</sup> ml (Table 3).

### Purification of lambda dpp phage

This procedure was utilized to obtain a concentrated phage suspension to infect S159 (ind-) for the <sup>35</sup>S-methionine labeling. Solid polyethylene glycol (PEG) was added to a final concentration of 10% w/v, dissolved by slow stirring on a magnetic stirrer at room temperature, and the mixture placed in an ice bath for 1 hr. After centrifugation at 11,000g for 10 min at 4°C, the supernate was discarded and the centrifuge bottles allowed to stand in an inverted, tilted position for 5 min to allow all of the solution to leave the pellet. The bacteriophage pellet was then gently resuspended in SM buffer (8 ml for each 500 ml of supernatant). An equal volume of chloroform was added to the bacteriophage suspension and the pellet gently vortexed for 30 sec. The organic and aqueous phases were then separated by centrifugation at 1600 g for 15 min at 4°C. The aqueous phase containing the bacteriophage was recovered. The volume of the supernate was then measured and 0.5 g/ml of solid cesium chloride was added. Once the cesium chloride dissolved, the bacteriophage suspension was layered onto cesium chloride step gradients that were performed in Beckman SW41 cellulose nitrate centrifuge tubes.

Cesium chloride solutions (100 ml) for step gradients prepared in SM

Density ( $\rho$ )	Cscl (g)	SM (ml)	Refractive index (n)
1.45	60	85	1.3768
1.50	67	82	1.3815
1.70	95	75	1.3990

A mark was made at the interface between the  $\rho$  1.50 layer and the  $\rho$  1.45 layer. The tubes were then centrifuged in the SW41 rotor at 22,000 rpm for 2 hr. A piece of cellophane tape was placed on the outside of the centrifuge tube spanning the  $\rho$  1.45-  $\rho$  1.50 boundary. A 21-gauge needle was used to puncture the tube through the tape and collect the band of bacteriophage particles. Enough cesium chloride solution (1.5 g/ml in SM) was added to the bacteriophage suspension to fill a cellulose nitrate tube that fits either a Type-50Ti rotor or an SW50.1 rotor. The suspension was then centrifuged at 35,000 rpm for 24 hr at 4°C. The band of bacteriophage appeared dark blue or black. The band of bacteriophage particles was collected as before and stored at 4°C in cesium chloride in a tightly capped tube (Maniatis et al., 1982).

#### $\Lambda$ DNA Isolation

Polyethylene glycol powder (PEG) was added to each of the lysates to 10% w/v. The PEG was dissolved completely

by shaking. The lysates with dissolved PEG were then placed on ice for 60 min. The pellet was collected by centrifugation at 600 g for 10 min at 4°C and then resuspended in TM buffer. Five to 10 ml of TM were used per liter of lysate. The PEG-phage suspension was extracted with an equal volume of chloroform in a screw-capped container by gently inverting the tube for 1 min at room temperature. This mixture was then centrifuged at 2000 g for 10 min. The aqueous upper layer containing the phage was pipetted off and placed in a sterile tube. A step gradient of glycerol was made in an ultracentrifuge tube and the aqueous layer containing phage was added on top of the gradient. The ultracentrifuge tubes were filled to the top with TM buffer and centrifuged using the SW4Ti rotor at 35,000 rpm for 60 min at 4°C. The supernatant was then decanted, and the pellet was resuspended in 1 ml of TM buffer. RNase and DNase were added to a final concentration of 10 ug/ml and 1 ug/ml to digest cellular RNA and DNA. For 30 min at 37°C 0.2 ml volumes of STEP buffer were added with thorough mixing and the mixture was placed in 50°C water bath for 15 min before being transferred to a sterile polypropylene tube. An equal volume of Tris-saturated phenol was added and the mixture was centrifuged at 1000 g for 10 min. After centrifugation, the phenol phase was removed and the aqueous phase was resuspended in 1/2 phenol and 1/2 24 parts of chloroform:1

part isoamyl alcohol. After centrifugation at 1000 g for 10 min the top aqueous layer was removed and transferred to a sterile tube. Two volumes of 95% ethanol were added to precipitate the DNA during 30 min incubation at 37 °C, and the DNA was pelleted by centrifugation for 5 min at 6000 g. The supernatant was removed and 70% ethanol was added. The 70% ethanol and the DNA were centrifuged again at 6000 g for 5 min and the supernatant was removed. The DNA pellet was dried and resuspended in 100 ul of TE buffer (Maniatis et al., 1982).

#### Visualization of DNA

0.25 gram of (HMP) high melting point agarose were added to 25 ml of either TAE or TBE buffer. This mixture was heated and stirred until clear and poured into a mold. Slots for loading DNA were made by pieces of plastic the size of the slots placed in the gel. The gel was allowed to solidify for 30 min (Maniatis et al., 1982). 10 ml of DNA solution and 2 ml of bromophenol blue marker were loaded and run for about 1/2 hr at 1000 to see if any DNA had been isolated from the DNA isolation procedure. This was stained with ethidium bromide and viewed under the ultraviolet light. If bands of DNA were present a BamHI digestion was run (Conway, pers. comm.).

### BamHI digestion

Five ul of phage DNA solution were mixed with 12 ul of sterile H<sub>2</sub>O, 2 ul BamHI buffer and 1 ul of BamHi enzyme and incubated for 1 hr at 37° C. The DNA was then mixed with 2 ul of bromo-phenol and then incubated at 65° C for 5 min. The DNA was loaded in the gel and run at 100 mv for 1/2 hr. As a control, DNA from wild-type  $\lambda$  dpp digested with BamHi was also loaded on the gel, along with a  $\lambda$  HindIII marker. The  $\lambda$  HindIII marker is a  $\lambda$  phage digested with HindIII. The size of the bands produced on a gel when  $\lambda$  HindIII is run have been determined so  $\lambda$  HindIII can be used to determine the approximate size of bands run next to it on a gel. The gel was then stained with ethidium bromide and viewed under ultraviolet light. The bands were compared to the wild type  $\lambda$  dpp bands to determine if any of the bands from the phage DNA had picked up the mini-Tn10. The band would appear 3 kb larger if it had picked up the mini-Tn10. The heavier the fragment the higher on the gel it will appear. (Conway, pers. comm.).

