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Regulation of the cardiolipin synthase gene in *Escherichia coli*

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REGULATION OF THE CARDIOLIPIN SYNTHASE GENE IN *ESCHERICHIA COLI*

By

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ABSTRACT

REGULATION OF THE CARDIOLIPIN SYNTHASE GENE IN *ESCHERICHIA COLI*

by

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Advisor: Professor Burton E. Tropp

To better understand the importance of *cls*, the structural gene for cardiolipin synthase, an extensive study was carried out. The first objective was to determine whether *cls* is essential and establish it's chromosomal location. Using Tn*10*dTet several insertions into *cls* were obtained. These insertions caused an increased resistance to 3,4-dihydroxybutyl-1-phosphonate and a deficiency in cardiolipin content. Using the tetracycline resistance gene inside Tn*10*dTet as a marker, three factor cross's were carried out to determine the location of *cls* with respect to neighboring genes. The gene order *hemA-narC-tyrT-adhC-cls* was established.

The second objective was to create operon and protein fusions, between *cls* and *lacZ*. Extensive *in vivo* attempts to fuse *cls* to *lacZ* were unsuccessful. It therefore became necessary to clone *cls* and create the

gene fusions *in vitro*. A two step approach was used to clone *cls*. The first step involved the insertion of a Tn*10*dCam next to *cls*. The second step was to clone *cls* by selecting for Tn*10*dCam. Plasmids containing a cloned *cls* increased the CL content of *cls* defective strains.

Using the cloned *cls*, *in vitro* operon and protein fusions between *cls* and *lacZ* were constructed. The gene fusions were transferred from multicopy plasmids into the *E. coli* λ attachment. Using β-galactosidase as an indication of *cls* expression, the regulation of *cls* was investigated under a number of conditions. Under most of the conditions studied, there was little or no change in *cls* expression. However, a two to three fold increase in expression of the operon and protein fusion was observed as the cells were grown to stationary phase, where grown in minimal media versus rich media, and when the bacteria were grown anaerobically. The anaerobic affect was further investigated using different electron acceptors. The better the electron acceptor the lower the *cls* expression. In the presence of two electron acceptors, *cls* expression reflected that of the better electron acceptor.

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ABBREVIATIONS

(APB)	Alkaline phosphatase buffer
(BAP)	Bacterial alkaline phosphatase
(BCIP)	Bromochloroindolyl phosphate
(BpB)	Bromphenol blue
(CIAP)	Calf intestine alkaline phosphatase
(CL)	Cardiolipin
(DBP)	3,4-dihydroxybutyl-1-phosphonate
(*E. coli*)	*Escherichia coli*
(IPTG)	Isopropylthio-β-D-galactoside
(LB)	Loading Buffer
(MDO)	Membrane derived oligosaccharides
(MOPS)	Morpholinopropane sulfonate
(NBT)	Nitroblue tetrazolium
(ONPG)	O-onitrophenyl-β-D-galactoside
(PE)	Phosphatidylethanolamine
(PEA)	Phenethyl alcohol
(PFU)	Plaque forming units
(PG)	Phosphatidylglycerol
(PGP)	Phosphatidylglycerophosphate
(PS)	Phosphatidylserine
(RB)	Running Buffer
(RBS)	Reservoir Buffer Stock
(SB)	Sample Buffer
(SDS)	Sodium dodecyl sulfate
(SG)	Spencer and Guest
(TTC)	Triphenyltetrazolium chloride
(UP)	Upper buffer
(XG)	5-bromo-4-chloro-indolyl-β-D-galactose
(XP)	5-bromo-4-chloro-3-indolyl-phosphate-p-toluidene

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CHAPTER 1

GENERAL INTRODUCTION

Cardiolipin (CL)[1] is one of three major phosphoglycerides in the *Escherichia coli* (*E. coli*)[1] cell envelope. Typically, CL comprises approximately 5% of the phosphoglycerides in the *E. coli* cell membrane (1). The two other predominant phosphoglycerides, phosphatidylethanolamine (PE)[1] and phosphatidylglycerol (PG)[1] are present at about 75% and 20% respectively (1). By virtue of their hydrophobic backbone and hydrophilic headgroups, these phosphoglycerides help to maintain the integrity of the cell membrane (Figure 1.1). However, individual phosphoglycerides may also have specific functions in biological processes (2,3). The isolation of mutations in the different steps of phosphoglyceride biosynthesis has been instrumental in the study of phosphoglycerides (Figure 1.2). These mutants have been used to vary the phosphoglyceride composition of the cell thereby providing valuable information on the phosphoglyceride biosynthetic pathways and the importance of individual phosphoglycerides.

Bacteria with an altered CL content have been isolated by mutating the genes coding for phosphatidylserine (PS)[1] synthase,

phosphatidylglycerophosphate (PGP)[1] synthase, and CL synthase. The main function of PS synthase is to catalyze the reaction of CDP-diacylglycerol and L-serine form PS (4). The enzyme may also catalyze the reaction of CDP-diacylglycerol and PG to form small amounts of CL (5). Temperature sensitive *pss* alleles have been isolated by colony autoradiography (6,7), and [^{3}H]serine suicide techniques (4). The latter selection was used to obtain OS2101 (*pss-1*).

Strain OS2101 does not grow in NBY medium at 42° C, but does grow at the restrictive temperature when the medium is supplemented with Na^+, K^+, NH_4^+, Mg^{2+}, or sucrose. The *pss*-1 allele causes a dramatic change in phosphoglyceride composition. When OS2101 is grown in NaCl-NBY medium at 42° C, it has a phosphoglyceride content of PE 26.8%, PG 39.6%, and CL 33.6% (4). When OS2124 (isogenic with OS2101 expect for a functioning *pss*) is grown under identical conditions, it has a phosphoglyceride content of PE 59.1%, PG 29.4%, and CL 11.5%. Under these conditions OS2101 grows slightly slower than OS2124. Thus, in wild type cells PE is either present in excess or it's main function is to maintain the lipid bilayer. A more conclusive argument that PE plays a largely structural role should be forthcoming. Dowhan has recently constructed a strain containing a null *pss* mutation, which has no observable PE (personal communication to B. Tropp and 3).

The enzyme PGP synthase catalyzes the formation of PGP from CDP-diacylglycerol and glycerol-3-phosphate (1,8,9). Several different mutations in the gene coding for PGP synthase, *pgsA*, have been isolated. The first PGP mutation isolated, *pgsA444*, was obtained by screening colonies for defects in incorporation of [^{32}P]glycerol phosphate (7). The *pgsA444* allele causes PGP synthase activity to drop to 5% of the wild type level but does not cause any alteration in phosphoglyceride composition. This could mean that PGP synthase is present in a 20 fold excess, or that the defective enzyme is more stable *in vivo* than it is *in vitro*.

A more restrictive *pgsA* allele was obtained by growing SD9 (*pss-1 cls-1*) at 37° C (10). The resulting spontaneous mutant SD103 (*pss-1 pgs3A cls-1*) is able to grow at 37° C and has no detectable PGP synthase activity. When SD103 is grown at 30° C, PE constitutes up to 95% of the total phosphoglycerides while PG plus CL make up approximately 2% of the cellular phosphoglycerides. Because SD103 is able to grow, it was not possible to determine whether PG or CL are essential phosphoglycerides or merely play a structural role.

To ascertain whether PG and CL are essential for cellular growth, Dowhan *et al.* attempted to transfer a disrupted *pgsA* gene onto the bacterial chromosome (11). A disrupted *pgsA* was created by inserting *kan*

into a cloned *pgsA*. The cloned *pgsA*::*kan* was integrated into the chromosome forming a merodiploid consisting of a functioning *pgsA* and a *pgsA30* allele (*pgsA*::*kan*). The *pgsA30* allele was transferred by P1 transduction into a strain containing a cloned *pgsA* gene. The plasmid containing the functioning *pgsA* had a temperature sensitive origin of replication. When the temperature of the growth medium was raised, the strain was cured of the cloned *pgsA*. Thus, the level of PGP synthase could be decreased by increasing the temperature. A decrease of PGP synthase to 25% of the wild type level resulted in a decrease in PG and CL (11). Further reduction of PGP synthase to approximately 10% of the wild type level caused a cessation of cell growth. These experiments indicate that PG, CL, or both are essential for growth (11). Strain SD103's ability to grow even though it contains only 2%, PG and CL, is due to a second site mutation in *lpp* (12) . Strains containing a mutation in *lpp* are deficient in a lipoprotein precursor which serves as an acceptor for glycerol moieties from PG.

Recently Dowhan's laboratory has studied the importance of acidic phosphoglycerides through the construction of a controlled *pgsA* mutation (13). By fusing the *pgsA* gene to the *lac* promoter, expression of *pgsA* can be easily regulated by the addition of isopropylthio-β-D-galactoside (IPTG)[1] (13). Initial studies with the controlled *pgsA* indicate there is a

seven fold excess in the PGP synthase level and confirm the importance of acidic phosphoglycerides in protein translocation (14,15). Possible essential roles for PG, CL, or both, include serving as a donor of glycerol moieties to cysteine, of several membrane proteins, and participating in the translocation of certain outer membrane proteins (11,14,15). While acidic phosphoglycerides are needed for activation of the DnaA protein (16,17), this protein is not essential. Bacteria deficient in RNAse H grow in the absence of the DnaA protein (18,19).

The failure of *pgsA* mutants to grow demonstrates the importance of acidic phosphoglycerides. It does not answer the question of whether CL is essential. To examine the importance of CL, mutants deficient in CL have been constructed. These mutants, which contain a defect in *cls*, the structural gene for CL synthase, have been isolated by *in vivo* mutagenesis and by insertional inactivation (20,21,22). CL synthase catalyzes the formation of CL, occurring by a phosphatidyl group transfer from one PG molecule to another (23,24). The *cls* mutants have a very low, but detectable CL content.