### EcoRI digestion

This digestion was performed like the BamHI digestion except that the EcoRI enzyme was used instead of the BamHi enzyme.

S159 ( $\lambda$  ind-) was inoculated in 10 ml of minimal salts methionine medium and grown overnight at 30° C.

20ug/ml of methionine was used. This culture was added to 375 ml of the same medium and grown at 37°C to an OD of 0.2. The cells were then centrifuged and the supernatant removed. The pellet was resuspended and washed in 15 ml of 10 uM MgCl<sub>2</sub> (Callahan and Parkinson, 1984). The 15 ml of washed cells were irradiated with U.V. light from a germicidal lamp at a distance of 35 cm for time intervals of 5, 10, 15, 20, 30, 45, 60 and 120 sec. The petri dish was rotated during irradiation. Samples removed at each time interval, plus unirradiated control cells, were serially diluted to 10<sup>-8</sup> and spotted on LB plates. The plates were incubated overnight at 30°C. Colonies were counted at suitable dilutions and a survival curve plotted (Manson, pers. comm.).

## RESULTS

The isolates 1105, 1107, 1108, and 1114 all transduced S159 (ind-) to tet which showed they contained the mini-Tn10 transposon due to the tetracycline resistance gene carried on mini-Tn10.

BamHI digests were made from isolates 1105, 1107, 1108 and 1114. The first isolates of tet  $\lambda$  dpp phage isolated after growth on dpp (mini-Tn10 strains MM1105, MM1107, MM1108, and MM1114) did not contain a digestion pattern similar to wild-type  $\lambda$  dpp (Fig. 4).

The first set of isolates 1105, 1107, 1108 and 1114 all grew on prolyl-leucine MMA plates. Comparison of growth of the isolates on MMA plates showed that they grew about the same amount as when titered (Table 2). The last set of lysates grew once on every prolyl-leucine plate for every 100 that transduced S159 (ind-) to Tet .

Comparison of EcoRI and BamHI digests together showed that the 1.7 kb fragment in isolate 1105 contained the mini-Tn10. The enzyme digests of the isolates 1107, 1108, and 1114 gave no indication at all where the mini-Tn10 is located (Figs. 4,5,6).

<u>Seconds</u>	<u>Concentrations</u>
0	$1 \times 10^{-6}$
5	$1 \times 10^{-6}$
10	$7 \times 10^{-5}$
15	$2 \times 10^{-5}$
20	$2 \times 10^{-4}$
30	$3 \times 10^{-3}$
45	none
60	$5 \times 10^{-2}$
120	$5 \times 10^{-1}$

The survival curve of the above data of irradiated E.coli cells was plotted with the times on the x-axis and the concentrations on the y-axis (Fig. 7). This curve showed that about 8 sec of radiation gave 1 log unit of killing. Eight was the slope of the curve. This indicated that times 8 sec apart should be chosen to irradiate the cells in a trial experiment in which the cells are infected with phage to determine at what U.V. dose bacterial protein synthesis is eliminated and substantial phage encoded protein synthesis is still observed.

## DISCUSSION

Four strains of E.coli, MM1105, MM1107, MM1108, and MM1114, each containing one mini-Tn10 located in the dpp locus were isolated. Each mini-Tn10 is probably inserted at a different site in each gene coding for their dpp loci because they were isolated independently. In strains MM1105 and MM1114 the mini-Tn10 may be in the gene coding for DBP because in these strains no DBP is produced.

To transfer the mini-Tn10 onto  $\lambda$  dpp the phage was used to make lysates on strains MM1105, MM1107, MM1108, and MM1114. By reciprocal homologous recombination some percentage of the  $\lambda$  dpp particles should pick up the mini-Tn10's (Fig. 8). Recombinant phage were identified by infecting S159 (ind-) with the lysate and then plating on tetracycline agar and incubating overnight at 30° C. Colonies were inoculated into nutrient liquid medium and grown at 37°C to 42°C to allow the few lysogens that are induced to produce phage to excise. These phage were used to form plaques on a lawn of non-lysogenic,  $\lambda$  sensitive MM500 E.coli strain. These plaques were transferred with toothpicks onto a lawn of MM500 and onto S159 (ind-) spread on tetracycline plates. The phage that transduced

S159(ind-) to tet was used to make lysates.

The DNA from these phage were extracted, purified, and digested with the BamHI enzyme. The fragment that is 3 kb larger than the wild type  $\Lambda$  dpp fragment after digestion should have picked up the mini-Tn10, demonstrating that some of the dpp locus is located on that fragment.

The first set of isolates 1105, 1107, 1108 and 1114 all grew on prolyl-leucine MMA plates. Growth on MMA plates was the same as growth on titered plates. This led us to believe that the mini-Tn10 was being transferred to the isolates due to complementation instead of reciprocal double recombination as was originally hypothesized. This explained how the mini-Tn10 could be transferred by means other than reciprocal double recombination (Table 1).

The last set of lysates isolated grew once on every prolyl-leucine plate for every 100 that transduced S159(ind-) to Tet. This lower frequency of growth on prolyl-leucine gave a much better indication that reciprocal double recombination was really occurring because reciprocal double recombination would have a low frequency of growth (Table 2).

The first set of isolates, 1105, 1107, 1108, and 1114 after a BamHI digest did not show digestion patterns similar to the wild-type  $\Lambda$  dpp.

The BamHI digest of  $\Lambda$  dpp yields five fragments because the phage has four BamHI sites. Two fragments are

the arms of the  $\lambda$  phage while the other three bands of 6.5, 1.7, and 5.6 kb contain the dpp spanning them somewhere. The position and extent of the dpp genes within the insert is unknown.

The last set of isolates upon digestion with the BamHI enzyme and the EcoRI enzyme had band patterns similar to  $\lambda$  dpp (Figs. 5,6). It was hard to tell where the mini-Tn10 was located from just looking at the BamHI digestion however, because EcoRI has a restriction site in the middle of the mini-Tn10, comparison of both digestions together showed that the 1.7 kb fragment in isolate 1105 contained the mini-Tn10. The actual evidence from the gels indicating this was that the 1.7 kb fragment in the 1105 BamHI digestion was not obtained and there should have been only one band. It definitely did not contain the mini-Tn10 in the 1.7 kb fragment but because its band pattern looked so similar to the wild-type  $\lambda$  dpp it may contain the mini-Tn10 in the 6.5 or the 5.6 kb fragment (Fig. 5) The enzyme digests of the isolates 1107 and 1114 give no indication at all of where the mini-Tn10 is located (Fig. 6).

A survival curve of the S159 (ind-) strain was done to determine the time course of cell death under ultraviolet light irradiation. The S159 ( $\lambda$  ind-) strain was used because it produces the  $\lambda$  CI repressor that only allows proteins coded by the chromosomal insert of  $\lambda$  dpp

to be produced after infection due to the repression of the  $\lambda$ -phage protein synthesis. S159 ( $\lambda$  ind-) was also used because it does not contain the gene that codes for recA protein. It is ura- so it cannot execute post-replication repair. If S159 ( $\lambda$  ind-) was ura+ it would enable the cells to repair themselves after U.V. irradiation (Manson, pers. comm.).

The proteins coded for by the dpp genes can be identified by  $^{35}\text{S}$ -methionine labeling. The first step was to determine a survival curve for S159(ind-). A survival curve is used to determine the death rate of S159(ind-) strain after ultraviolet light irradiation (Smith and Parkinson 1980). The dose of U.V. light must be large enough to destroy the ability of DNA to serve as a template for mRNA synthesis, and thus protein synthesis, which kills the cells. However, RNA polymerase, ribosomes, and tRNA of the cell must still function in transcription and translation of genes introduced on  $\lambda$  dpp, or the  $\lambda$  dpp mini-Tn10's, so that labeling of phage-encoded dpp proteins with  $^{35}\text{S}$ -methionine can be observed.

The survival curve must be done in the dark to prevent DNA repair by photoreactivation. The U.V. light causes the formation of thymine dimers in E.coli cells. These dimers cause the cell to die because a thymine dimer cannot act as a template for DNA or RNA polymerase. If the cells are exposed to light certain enzymes that act

only in the presence of light break covalent linkages between the thymine bases causing photoreactivation.

Suitably irradiated S159(ind- cells are infected with  $\lambda_{dLL}$  or  $\lambda_{dpp}$  mini-Tn10 phage (Silverman et al, 1976). After allowing 15 min for previously synthesized cellular mRNA to decay, S-methionine is added to label phage encoded proteins. Since the CI repressor present in these cells prevents expression of  $\lambda$ -specific genes, the majority of the labeled proteins will be specified by the dpp genes or other neighboring bacterial genes present on the bacterial DNA carried by  $\lambda_{dpp}$ . As a control, uninfected cells are also labeled (Fig. 9).

Labeled proteins can be separated on SDS-polyacrylamide gels. The protein bands from the labeled cells will then be compared with the bands of protein from the labeled cells infected with phage. Some dpp proteins will be eliminated by the mini-Tn10's because the mini-Tn10, upon insertion, disrupts the gene it enters allowing no protein synthesis.  $\lambda_{dpp}$  infected cells will show all the protein products from the inserted segment. Survival  $\lambda_{dLL}$  mini-Tn10 infected cells will show no protein products on certain parts of the gel due to the mini-Tn10. By comparing  $\lambda_{dLL}$ -infected cells and survival  $\lambda_{dLL}$  mini-Tn10-infected cells the dpp protein of the four different inserts can be determined by the absence of protein products from the mini-Tn10's (Fig. 10).

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Since 8 sec gives 1 log of killing, time intervals 8 sec apart should be chosen to irradiate the cells and the cells infected with phages because it gives the proper amount of killing. It should irradiate the cells long enough to destroy the DNA so that it cannot be used as a template but still leave functional organelles intact so that the cells can produce the dpp proteins. The labeling experiments will eventually lead to the elucidation of the dpp proteins.

Once the dpp proteins have been elucidated more knowledge about dpp and how it functions will be available. Right now the 1.7 kb fragment is being sequenced to determine the arrangement of the DNA amino acids in dpp. Understanding the dipeptide permease will aid in understanding more about bacterial chemotaxis and may eventually lead to a greater understanding of processes in cells.