The first *E. coli* mutant with a defect in CL synthesis was discovered by screening lipid extracts of temperature sensitive mutants for deficiencies in phosphoglycerides (20). Genetic analyses showed that the defect in CL synthesis and the temperature sensitive phenotype were

caused by independent mutational events. A second CL deficient mutant was isolated on the basis of its increased resistance to 3,4-dihydroxybutyl-1-phosphonate (DBP)[1] (21,25). The CL content in both these mutants was decreased to less than 1% of the total phosphoglyceride composition. The ability of these strains to synthesize CL suggested that either these alleles were leaky or there is an alternate pathway to produce CL. The latter possibility was confirmed when Nishijima *et al.* were able to transfer a *cls*::*kan* from a plasmid onto the bacterial chromosome (22). Bacteria containing the disrupted *cls* lack CL synthase activity but still synthesize small quantities of CL.

It is not known whether this small quantity of CL is essential to cell growth. Shibuya *et al.* proposed that the alternate route for CL synthesis is catalyzed by phosphatidylserine synthase (5). Presumably, CL is formed by a phosphatidyl group transfer from CDP-diacylglycerol to PG. Consistent with this proposal, *cls-1 pss-1* double mutants synthesize even less CL than do *cls-1* mutants (5). Consequences of a CL deficiency include a slightly longer doubling time (22), increased resistance to DBP (21), and decreased ability to grow at alkaline pH (25).

The *pss* (26,27), *pgsA* (28,29), and *cls* (30) genes, have all been cloned. The cloned *pss* gene causes an increase in the amount of PS synthase proportional to the gene dosage. However, this increase in

enzyme did not cause any change in phosphoglyceride composition (29). A cloned *pgsA* also led to enzyme over production in a fashion consistent with a direct gene dosage effect. Despite a 10 fold increase in PGP synthase production, only a 50% increase in PG, at the expense of PE, was observed (28). The cloned *cls* gene behaves in the same way as the cloned *pgsA* and *pss* genes, in that a large increase in enzyme activity is not accompanied by a corresponding increase in phospholipid product (30). A ten fold increase in cardiolipin synthase activity results in only a 50% increase in CL content at the expense of PG.

The inability of increased enzyme levels to significantly alter the phosphoglyceride composition indicates that regulation is occurring at the enzymatic level. However, it does not exclude the possibility that regulation also occurs at the genetic level. In fact, the *pss* gene has been found to be regulated by *pssR* (31). The use of gene fusions facilitates studies of genetic regulation, especially when the gene product is difficult to assay (32,33). Gene fusions can be constructed so that a target promoter along with part of its coding sequence is joined to a reporter gene, such as the *lacZ*. In this way, the expression of the target promoter can be measured by assaying the production of β-galactosidase. Production of β-galactosidase can also be used to readily select for regulatory mutants.

To better understand the importance of *cls*, an extensive study was carried out. The first objective was to determine whether *cls* is essential and establish it's chromosomal location (this project was started prior to Nishijima's construction of a *cls*::*kan*, 22). The second objective was to create operon and protein fusions to investigate genetic regulation of *cls*. Extensive *in vivo* attempts to fuse *cls* to *lacZ* were unsuccessful. It therefore became necessary to clone *cls* and create the gene fusions by recombinant DNA techniques. Finally, the gene fusions were used to study the regulation of *cls* and search for regulatory mutants.

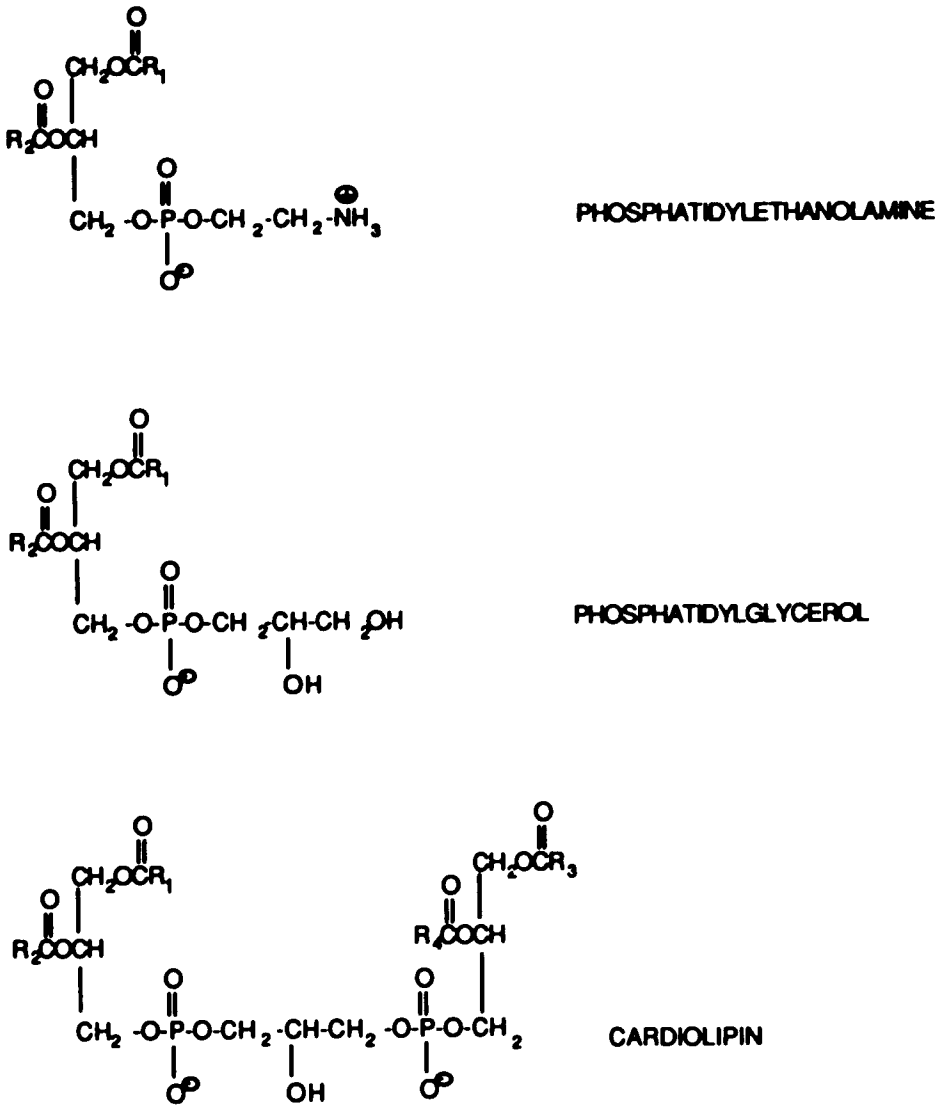

Figure 1.1. Structure of phosphatidylethanolamine, phosphatidylglycerol, and cardiolipin.

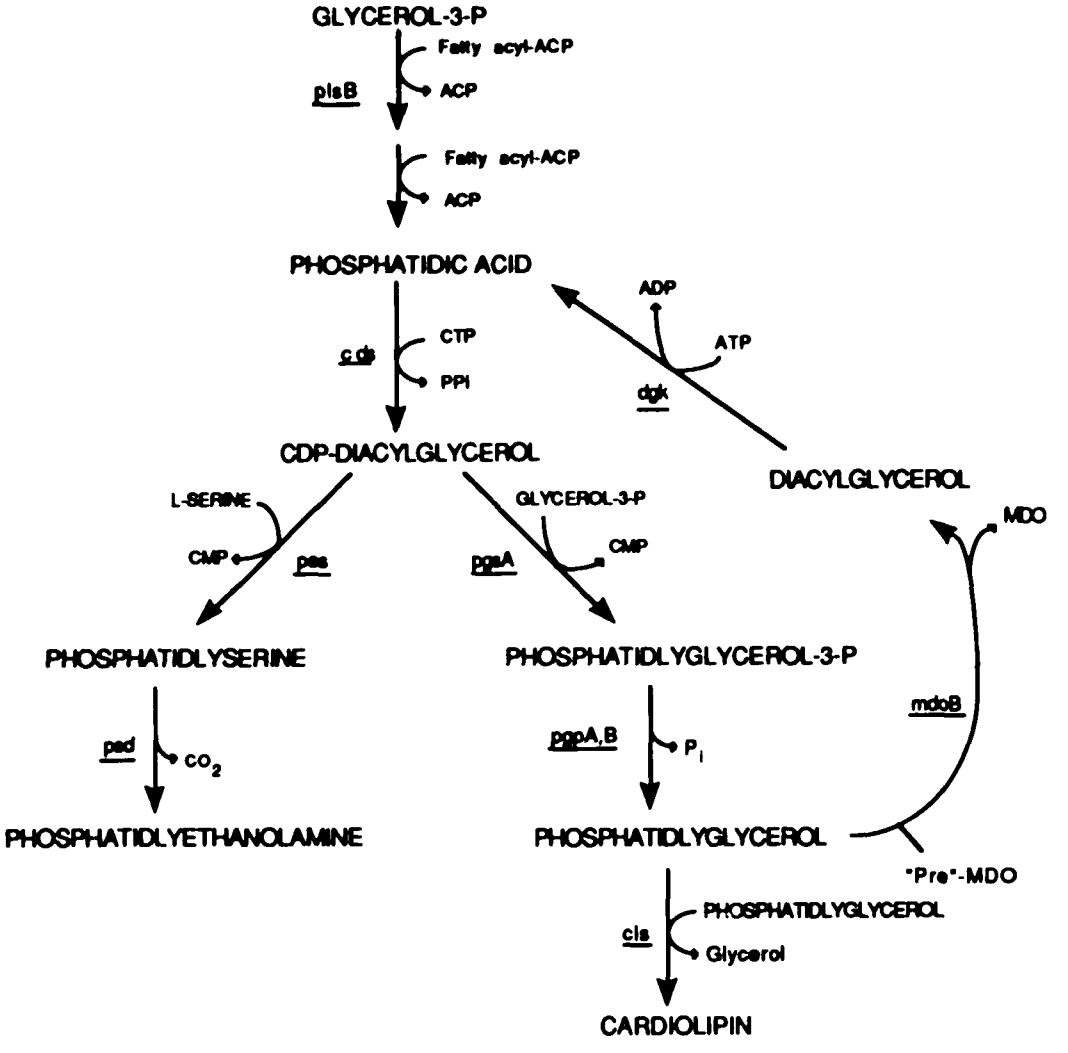

Figure 1.2. Phosphoglyceride biosynthesis in *Escherichia coli*.

CHAPTER 2

Insertion Of Tn*10*dTet Inside *cls*

INTRODUCTION

As a first step in studying *cls*, several different *cls*$^-$ insertions were created by inserting a defective transposon into *cls*. The insert and surrounding chromosomal DNA from one of the *cls*::Tn*10*dTet colonies was subcloned into pIBI20. Restriction analysis revealed that the transposon is inserted between the SphI and HindIII restriction sites of the *cls* gene. The ability to isolate mutants containing a disrupted *cls* indicates that *cls* is not an essential gene. It therefore should be possible to create *in vivo* gene fusions between *cls* and the *lac* operon.

Construction of gene fusions can be facilitated using specially constructed λ phages. Phages λp*lac*Mu9 and λp*lac*Mu53 were used in attempts to create gene fusions between *cls* and *lacZ*. These phage have a promoterless *lac* operon flanked by parts of bacteriophage Mu (34,35). There is enough Mu present to enable the hybrid bacteriophage to transpose at a high frequency only if the transposase is also present. Phage λp*lac*Mu53, containing the *trp* translational initiation site just

upstream from *lacZ*, is used to create operon fusions. While λ*plac*Mu9, lacking a translational initiation site, is used to create protein fusions. Surprisingly, despite extensive efforts neither λ*plac*Mu9 or λ*plac*Mu53 insertions into *cls* were obtained.

A secondary reason for inserting Tn*10*dTet inside *cls* is to provide a means to readily select for *cls*⁻. This greatly facilitates the study of *cls* in different genetic backgrounds. In addition, the disrupted *cls* can be used to precisely map the chromosomal location of *cls*. While the *cls* gene is located at minute 27 on the *E. coli* genetic map, its location with respect to neighboring genes has never been determined (36). The major obstacle to precisely mapping *cls* has been the lack of a selectable phenotype. The Tn*10*dTet inside of *cls* provides an easily detectable phenotype. Using tetracycline resistance as a marker for *cls*, three factor crosses were carried out to determine the position of *cls* with respect to neighboring genes. A gene order of *hemA-narC-tyrT-adhC-cls* was established.

MATERIAL AND METHODS.

Chemicals: *rac*-3,4-Dihydroxybutyl-1-phosphonate was synthesized as previously described (37). Fusaric acid, triphenyltetrazolium chloride (TTC)[1], δ-aminolevulinic acid, ampicillin, tetracycline, chloramphenicol, nalidixic acid, and *L*-amino acids were purchased from Sigma Chemical Co., St. Louis, Mo. Bacto-tryptone, Bacto-yeast extract, Bacto-agar, Bacto-peptone, and proteose peptone were products of Difco Laboratories, Detroit, MI. Ethidium bromide, restriction enzymes, and T4 DNA ligase were obtained from BRL., Gaithersburg, MD., or IBI., New Haven. CT. Carrier free [^{32}P]phosphate was purchased from ICN., Irvine, CA. Polygram Sil G TLC plates were a product of Brinkmann Instruments, Inc., Westbury, NY. HPTLC-alufolien Kieselgel 60 plates were obtained from EM Science, Cherry Hill, NJ. All other chemicals were reagent grade or better.

Media and growth conditions: LB broth consisted of 1.0% tryptone, 0.5% yeast extract, and 0.5% sodium chloride. Unless otherwise stated, LB broth was adjusted to pH 7.4 with sodium hydroxide. GL minimal medium was that of Garen and Levinthal but lacked casein hydrolysate (38). This medium was supplemented with 0.4% of the

specified carbon source and 20 μg/mL of required *L*-amino acids. Solid medium was solidified with 1.5% bacto-agar. DBP test plates contained GL medium supplemented with 0.4% potassium succinate and 30 μM *S*-DBP. TTC-nitrate plates contained LB broth, 1.0% potassium nitrate, and 0.005% TTC (39). TTC-ethanol plates contained 0.7% dibasic potassium phosphate, 0.3% monobasic potassium phosphate, 0.01% magnesium sulfate, 0.2% proteose peptone, 0.5% ethanol, and 0.0025% TTC (40). PN agar plates contained 1.7% bacto-peptone, 0.3% proteose peptone, 0.5% sodium chloride, and 1.0% potassium nitrate (41). Fusaric acid plates were prepared in the following manner (42): a solution containing 1.5% Bacto-agar, 0.5% Bacto-tryptone, 0.5% Bacto-yeast extract, 1.0% sodium chloride, and 0.005% chlortetracycline was autoclaved for 20 minutes (the pH was not adjusted). After the solution cooled to about 55° C, in a 55° C water bath, 6.0 mL of 20 mM zinc chloride (filter sterilized), 17 ml 6 g/mL monobasic sodium phosphate (filter sterilized) and 6 grams of fusaric acid were added. When needed, δ-aminolevulinic acid, ampicillin, tetracycline, and chloramphenicol, were added to final concentrations of 40, 100, 20, and 15 μg/mL, respectively.

Bacterial strains. *E. coli* strains used are listed in Table 2.1. The construction of these strains and subsequent mapping were done by standard conjugation and P1 transduction (43,30). Strain JM246

(*cysI53(am)*) was found to have a defect preventing anaerobic growth on PN agar and causing a red appearance on TTC-nitrate plates. A wild type derivative, SOH11 [*cysI53(am)*] was constructed by selecting for anaerobic growth on PN agar after transduction with a P1 lysate from Lin8.

Insertion of Tn*10*dTet into *cls*. Several different random Tn*10*dTet pools were constructed by infecting GE886 (K12/pNK972) with a P1 lysate from GE1031 (*supE42/Fzzf*-1831::Tn*10*dTet). The defective transposon Tn*10*dTet can not transpose unless supplied with a transposase, which is present in pNK972 (44,45,46). Once Tn*10*dTet is introduced into GE886 it randomly inserts into the bacterial chromosome. Independent pools, consisting of approximately ten thousand GE886 tetracycline resistant colonies, were collected and infected with $P1_{vir}$. The resulting P1 lysates were used to infect SOH5 (*glpR glpD narC*). Colonies acquiring Tn*10*dTet and a wild type *narC* appeared white on TTC-nitrate plates supplemented with tetracycline. These colonies were purified and tested for DBP resistance on DBP test plates. A P1 lysate prepared from each DBP-resistant colony was used to transduce SOH2 (*glpR glpD*). Transductants that were resistant to both tetracycline and DBP were tested for the ability to synthesize CL.

Insertion of Tn*10*dCam near *hemA*. Random Tn*10*dCam pools were generated by similar procedures to those described for the Tn*10*dTet

pools (44). However, GE1033 (*supE42/Fzzf*-1836::Tn*10*dCam) was used as the transposon donor and chloramphenicol resistance was used as the selected marker. The resulting P1 lysate, made by infecting the random Tn*10*dCam pool with $P1_{vir}$, was used to place a Tn*10*dCam near *hemA* in S730 (*hemA*). Strain SOH15 (*cysI53(am) narC::*Tn*10 adhC tyrT*) was infected with a P1 lysate, made from a strain containing Tn*10*dCam near *hemA*. Selecting for chloramphenicol resistance led to the construction of SOH16 (*hemA adhC tyrT cysI53(am) zcd::*Tn*10*dCam)

DNA preparation and manipulation. Plasmid DNA was isolated from bacteria grown shaking overnight in LB broth, supplemented with the appropriate antibiotic, at 37° C. The rapid SDS-alkaline lysis method was used to obtain plasmid DNA from 1.5 mL of culture (47). This procedure was scaled up, to accommodate 5 to 10 mL of culture, in the following manner (48): cells were grown in 5-10 mL of LB broth, supplemented with the appropriate antibiotic, shaking at 37° C, overnight. The following morning, the cells were harvested, and resuspended in 150 µL GTE (50 mM glucose, 25 mM Tris-HCl, and 10 mM EDTA; pH 8.0). The suspension was transferred to a microfuge tube, incubated at room temperature for 5 minutes, and then 300 µL of NaOH/SDS (freshly prepared, 1% SDS in 0.2 N NaOH) were added. The mixture was agitated, by inverting the microfuge tube 6 times, and then incubated on

ice for 5 minutes. A solution of 225 µL KAc buffer (prepared by mixing 600 mL 5 M KAc, 115 mL glacial acetic acid, and 285 mL of water) was added. The mixture was kept on ice for 5 minutes and then centrifuged for 10 minutes in a cold microfuge. The supernatant was divided into two 300 µL samples and placed in new microfuge tubes. A solution containing 300 µL of CPI (chloroform: phenol: isoamyl alcohol:: 24:25:1) was added to the mixture. The CPI was prepared by mixing 150 µL Tris-buffered phenol with of 150 µL chloroform: isoamyl alcohol mixture (48). The mixture was vigorously vortexed and then centrifuged for 5 minutes at room temperature. The top aqueous layer was transferred to a new microfuge tube and 600 µL of absolute ethanol were added to it. The mixture was then centrifuged for 5 minutes at room temperature. The supernatant was removed and the tubes drained on a paper towel. A 1.0 mL solution of 70% ethanol was added to the precipitate, and the mixture was briefly vortexed. The mixture was centrifuged for 5 minutes at room temperature. The supernatant was discarded and the tubes were drained on a paper towel. The pellet was dried briefly in a Savant evaporator (5-15 minutes), and resuspended in 50-100 µL of TE buffer (10 mM Tris-base, 1 mM EDTA; pH 8.0), containing 20 µg/mL boiled RNAse.