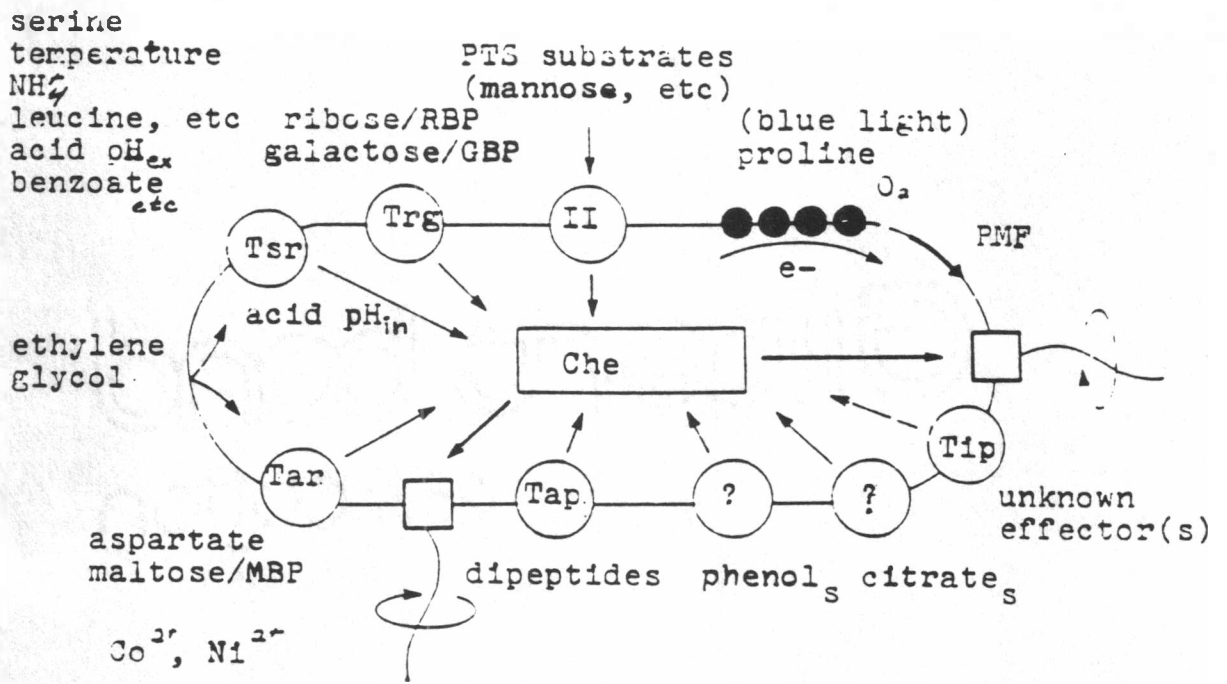


Fig. 1. Sensory stimuli for a cell of *E. coli*. The information is relayed from the transducers to the chemotaxis apparatus (Che) and transmitted to the flagella, where the sense of rotation is modulated to cause migration of the cell to more favorable environments (Macnab, 1987).

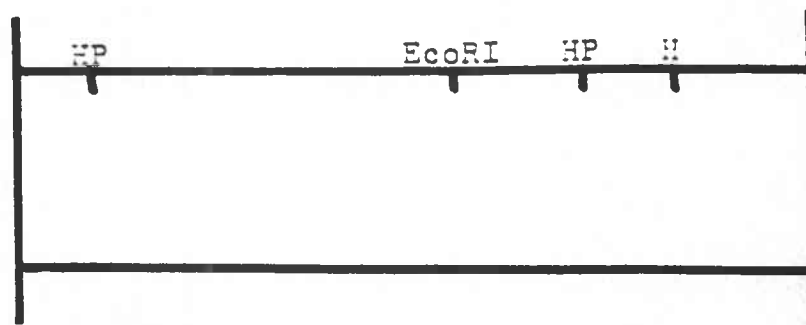


Fig. 2. Restriction sites in the  $\Delta 16 \Delta 17$  mini-Tn10 transposon.

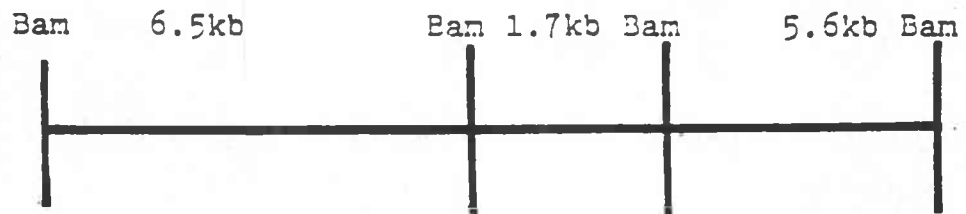


Fig. 3. The DNA insert containing the gene dpp. The restriction sites where the BamHI enzyme cuts are shown along with the three fragments (6.5kb, 5.6kb, 1.7kb) that result due to the Bam digest.

Isolates of E.coli

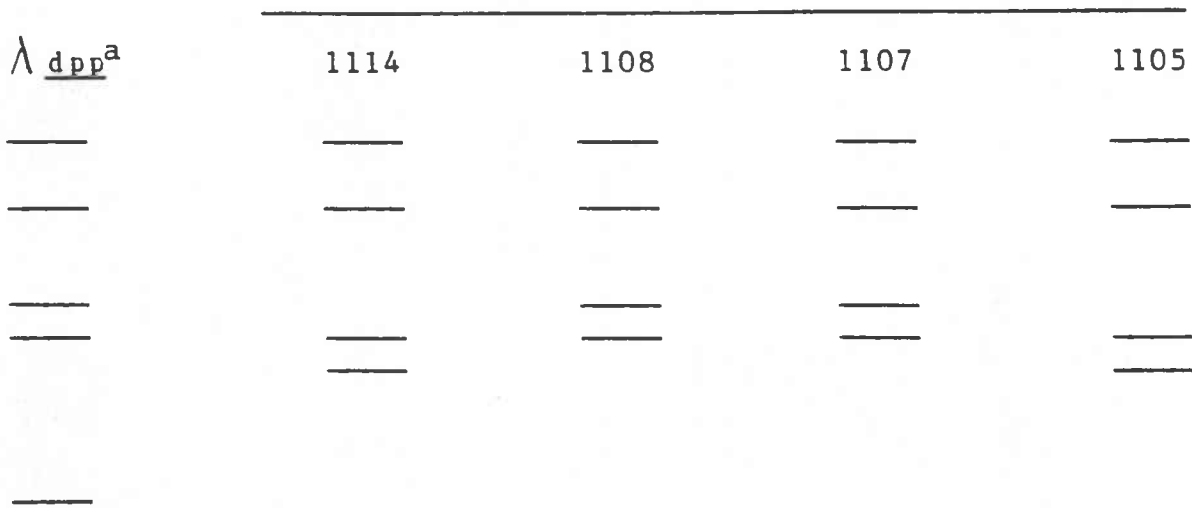


Fig. 4. DNA bands from gel separation of first BamHI digest. The 1.7 kb fragment is missing in 1114, 1108, 1107, and 1105.