Chromosomal DNA was isolated from cells cultured shaking overnight in LB broth at 37° C (32). Ligations between chromosomal and

plasmid DNA were carried out by overnight incubation at 11° C in 1x ligation buffer (supplied by the manufacturer) containing 1 unit of T4 DNA ligase. Plasmid and chromosomal DNA were stored in TE buffer containing 20 µg/ml RNase, at -20 ° C.

Restriction enzymes and T4 DNA ligase were used according to their manufacturers' specifications. DNA bands were stained using 5 µg/mL ethidium bromide and visualized using UV light. Staining took place in 1x electrophoresis buffer for 45 minutes. Two types of buffers were used during the electrophoresis: TAE (40 mM Tris-HCl, 20 mM sodium acetate, and 2 mM EDTA; pH 8.3) and TBE (80 mM Tris-HCl, 40 mM boric acid, and 2 mM EDTA; pH 8.0). TAE buffer was used for overnight electrophoresis or when the DNA, separated by gel electrophoresis, was to be used in future ligations. TBE buffer was used for over day electrophoresis (typically 2-6 hours). Destaining took place for 20 minutes in either water or 1x electrophoresis buffer (electrophoresis buffer was used when the DNA was needed for future ligations).

Insertion of λ*plac*Mu9 and λ*plac*Mu53 into the bacterial chromosome: λ*plac*Mu9 and λ*plac*Mu53 were inserted into the *E. coli* chromosome. Insertions were obtained using the following protocol: the recipient strain was grown overnight in LB broth. The next morning 1.0 mL of cells was mixed with 10^8 plaque forming units (PFU)[1] of λ*plac*Mu

and incubated at room temperature for 30 minutes. Five mL of LB broth were added, and the cells were harvested and washed pellet three times with 5.0 mL of LB broth. Several dilutions (10^{-1} to 10^{-4}) were plated onto selective plates. No out growth was required when selecting for kanamycin resistance or the ability to grow using lactose as the sole carbon source. To increase transposition frequency, λpMu507 was used to supply λp*lac*Mu with a transposase. The above procedure was followed when using λpMu507, except the fresh overnight was mixed with 10^8 PFU of λp*lac*Mu and 10^9 PFU of λpMu507.

Several different approaches were used to select for the insertion of λp*lac*Mu9 or λp*lac*Mu53 into *cls*. Direct selection was attempted by selecting for DBP^r alone or in conjunction with cold sensitivity or deoxycholate sensitivity. Indirect selection was performed by infecting bacteria with a P1 lysate, made from random insertions of λp*lac*Mu9 or λp*lac*Mu53 into the chromosome, and then selecting for a chromosomal marker near *cls*. All attempts to insert λp*lac*Mu9 or λp*lac*Mu53 into *cls* were unsuccessful. However, during these efforts a colony, SOH53, overproducing CL was obtained.

Lipid analysis. Unless otherwise specified, phospholipids were labeled with 5 to 10 μCi/mL of carrier-free [^{32}P]phosphate. Radioactive phosphate was added when the cells were at a density of 1-3 Klett units.

After allowing the bacteria to grow to the desired cell density, cells were collected by centrifugation and resuspended in 1 mL of distilled water. Phospholipids were isolated as previously described (21), and separated by one or two dimensional chromatography. The former was performed using HPTLC chromatographic plates in a chloroform-acetone-methanol-acetic acid-water 30:40:10:10:5 solvent system (49). The latter was performed using Sil G TLC plates and with chloroform-methanol-ammonia (65:35:5) as the solvent system in the first dimension, and chloroform-methanol-acetic acid (65:25:8) as the solvent system in the second dimension (49). Radioactive spots were identified by autoradiography. Results were quantitated by cutting radioactive spots from the TLC plates and counting them in an Isocap 300 scintillation counter.

RESULTS

Isolation of *cls*::Tn*10*dTet. The strategy used for constructing strains with a Tn*10*dTet insertion within *cls* is based upon the observation that *cls* mutants are more resistant to DBP than are wild type strains (21,25). While *cls* mutants are inhibited on DBP test plates, DBP resistance is not "tight" enough to be used as an effective selection. However, it is possible to enrich for the desired insertion mutants by selecting for insertions near *narC* (an easily scored marker which maps near *cls*, 36).

The defective transposon Tn*10*dTet was used as the insertion element because it contains a tetracycline resistance gene and cannot transpose or cause gene rearrangements unless supplied with an external transposase (44,45,46). Random Tn*10*dTet insertion pools, each containing several thousand transductants, were prepared as described in Materials and Methods. A P1 lysate of each pool was used to infect SOH5 (*glpR glpD narC*). Fewer than 1% of the tetracycline transductants appeared as white colonies on TTC-nitrate plates containing tetracycline. A total of 331 *nar*$^+$ colonies were purified and subjected to further analysis. Nine of the 331 white colonies grew on DBP test plates. Five of these mapped near *trp*. Strains SOH7 (*cls*::Tn*10*dTet2A) and SOH8 (*cls*::Tn*10*dTet2B) originated

from the same Tn*10*dTet pool. The three other mutants originated from different pools.

All five putative insertion mutants were cultured in the presence of carrier-free [^{32}P]phosphate and their lipids were extracted and analyzed by one-dimensional thin layer chromatography using HPTLC plates. All five mutants synthesized 2-4 fold less CL than SOH2 (*cls*$^{+}$) (Table 2.2). However, one dimensional chromatography may be inadequate to separate CL from contaminating minor lipids. To more accurately access the lipids formed, [^{32}P]phosphate labeled lipid extracts from SOH9 (*cls*::Tn*10*dTet3) and SOH2 (*cls*$^{+}$) were analyzed by two dimensional thin layer chromatography (Table 2.3). Strain SOH9 contains less than 10% of the CL content of its wild type parent. As expected, the defect in CL formation, tetracycline resistance, and DBP resistance are 100% cotransducible. The insertion mutation obtained in SOH9 was subjected to further genetic analysis.

Verification of Tn*10*dTet insertion in *cls*. To establish that the Tn*10*dTet insertion causing the defect in CL was inserted within *cls*, the chromosomal DNA containing the defective transposon was cloned into pIBI20. DNA was isolated from SOH12 (Lin8 *cls*::Tn*10*dTet3), digested with BglII, and then mixed with a preparation of pIBI20 that had been digested with BamHI. After treatment with T4 DNA ligase the mixture

was used to transform JM105. Several tetracycline and ampicillin resistant colonies were obtained and the plasmid DNA isolated. The plasmid pSH9, from one of these transformants, was retained for further study.

A restriction map of pSH9 was obtained by cutting the plasmid with different combinations of restriction enzymes (Figure 2.1). Comparison of the restriction maps of pSH9 and of that reported for *cls* (22,30), and Tn*10*dTet (44,45,46), indicates that Tn*10*dTet is inserted between the SphI and HindIII sites of *cls*.

Chromosomal mapping of *cls*::Tn*10*dTet3. The presence of a tetracycline resistance gene within *cls* facilitated mapping of *cls* with respect to neighboring genes. A P1 lysate of SOH17 (*hem*$^+$ *narC tyr*$^+$ *adh*$^+$ *cls*::Tn*10*dTet3 *cysI53am*) was used to infect SOH16 (*hemA nar*$^+$ *tyrT adhC cls*$^+$ *cysI53am*). Tetracycline resistant colonies were scored for *hemA*, *tryT*, *adhC* and *narC*. The arrangement of the genes was determined by three factor analysis (Table 2.4). Reciprocal crosses were also performed in which SOH16 was the donor and SOH17 was the recipient (Table 2.5). The data from both crosses are consistent with a clockwise gene order of *hemA-narC-tyrT-adhC-cls*::Tn*10*dTet3. In so far as there is overlap, this order is consistent with the results of Cunningham and Clark (50). These workers reported a clockwise gene order of *narC-tyrT-bgl-galU-adhC-trp*.

Growth of strains with a defective cls is inhibited at high pH. CL mutants are much more sensitive to alkaline pH culture conditions than are their wild type parents (25). As illustrated in Figure 2.3, HW55 (*glpR cls*-1) and SOH13 (*glpR cls*::Tn*10*dTet3) grow normally at pH 7.3 but fail to do so at pH 9.3. In contrast, the wild type parent HW56 (*glpR*) grows reasonably well at both pH's (these pH experiments were performed by L. Ragolia).

Isolation of a colony overproducing CL: While no gene fusions between *cls* and *lacZ* were obtained by inserting λp*lac*Mu9 or λp*lac*Mu53 into the *E. coli* chromosome, a mutant overproducing *cls* was isolated. Strain SOH19 (MC4100 *glpR glpD narC*::Tn*10*) was infected with λp*lac*Mu53. Kanamycin resistant colonies (about 5 x 10^4), were pooled and infected with $P1_{vir}$. The resulting P1 lysate was used to infect SOH2 (MC4100 *glpR glpD*). Approximately ten thousand tetracycline resistant colonies were isolated. Upon replica plating these colonies onto kanamycin plates 100 cells survived. Kanamycin resistant colonies were replica plated onto GL-DBP plates and finally onto tetracycline plates containing XG. Two colonies, one white and one blue (a blue color indicates β-galactosidase activity), were obtained. The blue colony had a normal phosphoglyceride composition. The white colony, SOH53, was unable to grow on minimal media and was resistant to both kanamycin

and tetracycline. The ability of the SOH53 to survive a selection on a minimal medium plate (DBP- succinate), may have been due to plating a large concentration of cells onto the plate. The phosphoglycerides from SOH53 were labelled with [^{14}C]acetate and separated in one dimension using Whatman SG81 chromatography paper. The labelled phosphoglycerides were identified using a radiochromatogram (21). Three peaks of radioactivity were observed (Table 2.6). These peaks migrated were PE, PG and CL would be expected to be. Strain SOH53 had a 50% increase in CL compared to its wild type derivative. The increase in CL was at the expense of PG.

DISCUSSION

Four independent *cls*::Tn*10*dTet insertion mutants were isolated. Each maps near *narC*, is resistant to DBP, and synthesizes 2-10 fold less CL than it's wild type parent (Tables 2.2 and 2.3). The insertion mutation from SOH9 (*cls*::Tn*10*dTet3), has been examined in considerable detail. Restriction analyses reveal that the insertion lies well within the essential region of the *cls* gene (22,30). The availability of a strain containing a *cls*::Tn*10*dTet provides a selection for *cls*$^-$. Using the tetracycline resistance as a marker for *cls* the position of *cls* with respect to neighboring gene was determined. As indicated by the data in Tables 2.4 and 2.5, the gene order is *hemA-narC-tyrT-adhC-cls* (Figure 2.2). This order clarifies the position of *cls*, which had been tentatively placed between *hemA* and *trp* (36).

The reason for not being able to insert λp*lac*Mu53 or λp*lac*Mu9 into *cls* is not readily apparent. Selecting for DBP^r or markers as close as 0.5 Kb to *cls*, using SOH25 (*zch*::Tn*10*dCam, see Table 3.1), failed to produce a gene fusion between *cls* and *lacZ*. The ability to create SOH20 (*narC*::Tn*5* *cls*::Tn*10*dTet) suggests that kanamycin resistance and a *cls*$^-$ genotype are compatible (Tn*5* contains a gene for kanamycin resistance). A possible explanation for the failure to obtain a *cls* to *lacZ* gene fusion

is that *cls* is part of an operon containing an essential gene downstream from *cls*. The ability of Tn*10*dTet to insert inside *cls* may be due to some promoter sequence at the terminus of Tn*10*dTet which allows transcription of downstream genes (46). Thus a Tn*10*dTet insert may not completely inhibit transcription of downstream genes.

The appearance of a mutant overproducing CL is also a mystery. Strain SOH53 is unable to grow on minimal media and grows poorly on rich medium. The only evidence that SOH53 is indeed *E. coli* is the appearance of three major phosphoglycerides migrating were PE, PG and CL are expected to migrate. A possible explanation for this mutant is a deletion causing *cls* to be under control of a strong promoter.

In comparing the results from bacteria incubated with either [^{32}P]phosphate or [^{14}C]acetate (Tables 2.2, 2.3, and 2.6), a difference in the percent of labelled CL was observed. Bacteria labelled with [^{14}C]acetate had a higher percentage of labelled CL, than bacteria labelled with [^{32}P]phosphate. There are several factors which may have contributed to this difference; (*i*) cells were incubated with [^{14}C]acetate at a density of 100 Klett units for one hour, as opposed to [^{32}P]phosphate being added to the cells at a 1-3 Klett units and present throughout cell growth; (*ii*) the stability of [^{14}C]acetate labelled phosphoglycerides compared to [^{32}]phosphate labelled phosphoglycerides; and (*iii*) differences in

chromatography ([^{14}C]acetate labelled phosphoglycerides were separated in one dimension using SG81 chromatography, as opposed to [^{32}P]phosphate labelled phosphoglycerides being separated either by one dimensional chromatography using HPTLC or by two dimensional chromatography).

TABLE 2.1

Strains, Phage, and Plasmids Used

Strain	Relevant properties	Source or reference
EE105	narG::Tn5	39
GE886	K12/pNK972	G. Weinstock
GE1031	supE42/Fzzf-1831::Tn10dTet	G. Weinstock
GE1033	supE42/Fzzf-1836::Tn10dCam	G. Weinstock
HW50	HfrC zch::Tn10 trp45 glpR glpD	21
HW55	HfrC glpR cls-1	21
HW56	HfrC glpR	21
JM105	Δ(lac proA,B) hsdR4 thi/F' proA,B lacI^{q}ZΔm15	51
JM246	cysI53(am) fails to grow on PN agar	B. Bachmann
Lin8	λ glpR glpD ΔphoA	52
MC4100	F$^-$ araD139 Δ(argF-lac)U169 rpsL150 relA1 deoC1 ptsF25 rbsR flbB5301	G. Weinstock
OS2101	pss-1 strA118	4,b
PRC607	narC::Tn10 adhC tyrT fadR mel	50
S730	F$^-$ purB51 hemA30 trp45 his68 tyrA2	B. Bachmann
SSR100	narC rpsL	39
SOH1	MC4100 zhe::Tn10 glpR glpD	(P1) HW11 X MC4100
SOH2	MC4100 glpR glpD (derived from SOH1 by selecting for fusaric acid resistance)	This study
SOH3	MC4100 glpR glpD trp45 zch::Tn10	(P1) HW50 X SOH2

Table 2.1

SOH4	MC4100 glpR glpD cls-1	(P1) HW55 X SOH3
SOH5	MC4100 glpR glpD narC	(P1) SSR100 X SOH3
SOH6	MC4100 glpR glpD cls::Tn10dTet1	a
SOH7	MC4100 glpR glpD cls::Tn10dTet2A	a,d
SOH8	MC4100 glpR glpD cls::Tn10dTet2B	a,d
SOH9	MC4100 glpR glpD cls::Tn10dTet3	a
SOH10	MC4100 glpR glpD cls::Tn10dTet4	a
SOH11	cysI53(am)	(P1) Lin 8 X JM246
SOH12	Lin 8 cls::Tn10dTet3	(P1) SOH9 X Lin8
SOH13	HW56 cls::Tn10dTet3	(P1) SOH9 X HW56
SOH14	narC cls::Tn10dTet3	(P1) SOH12 X SSR100
SOH15	cysI53(am) narC::Tn10 adhC tyrT	(P1) PRC607 X SOH11
SOH16	hemA adhC tyrT cysI53(am) zcd::Tn10dCam	a
SOH17	narC cysI53(am) cls::Tn10dTet3	(P1) SOH14 X SOH11
SOH18	Lin8 narC cls::Tn10dTet3	(P1) SOH9 x SOH5
SOH19	MC4100 glpR glpD narC::Tn10	c
SOH20	MC4100 glpR glpD narG::Tn5 cls::Tn10dTet3	(P1) EE105 x SOH9
SOH53	narC::Tn10 λp*lac*Mu53	a

Phage

λplacMu9	imm λ 'lacZ lacY$^+$ lacA' 'ara' xho::kan Mu[cIts62ner$^+$A$^+$'s]	34

Table 2.1

λplacMu53	imm λ 'trp' 'lacZ$^+$ lacY$^+$ lacA' 'ara' 'uvrD'xho::kan Mu[cIts62ner$^+$A$^+$'s]	35
λpMu507	cI857 Sam7 Mu[cIts62ner$^+$A$^+$B$^+$]	34,35
Plasmids		
pIBI20	amp^r	e
pNK972	amp^r transposase	G. Weinstock
pSH9	amp^r cls::Tn10dTet3	a

Table 2.1.

a- Isolated as described in Materials and Methods.

b- B. Bachmann, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

c- Created by inserting Tn10 from λNK370 into *nar* (see Hwang pH.D thesis for procedure)

d- SOH7 and SOH8 were created using the same random Tn*10*dTet P1 lysate.

e- Product of IBI.

TABLE 2.2

Phospholipid Distribution Of *cls*::Tn*10*dTet Determined In One Dimension

	Mole %		
Strain	**PE**	**PG**	**CL**
SOH2 (*cls*$^+$)	73.3	23.2	2.5
SOH4 (*cls*-1)	74.0	25.0	1.0
SOH6 (*cls*::Tn*10*dTet1)	72.4	26.4	1.2
SOH7 (*cls*::Tn*10*dTet2A)	73.1	26.0	0.9
SOH8 (*cls*::Tn*10*dTet2B)	76.2	23.1	0.7
SOH9 (*cls*::Tn*10*dTet3)	74.6	24.8	0.6
SOH10 (*cls*::Tn*10*dTet4)	73.3	25.9	0.8

Table 2.2. Phospholipid distribution, as determined by labeling with 5-10 μCi/mL [^{32}P]phosphate. Radioactive phosphate was added to bacteria at a cell density of 1-3 Klett units. Bacteria were cultured shaking in 5.0 mL of LB broth at 37 °C. After the bacteria reached stationary phase the phospholipids were isolated and separated by one dimensional chromatography using a HPTLC plate.

TABLE 2.3

Phospholipid Distribution Of SOH9

Strain	Mole % PE	PG	CL
SOH2 (*cls*$^+$)	72.3	24.0	2.8
SOH9 (*cls*::Tn*10*dTet3)	74.0	25.8	0.2

Table 2.3. Phospholipids distribution, as determined by labeling with 5-10 μCi/mL [^{32}P]phosphate. Radioactive phosphate was added to bacteria at a cell density of 1-3 Klett units. Bacteria were cultured shaking in 5.0 mL of LB broth at 37 °C. When the bacteria reached stationary phase the phospholipids were isolated and separated by two dimensional chromatography.