a.  $\Lambda$  phage with dpp insert

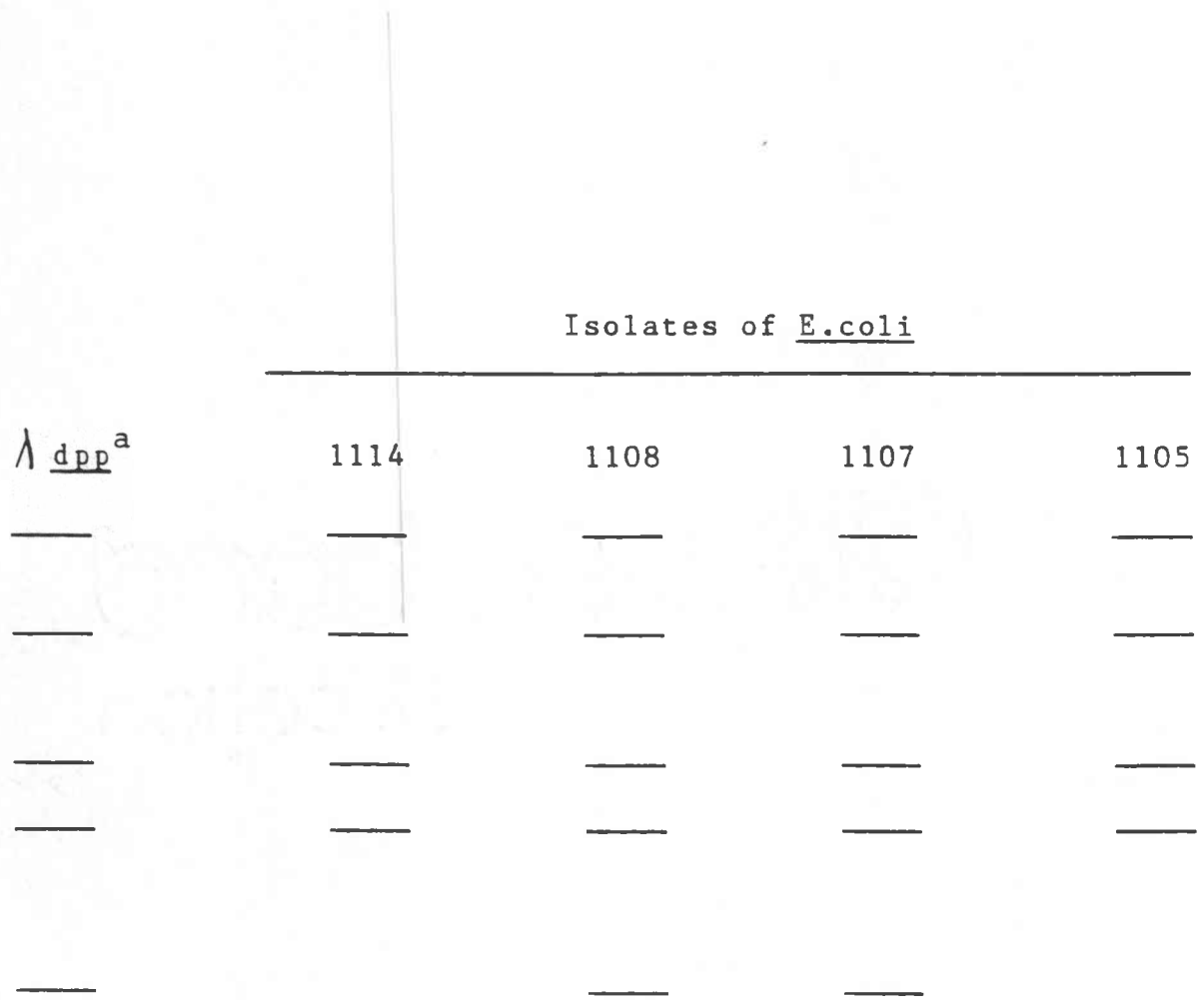


Fig. 5. DNA bands from gel separation of second BamHI digest. The 1.7 kb fragment is missing in the 1114 and 1105.

a.  $\lambda$  phage with dpp insert

Isolates of E.coli

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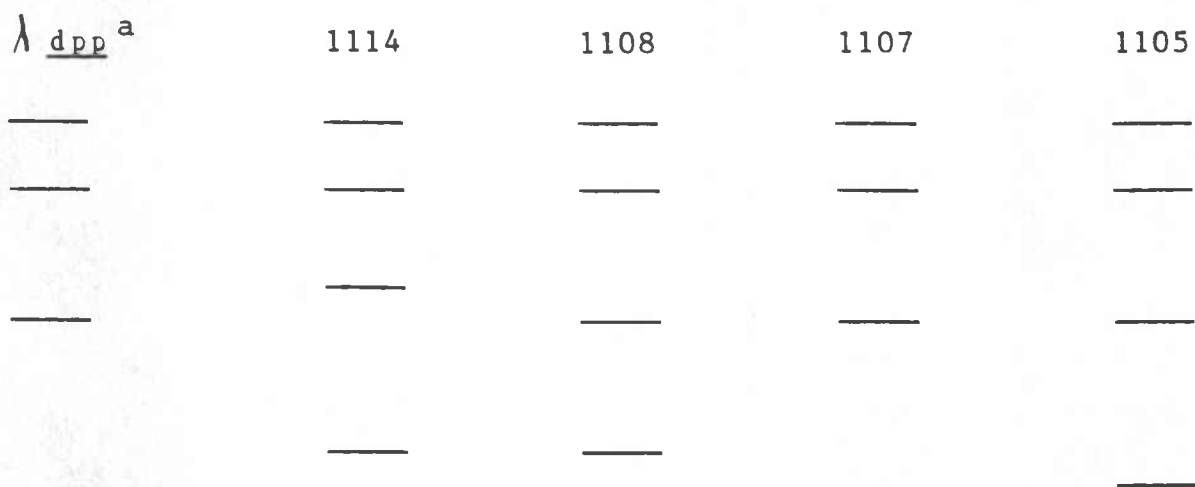