TABLE 2.4

Transductional Analysis Of Tn*10*dTet3

Donor	Recipient	Selected Marker	Unselected Markers	Number of Cotransductants
SOH17	SOH16	Tet	*adhC*, *tyrT*	7
			adhC, *tyr*$^+$	0
			adh$^+$, *tyrT*	21
			adh$^+$, *tyr*$^+$	218
SOH17	SOH16	Tet	*hem*$^+$, *nar*$^+$	2
			hem$^+$, *narC*	38
			hemA, *nar*$^+$	42
			hemA, *narC*	164
SOH17	SOH16	Tet	*adhC*, *narC*	0
			adhC, *nar*$^+$	7
			adh$^+$, *narC*	206
			adh$^+$, *nar*$^+$	33

Indicated gene order *hemA-narC-tyrT-adhC-cls*::Tn*10*dTet3

Table 2.4. Transduction of SOH16 (*hemA*, *nar*$^+$, *tyrT*, *adhC*, *cls*$^+$, *cysI53*am) with a P1 lysate from SOH17 (*hem*$^+$, *narC*, *tyr*$^+$, *adh*$^+$, *cls*::Tn*10*dTet3, *cysI53*am). The indicated gene order was determined by three factor analysis. Except when testing for *hemA* all test and selection plates contained 4 μg/mL δ-aminolevulinic acid. Colonies were scored as follows: *cls*::Tn*10*dTet3- tetracycline resistant, *adhC*- red on TTC-ethanol plates, *tyrT*- grows on minimal plates without cysteine, *narC*- red on TTC-nitrate plates, *hemA*- requires δ-aminolevulinic acid for growth on LB plates. The overall gene order is shown in Figure 2.2.

TABLE 2.5

Transductional Analysis Of Tn*10*dTet3

Donor	Recipient	Selected Marker	Unselected Marker	Number of Cotransductants
SOH16	SOH17	*nar*$^+$	*adhC*, *tyrT*	272
			adhC, *tyr*$^+$	0
			adh$^+$, *tyrT*	53
			adh$^+$, *tyr*$^+$	11
SOH16	SOH17	*nar*$^+$	*tyrT*, Tet^r	61
			tyrT, Tet^s	264
			tyr$^+$, Tet^r	11
			tyr$^+$, Tet^s	0

Indicated Gene Order *narC-tyrT-adhC-cls*::Tn*10*dTet3

Table 2.5. Transduction of SOH17 (*hem*$^+$, *narC*, *tyr*$^+$, *adh*$^+$, *cls*::Tn*10*dTet3, *cysI53*am) with a P1 lysate from SOH16 (*hemA*, *nar*$^+$, *tyrT*, *adhC*, *cls*$^+$, *cysI53*am). The indicated gene order was determined by looking for rare quadruple recombinants. All test and selection plates contained 4 µg/mL δ-aminolevulinic acid. Colonies were scored as follows: *cls*::Tn*10*dTet3- tetracycline resistant, *adhC*- red on TTC-ethanol plates, *tyrT*- grows on minimal plates without cysteine, *narC*- red on TTC-nitrate plates, and *hemA* requires δ-aminolevulinic acid for growth on LB plates. The overall gene order is shown in Figure 2.2.

Table 2.6

Phospholipid Distribution
Of A Strain Overproducing CL

	Mole %		
Strain	**PE**	**PG**	**CL**
SOH53	65.4	18.7	16.8
SOH19	63.0	26.0	11.0
QC120	71.9	27.4	0.7

Table 2.6. Bacteria were grown shaking in 5.0 mL of LB broth at 37° C. At a cell density of 100 Klett units 1 μCi/mL [^{14}C]acetate was added. After an additional hour of incubation the cells were centrifuged and the phosphoglycerides were isolated and separated by one dimensional chromatography (21).

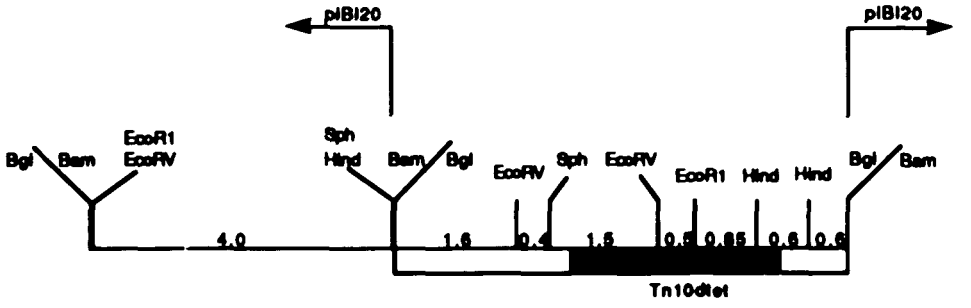

Figure 2.1. Restriction enzyme analysis of pSH9. The following restriction enzymes were used: Bam (BamH1), Bgl (BglII), Hind (HindIII), EcoR1, EcoRV, Sph (SphI). Solid line: pIBI20, open box chromosomal DNA, closed box Tn*10*dTet. Distances refer to Kilobases.

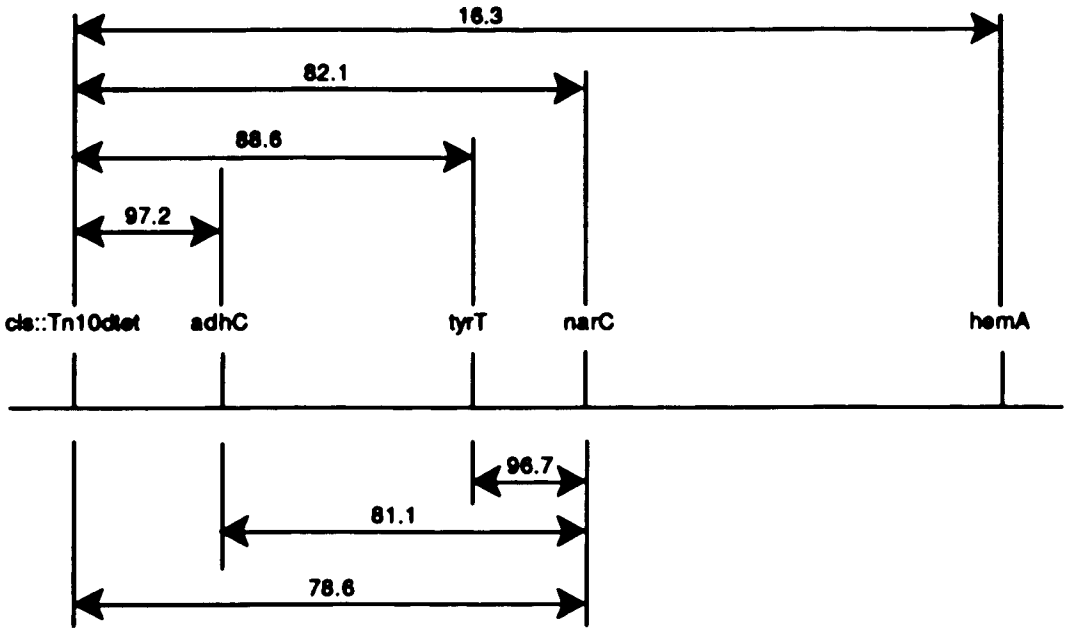

Figure 2.2. Chromosomal location of *cls*::Tn*10*dTet. Gene order was determined by three factor analysis. Numbers refer to cotransductional frequency obtained from the data in Tables 2.4 and 2.5.

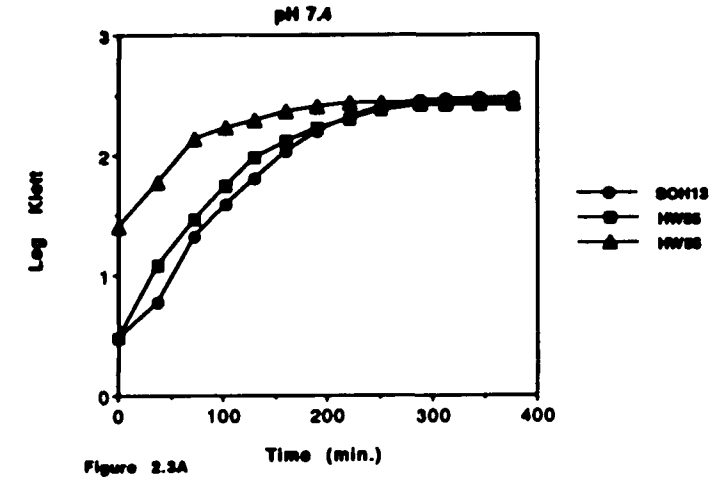

Figure 2.3A

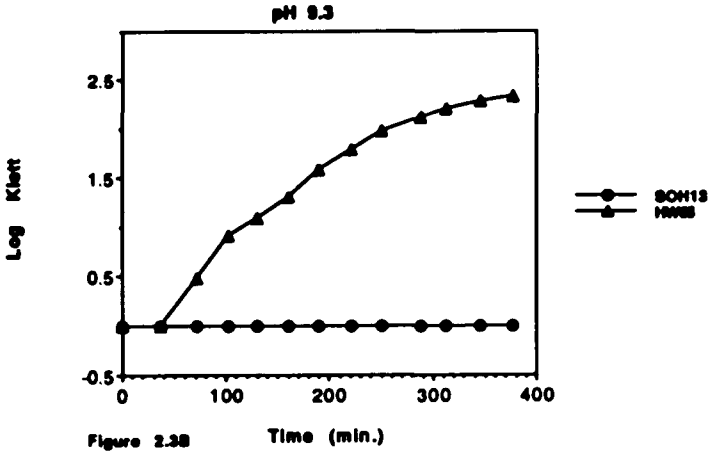

Figure 2.3B

Figure 2.3A. Growth of HW56 (*glpR*), HW55 (*glpR cls-1*), and SOH13 (*glpR cls*::Tn*10*dTet3), in LB broth at pH 7.4. Fresh LB was inoculated to 3 Klett units with cells from an overnight culture of the same medium.

Figure 2.3B. Growth of HW56 (*glpR*), SOH13 (*glpR cls*::Tn*10*dTet3), in LB broth at pH 9.3. Fresh LB broth was inoculated to 3 Klett units with cells from an overnight culture in LB broth at pH 7.4. HW55 responded in exactly the same fashion as SOH13 (data not shown).

CHAPTER 3

Cloning *cls*

INTRODUCTION

Despite extensive efforts to exploit λp*lac*Mu53 and λp*lac*Mu9 to isolate gene fusions between *cls* and *lacZ*, none were obtained. Recombinant DNA technology offers an alternative approach for constructing the desired fusions. However, it is first necessary to clone the *cls* gene. Unfortunately, there is no simple selection technique for the *cls* gene. While a cls^- genotype confers increased resistance to DBP, it is doubtful that DBP resistance could be used to select for cls^+ clones. Two serious obstacles are DBP resistance being a negative selection and resistance to the drug being very leaky (25).

A better selection procedure for cloning *cls* makes use of the inability of *pss*-1 *cls*-1 double mutants to grow at 42° C (30). If either a wild type *cls* or *pss* gene is present the mutant can grow at the elevated temperature. While this selection procedure has been used successfully to clone *cls* (30), growth at 42° C due to the reversion of *pss*-1 to pss^+ is a major obstacle.

A third scheme for cloning *cls*, which may be used as a general procedure to clone genes which lack a readily selectable phenotype, is to clone the gene of interest by selecting for a neighboring gene. For this strategy to work the nearby gene must be readily selectable and close to the gene of interest. If there are no neighboring genes fulfilling these requirements a transposon can be inserted next to the gene of interest.

The first step in successfully cloning *cls* was to insert Tn*10*dCam next to *cls*. A partial Sau3A digest of chromosomal DNA from SOH25 (*zch::*Tn*10*dCam) was ligated to pBR322. Transformation of HB101 (*recA13*) with the ligation mix resulted in the formation of a chloramphenicol resistant colony. This colony was found to contain a plasmid, which was named pSH101. To determine whether pSH101 contains a functioning *cls*, the plasmid was transferred into LR120 (*cls*$^-$ *recA56 srl::*Tn*10*). The percentage of CL inside LR120/pSH101 is 17 fold greater than in LR120. Restriction enzyme sites in pSH101 correspond to those reported for *cls* (22,30).

The *cls* gene was subcloned to yield two smaller plasmids, pSH102 and pSH103. Both of these plasmids enabled *cls*$^-$ strains to synthesize elevated levels of CL. Bacteria containing a cloned *cls* and CL overproducing colonies, QC120/pSH105x and SOH53 (see Chapter 2 for a description of SOH53), grow poorly on minimal media.

MATERIAL AND METHODS.

Chemicals: Ampicillin, tetracycline, chloramphenicol, and triphenyltetrazolium chloride (TTC)[1] were purchased from Sigma Chemical Co., St. Louis, Mo. Bacto-tryptone, Bacto-yeast extract, Bacto-agar were products of Difco Laboratories, Detroit, Mich. Agarose, low melting point agarose, bacterial alkaline phosphatase, proteinase K, restriction enzymes, restriction enzyme buffers, T4 DNA ligase, and T4 ligase buffer were obtained from BRL., Gaithersburg, MD., or IBI., New Haven. CT. Carrier free [^{32}P]phosphate was purchased from ICN., Irvine, CA. Polygram Sil G TLC plates were a product of Brinkmann Instruments, Inc., Westbury, N.Y. GENECLEAN kit was obtained from BIO 101 Inc. P.O. Box 2284 La Jolla, CA. All other chemicals were reagent grade or better.

Bacteria and Plasmids: Bacteria and plasmids used are described in Table 3.1. Strain HB101 (*recA*13) was used as the recipient in transformations involving newly constructed plasmids. When made competent, HB101 is transformed at a high frequency (53). Two copies of pSR105/SSR100 were obtained from DeMoss. DNA obtained from the first copy is denoted pSR105x.

Media and growth conditions: LB broth consisted of 1.0% tryptone, 0.5% yeast extract, and 0.5% sodium chloride. Unless otherwise

stated, LB broth was adjusted to pH 7.4 with sodium hydroxide. TTC-nitrate plates contained LB broth, 1.0% potassium nitrate, and 0.005% triphenyltetrazolium chloride (TTC) (39). TYM consisted of 2.0% bacto-tryptone, 0.5% yeast extract, 0.1 M sodium chloride and, 10 mM magnesium sulfate. GL minimal medium was that of Garen and Levinthal but lacked casein hydrolysate (38). Minimal medium M9 was made as described by Miller (43). Minimal medium was supplemented with 0.4% of the specified carbon source, and 20 μg/μL of required *L*-amino acids. Liquid media was solidified using 1.5% bacto-agar. Ampicillin, tetracycline, chloramphenicol, kanamycin, and nalidixic acid, were used at 100, 20, 20, 35 and 20 μg/mL, respectively. Unless otherwise stated, bacteria were cultured at 37° C.

Insertion of Tn*10*dCam next to *cls*. Random Tn*10*dCam pools were constructed using GE886 (K12/pNK972) as described in Chapter 2. The P1 lysates, obtained by infecting the pools with $P1_{vir}$, was used to transduce SOH5 (*narC*). Transductants were selected on TTC-nitrate plates supplemented with chloramphenicol. Colonies having Tn*10*dCam inserted near *narC*, and consequently near *cls* were selected as white chloramphenicol resistant transductants. The proximity of Tn*10*dCam to *cls* was determined by transducing SOH14 (*narC cls*::Tn*10*dTet3) with a P1 lysate from each of the white chloramphenicol resistant bacteria. The

Tn*10*dCam from two purified colonies, MEG9 and MEG19, cotransduced with *cls* at a frequency greater than 95%. The insert from MEG9 was transferred to LK16 (MC4100 *glpR*), by P1 transduction, to form SOH25 (MC4100 *glpR* zch::Tn*10*dCam). Chromosomal DNA from SOH25 was used to clone *cls*.

DNA preparation and manipulation. Unless otherwise stated chromosomal and plasmid DNA were isolated as described in the Material and Methods section in Chapter 2 (32,47,48). Plasmid and chromosomal DNA were stored in TE buffer containing 20 μg/ml RNase, at -20° C (TE buffer consisted of 10 mM Tris-HCl and 1 mM EDTA; pH 8.0). DNA fragments were separated by agarose gel electrophoresis (typically 0.9% agarose was used). Two types of buffers were used during the electrophoresis: TAE (40 mM Tris-HCl, 20 mM sodium acetate, and 2 mM EDTA; pH 8.3) and TBE (80 mM Tris-HCl, 40 mM boric acid, and 2 mM EDTA; pH 8.0). TAE buffer was used for overnight electrophoresis or when the DNA, separated by electrophoresis, was to be used in future ligations. TBE buffer was used for over day electrophoresis (typically 2-6 hours). Restriction enzyme digests used 1-10 μg DNA and took place in 1x buffer (supplied by BRL or IBI). DNA concentration was estimated either on plates (comparing intensity of ethidium bromide staining against

DNA standards, 32), or by A_{260} (one A_{260} unit corresponds to 50 μg/mL double stranded DNA, 48).

DNA fragments used in subcloning were isolated from low melting point agarose. DNA was digested with restriction enzymes and loaded onto an agarose gel consisting of 1.0% low melting agarose. Different size DNA fragments were separated by electrophoresis in TAE buffer either overday (2 to 6 hours at 50-150 volts) or overnight (15-20 volts). DNA bands were stained with ethidium bromide and visualized using UV light. Individual bands were cut out of the gel with a razor blade and stored at 5° C.

When needed for ligations, DNA fragments could be eluted from low melting point agarose. No further purifications of the DNA obtained from low melting point agarose was necessary (ethidium bromide was not removed from the agarose). The agarose was liquified by heating for 15 minutes at 65° C. The DNA was removed, using a pipetman, and transferred to a microfuge containing 25-50 μL of 1x ligation buffer. Typically 25 uL of liquified agarose were used, though the amount varied depending upon the concentration of the DNA in the agarose.

Ligations between chromosomal and vector DNA were carried out by overnight incubation at 11° C in 1x ligation buffer (BRL ligation buffer contained 66 mM Tris-HCl pH 7.6, 6.6 mM $MgCl_2$, 10 mM DTT, and

0.4 mM ATP), containing 1 unit of T4 DNA ligase. Ligations involving two "sticky ends", were performed overnight at 11° C in 1x ligation buffer and 0.01 units of T4 DNA ligase. Ligations involving two "blunt ends", or both a blunt end and a sticky end, took place at room temperature for two hours in 1x ligation buffer containing 1.0 unit of T4 DNA ligase.

DNA was transferred into bacteria using either the high efficiency transformation protocol of Mike Scott (personal communication from Brian Seed), or a low efficiency colony transformation (53). The high efficiency procedure was carried out as follows: cells were grown overnight on a LB plate. The following morning a single colony was transferred from the LB plate into a 250 mL flask containing 20 mL of TYM broth. The bacteria were cultured shaking at 37° C. When the cells reached mid log phase (25-70 Klett units), they were transferred into a 2.0 L flask containing 100 mL TYM and incubated shaking at 37° C. Upon reaching an OD_{600} of 0.5-0.9, an additional 380 mL of TYM were added to the culture. The cells were again incubated shaking at 37° C. When OD_{600} reached 0.6, the cells were rapidly cooled in an ice water bath, centrifuged at 5° C, and resuspended in 100 mL cold TfBI (TfBI consisted of 30 mM potassium acetate, 50 mM manganese chloride, 100 mM potassium chloride, 10 mM calcium chloride and 15% (v/v) glycerol) by shaking gently in an ice water bath. This suspension was centrifuged and the

pellet resuspended (as in the previous step), in 20 mL of cold TfBII (TfBII consisted of 10 mM sodium-MOPS, pH 7.0; 75 mM calcium chloride; 10 mM potassium chloride; and 15% (v/v) glycerol). Cell aliquots ranging from 0.1-0.6 mL were transferred to prechilled sterile microfuge tubes and rapidly frozen in liquid nitrogen. Competent bacteria were stored at -70° C.