Fig. 6. DNA bands of gel separation of EcoRI digest. The 1.7 kb fragment is missing in 1107.

a.  $\lambda$  phage with dpp insert

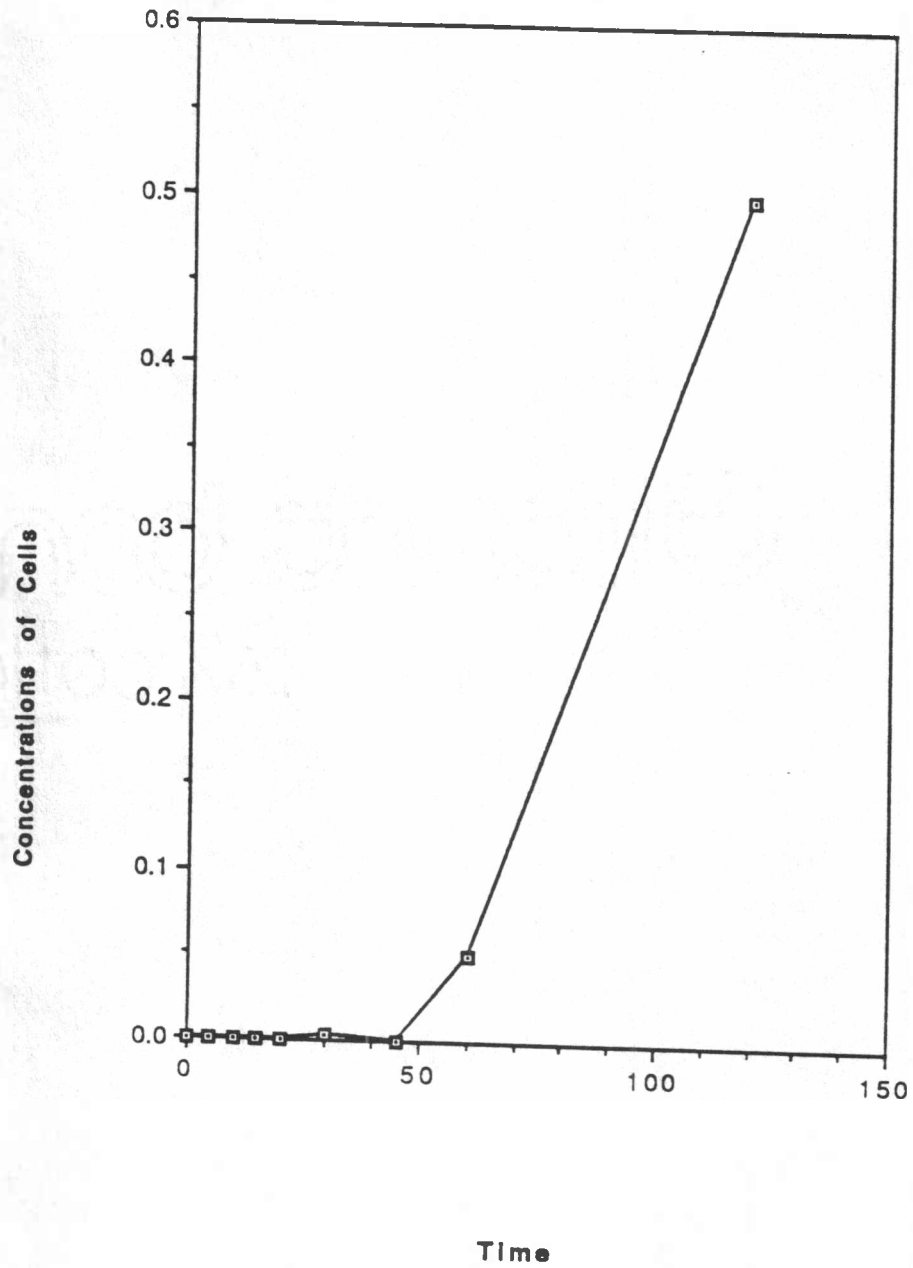


Fig. 7. Survival curve of irradiated E.coli cells. The concentrations of cells in cells/ml are plotted against time.

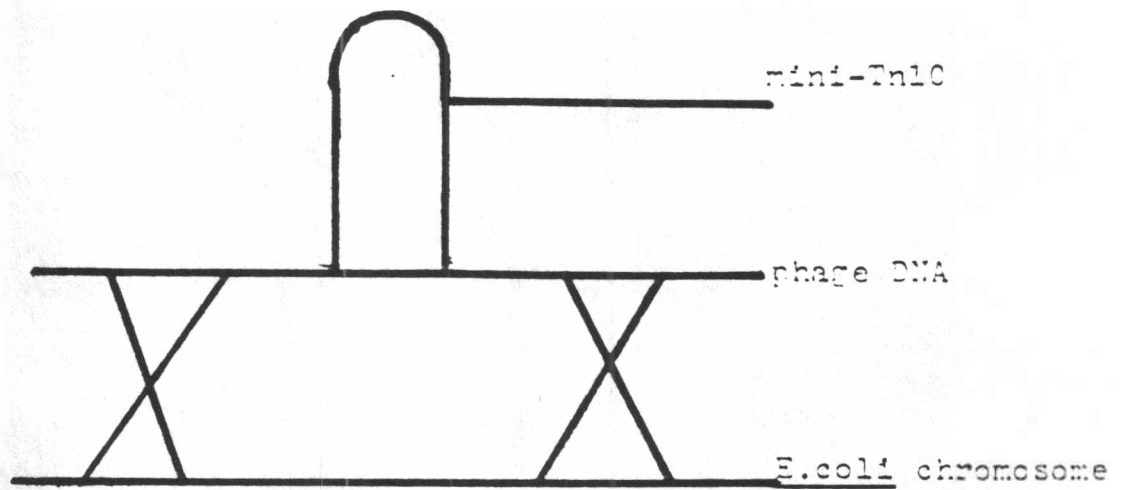


Fig. 8. Illustration of reciprocal double recombination of the mini-Tn10 occurs from the E.coli chromosome to the phage DNA. The mini-Tn10 is shown between two crossovers.

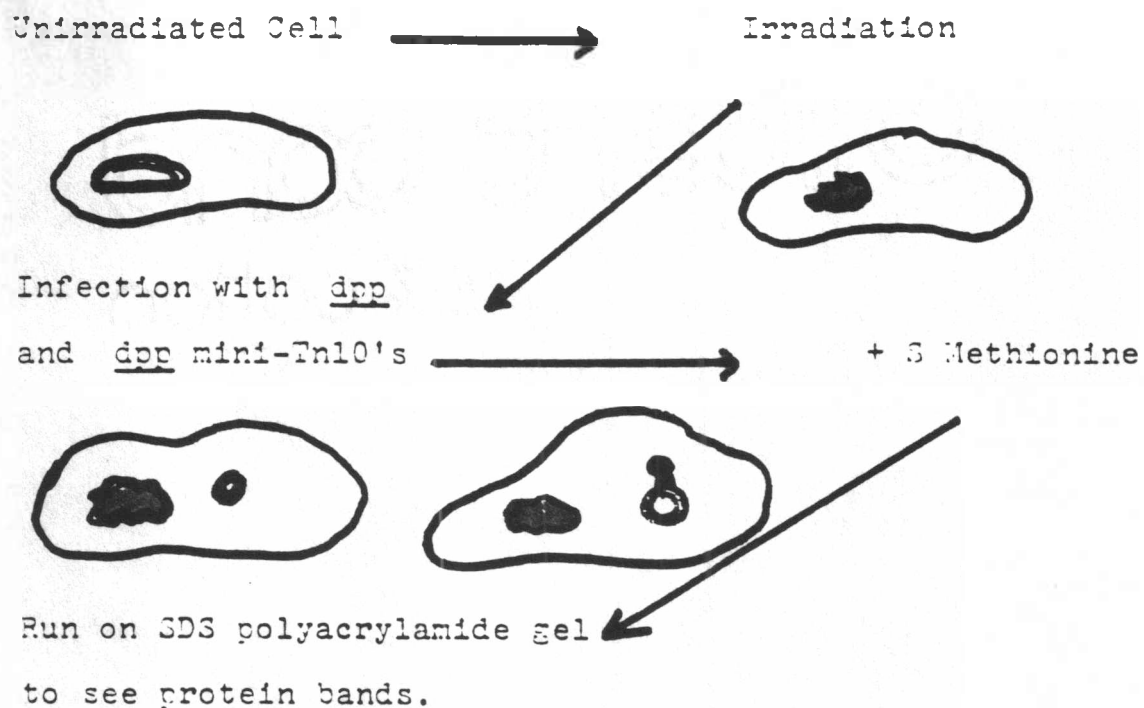


Fig. 9. Labeling of proteins for gel electrophoresis of unirradiated E.coli cell with active DNA (red). The cells are irradiated destroying the DNA so that it cannot be used as a template (blue) and then infected with phage containing dpp and dpp with mini-Tn10's (green). The proteins are then labeled with S-methionine and aliquots of irradiated and unirradiated cells are separated on SDS-polyacrylamide gels.



Table 1

Titers of small scale  $\Lambda$  dpp phage isolates. These isolates were done using only 5 ml of LB. The numbers represent the concentrations of phage in each ml.

$\Lambda$  Phage Isolates

1105 phage/ml	1107 phage/ml	1108 phage/ml	1114 phage/ml
1. $9 \times 10^{10}$	$1 \times 10^9$	$1 \times 10^{10}$	$7 \times 10^{10}$
2. $100 \times 10^8$	$20 \times 10^8$	$20 \times 10^8$	$50 \times 10^8$
3. $1.8 \times 10^9$	$5 \times 10^7$	$2 \times 10^{10}$	$1.4 \times 10^9$

Table 2

Growth of phage-infected E.coli cells on MMA and LB plates. All four phage strains, each having dpp insert, with a mini-Tn10 located in a unique place in the insert, are shown.

Isolates <sup>a</sup>	Growth on MMA plates	Titers on LB plates
	(cells/ml)	(phage/ml)
1105	$1 \times 10^6$	$1.8 \times 10^9$
1107	$1 \times 10^4$	$5 \times 10^7$
1108	$1 \times 10^6$	$2 \times 10^{10}$
1114	$1 \times 10^6$	$1.4 \times 10^9$

a.  $\lambda$  phage with dpp insert

Table 3

Titers of large scale  $\Delta$  dpp isolates grown in 250-500 ml of LB.  
The concentration is in phage per ml.

Isolates of / <u>dpp</u>			
1105 phage/ml	1107 phage/ml	1108 phage/ml	1114 phage/ml
1. $30 \times 10^{10}$	$2 \times 10^{10}$	$50-100 \times 10^9$	$2 \times 10^9$
2. $4 \times 10^{10}$	$2 \times 10^9$	$5 \times 10^9$	$2 \times 10^9$

#### REFERENCES CITED

- Callahan, A.M., and J.S. Parkinson. 1985. Genetics of Methyl-Accepting Chemotaxis Proteins in *Escherichia coli*: *ched* Mutations Affect the Structure and Function of the Tsr Transducer, J. Bact. 161:96-104.
- Conway, C., personal interview, Texas A&M University, June-July 1989.
- Dahl, Michael K., W. Boos, and M.D. Manson. 1989. Evolution of Chemotactic-Signal Transducers in Enteric Bacteria, J. Bact. 171:2370-2371.
- Dahl, M.K. Evolution der Maltosetransport-und chemotaxis-systeme in Enterobacteriaceae. (Konstanz, 1988) 23-27.
- Freifelder, D., Microbaial Genetics. (California: Jones and Bartlett Publishers, Inc., 1987) p. 107, 162, 199-200, 299-303, 399, 402, 432-433.
- Higgins, C.F., M.M. Hardie, D. Jamieson, and L.M. Powell. 1983. Genetic Map of the *opp* (Oligopeptide Permease) Locus of *Salmonella typhimurium*, J. Bact. 153:1548-1551.
- Macnab, R.M., Escherichi coli and salmonella typhimurium Cellular and Molecular Biology. (Washington, D.C.: American Society for Microbiology, 1987) pp. 733-734, 754, 43-44.
- Maniatis, T., E.F. Fritsch and J. Sambrook. Molecular Cloning A Laboratory Manual. (New York: Cold Spring Harbor Laboratory, 1982) pp. 80-82, 93-94, 95, 140-141, 142-143, 180, 252-253, 440.
- Manson, M.D., V. Blank and G. Brade. 1986. Peptide chemotaxis in *E.coli* involves the Tap signal transducer and the dipeptide permease, Nature 321:253-256.
- Manson, M.D., personal interview, Texas A&M University, June-July 1989.

Schaechter, M. and F.C. Neidhardt. Escherichia Coli and Salmonella Typhimurium Cellular and Molecular Biology. (Washington, D.C.: American Society for Microbiology, 1987) p. 1.

Silhavy, T.J., M.L. Berman, and L.W. Enquist, Experiments with Gene Fusions. (New York: Cold Spring Harbor Laboratory, 1984)

Silverman, M., P. Matsumura, and M. Simon. 1976. The identification of the mot gen product with Escherichia coli-lambda hybrids, Proc. Natl. Acad. Sci. U.S.A. 73:3126-3130.

Smith, R.A. and J.S. Parkinson. 1980. Overlapping genes at the cheA locus of Escherichia coli, Proc. Natl. Acad. Sci. U.S.A. 77:5370-5374.

Way, J.C., M.A. Davis, D. Morisato, D.E. Roberts and N. Kleckner. 1984. New Tn10 derivatives for transposon mutagenesis and for construction of lac Z operon fusions by transposition, Gene 32:369-379.

## APPENDIX A

### Labeling of dpp gene products

The concentrated  $\lambda$  dpp and  $\lambda$  dpp mini-Tn10 phage will first be dialyzed against SM to remove the cesium chloride, which will kill the cells (Manson, 1989). Cells of strain S159 ( $\lambda$  ind-) will then be irradiated. Infection of the cells with  $\lambda$  dpp and  $\lambda$  dpp mini-Tn10's phage follows irradiation. Cells of strain S159 ( $\lambda$  ind-) will be infected with phage at a ratio of 1 phage for every 10 cells and incubated at 37°C in methionine U.V. medium (MUV) for 40 min. before addition of the <sup>35</sup>S-methionine label (Smith et al., 1980). 40 min. allows enough time for the phage to produce dpp proteins. Proteins are labeled by addition of 5 to 15 Mci of <sup>35</sup>S-methionine and incubated 15 min. at 37°C (Silverman et al., 1976). Aliquots of irradiated infected cells with  $\lambda$  dpp and  $\lambda$  dpp mini-Tn10's and irradiated uninfected cells will be taken and run on SDS-polyacrylamide gels (Manson, pers. comm.).

The bands of protein from the labeled cells uninfected and infected with  $\lambda$  dpp and  $\lambda$  dpp mini-Tn10's will be observed. No cell protein bands will appear due to irradiation and most of the phage proteins won't be

synthesized due to the CI repressor and so won't appear on the gel. Only those proteins from the 141cb insert will appear as bands. Some of the bands of dpp proteins will be eliminated by the mini-Tn10's because the mini-Tn10, upon insertion, disrupts the gene it enters allowing no protein synthesis (Fig. 10).

## APPENDIX B

Luria broth: for 1 liter

10g peptone

5g yeast extract

5g NaCl

1000ml H<sub>2</sub>O

Autoclave 20 minutes at 120° C

For LB plates add 15g agar

For tet plates add tet to a final concentration of 5ug/ml  
(Maniatis 1982)

Tryptone broth: for 1 liter

peptone 10g

NaCl 8g

For TB plates also add 10g agar

Autoclave 20 minutes at 120° C then cool and add MgSO<sub>4</sub> 1M-  
10ml

(Maniatis 1982)

Minimal Media Plates: for 1 liter

15g agar to 900ml H<sub>2</sub>O-autoclave 120° C for 20 minutes then  
add:

100ml mM 10x

1ml 1M MgSO<sub>4</sub>

Thiamine to a final conc. of 1ug/ml

Methionine, Threonine, and Histidine to a final conc.

20ug/ml

(Maniatis 1982)

Prolyl-Leucine to a final conc of 10ug/ml

add glucose to 0.2% of final conc.

(Manson 1989).

Minimal Salts Medium--375ml

37.5ml MUV medium

Methionine to a final conc. of 10ug/ml

Thiamine to a final conc of 1ug/ml

1% glycerol

0.5% maltose

3mM MgCl<sub>2</sub>

3uM FeCl<sub>2</sub>

0.1mM CaCl<sub>2</sub>

1mM MgSO<sub>4</sub>

50mM Tris-hydrochloride

25mM Na<sub>2</sub>HPO<sub>4</sub>

22mM KH<sub>2</sub>PO<sub>4</sub>

20mM NH<sub>2</sub>Cl

Autoclave these last four reagents for 20 minutes at 120° C  
then add other ingredients (Silverman et al., 1976).

Add 303.9ml of sterile H<sub>2</sub>O to make a final volume of 375ml  
(Manson 1989).