Highly competent bacteria were transformed, in a microfuge tube, by thawing a frozen aliquot of cells at room temperature, adding DNA (from 1-10 μg in a volume less than 10 μL) to 200 μl of cells, and incubating on ice. After 15-30 minutes, the cells were heat shocked at either 37° C or 42° C for 3 min, followed by the addition of 1.0 mL of LB broth. The bacteria were then incubated at 37° C for 90 minutes. After this outgrowth the cells were centrifuged for 15 seconds in a microfuge, resuspended in 0.1 mL LB broth, and spread on to a selective plate.

Cloning the *cls* gene. Initial efforts to clone *cls* focused on the neighboring *narC* gene. Plasmid pSR105 containing *narC*, and 10 Kb of nearby chromosomal DNA, was obtained from DeMoss (39). Strain QC120 (*cls*$^-$) was transformed with DNA from SSR100/pSR105x. The DNA was isolated according to Klein *et al.* (54), and bacteria were made competent according to Silhavy *et al.* (32). Transformed colonies were selected on LB plates containing either tetracycline, ampicillin or

kanamycin. Transformed cells were checked for the two drug markers not used in the initial selection. Colonies resistant to all three drug markers were grown with [^{14}C]acetate and their phospholipids were isolated (21). The phospholipids were separated by one dimensional chromatography using Whatman SG81 paper in a chloroform:methanol:acetic acid 65:25:8 solvent system. Radioactive phospholipids were located using a radiochromatogram. Bacteria transformed with DNA from SSR100/pSR105x overproduced a phospholipid migrating where CL would be expected to migrate (Table 3.2). DNA isolated from QC120/pSR105x was transferred back into QC120. Recipient colonies which became resistant to ampicillin, kanamycin, and tetracycline also overproduced CL. Thus pSR105x appeared to contain the *cls* gene.

Unfortunately, further analysis of pSR105x revealed several disturbing results. Not all colonies transformed with DNA from SSR100/pSR105x or QC120/pSR105x were resistant to ampicillin, kanamycin and tetracycline. More troubling was the inability to observe plasmid DNA isolated from SSR100/pSR105x or QC120/pSR105x, after agarose gel electrophoresis. Two additional phenotypes were observed for QC120/pSR105x: they formed red colonies on TTC-Nitrate plates and grew poorly on minimal media plates.

Another copy of SSR100/pSR105 was obtained from DeMoss. Plasmid DNA from this copy was isolated as described in the Material and Methods section of Chapter 2. The presence of a plasmid was confirmed by visualization on agarose gel. Bacteria transformed with pSR105 were white on TTC-nitrate plates. Unfortunately QC120 (*cls*$^-$) transformed with the newly obtained pSR105 was still deficient in CL. Thus the overproduction of CL was not due to a cloned *cls* gene.

A second effort to clone *cls*, making use of the proximity of *cls* to *narC*, was performed using a random λSE6 pool. This pool contained random inserts of chromosomal DNA, 15 Kb in length, inserted inside λSE6. Strain SOH18 (Lin 8 *narC cls*::Tn*10*dTet3) was infected with the λSE6 pool and white kanamycin resistant colonies were isolated (λSE6 contains a kanamycin resistance gene). None of these colonies produced wild type levels of CL.

To maximize the probability of cloning of *cls*, by selecting for a neighboring gene, a Tn*10*dCam was inserted to within 0.5 Kb of *cls*. DNA from the resulting strain, SOH25 (zch::Tn*10*dCam), was used in the subsequent cloning of *cls*. DNA ranging in size from 10-18 Kb was obtained by partial digestion with Sau3A. Optimal conditions for obtaining the desired fragments were established using different concentrations of Sau3A as described by Silhavy *et al.* (32). The digested

DNA was separated by agarose gel electrophoresis. DNA fragments ranging from 10-18Kb were cut from the gel using a razor blade. DNA was eluted from the gel using a GENECLEN kit as described by the manufacturer (the GENECLEN kit works by binding DNA to a silica matrix, washing out contaminants, and eluting the purified DNA from the matrix).

Purified chromosomal DNA was ligated overnight to BamH1 digested pBR322. The next day highly competent HB101 (*recA13*) was transformed with the ligation mix and spread onto LB plates containing chloramphenicol. After 36 hours of incubation a single colony grew on the chloramphenicol plate. This colony was ampicillin resistant and tetracycline sensitive. The colony was found to contain a recombinant plasmid which was called pSH101. The ability of pSH101 to code for *cls* was determined by transferring the plasmid into LR120 (*cls-1 recA56 srl::*Tn*10*) and measuring the production of CL (Table 3.2). Digesting pSH101 with different combinations of restriction enzymes led to the identification of several restriction sites (Figure 3.1).

Subcloning *cls*. Based upon the restriction map of pSH101, a 2.15 Kb PvuII-PvuII fragment was predicted to contain *cls*. This fragment was cloned into pBR322 which had been digested with PvuII and EcoRV. The newly constructed plasmid, pSH102, contains *cls* and a part of Tn*10*dCam

upstream from the *cls* promoter. An additional plasmid, pSH103, was derived from pSH102 by transferring a 1.75 Kb HpaI-PvuII fragment containing *cls*, from pSH102 into the PvuII-EcoRV sites in pBR322. Construction of these plasmids is summarized in Figure 3.1.

Lipid analysis. Phospholipids from QC30 (*pss-1*) and QC300 (*cls-1 pss-1*) were labeled with [^{32}P]phosphate in the following manner: bacteria were grown in 2-8 mL of LB broth overnight at 30° C. The overnight culture was used to inoculate 5 mL of fresh LB broth to a density of 2-5 Klett units. Bacteria were cultured shaking at 30° C. After one hour 5 μCi/mL [^{32}P]phosphate was added. Cells were allowed to continue growing at 35° C. Unless otherwise stated, the phospholipids from bacteria containing a wild type *pss* were labeled as described in the Material and Methods section of Chapter 2.

Labelling of the phospholipids was stopped at mid log phase (100-200 Klett units) by centrifuging the cells and resuspending them in 1 mL of distilled water. Phospholipids were isolated as previously described (21). Separation and detection of phospholipids were as described in Chapter 2.

RESULTS

Cloning *cls*: The Tn*10*dCam inside SOH25 (zch::Tn*10*dCam) cotransduces with *cls* at a frequency greater than 95%. This cotransductional frequency corresponds to approximately 0.5 Kb (based upon the relationship between cotransduction frequency and map distance, 55). This distance between the Tn*10*dCam insert and *cls* is small enough to ensure an excellent chance of cloning *cls* by selecting for Tn*10*dCam.

Strain HB101 (*recA13*) was transformed using an overnight ligation mixture containing BamH1 digested pBR322 and SOH25 chromosomal DNA, ranging in size from 10-18 Kb, that had been obtained by a partial Sau3A digest. One chloramphenicol resistant colony appeared after 36 hours incubation at 37° C on a LB plate containing chloramphenicol. This strain was found to contain a plasmid, pSH101, which was able to confer ampicillin and chloramphenicol resistance, but not tetracycline resistance. The inactivation of the tetracycline resistance gene was due to insertion of chromosomal DNA into the BamH1 site of pBR322.

Plasmid pSH101 was introduced into LR120 (*cls-1 recA56 srl::*Tn*10*). When LR120/pSH101 was grown in LB broth, the bacteria grew poorly and cell debris was observable. Lipid analysis of LR120/pSH101 demonstrates the presence of a functioning *cls* in pSH101

(Table 3.3). The restriction map pSH101 contains restriction sites previously reported for *cls* (22,30) (Figure 3.1).

Subcloning *cls*: Plasmid pSH101 is over 18 Kb and contains a Tn*10*dCam upstream from the *cls* promoter (Fig 3.1). To better study *cls*, a 2.15 Kb PvuII-PvuII fragment containing *cls* and part of Tn*10*dCam was subcloned into pBR322. The resulting plasmid pSH102 is 4.8 Kb and contains only 0.2 Kb of Tn*10*dCam. To remove all traces of Tn*10*dCam and eliminate a HpaI site, pSH102 was digested with an excess of PvuII and partially digested with HpaI (removal of the HpaI site is important in the subsequent creation of gene fusions between *cls* and *lacZ* see Chapter 4). A 1.75 Kb HpaI-PvuII fragment from pSH102, isolated in low melting point agarose and predicted to contain *cls*, was cloned into pBR322. The resulting plasmid, pSH103, is approximately 4.35 Kb and contains no Tn*10*dCam DNA. Both pSH102 and pSH103 when transferred into LR120 (*cls-1 recA56 srl::*Tn*10*), caused a 8-20 fold increase in the level of CL (Table 3.4). An even more dramatic CL increase was observed when pSH102 or pSH103, were inserted into QC300 (*cls-1 pss-1*) (Table 3.5).

Additional properties of plasmids containing *cls*: Bacteria containing pSH101, pSH102, and pSH103 were checked for additional phenotypes on rich medium and minimal medium. While strains containing pSH102 or pSH103 grow well in LB broth, bacteria containing

pSH101 grow slowly in LB broth and on LB plates. The effect of DBP on LR120 (*cls-1 recA56 srl::*Tn*10*) containing pSH102 or pSH103 was checked on GL plates supplemented with 0.4% potassium succinate, 20 μM *S*-DBP, and 100 μg/ml ampicillin. As expected, bacteria containing a cloned *cls* gene grew poorly or not at all on this minimal medium. However, bacteria containing a cloned *cls* also failed to grow in the absence of DBP. Growth inhibition was also observed using glucose as the carbon source and with minimal medium M9. Strain LR120 transformed with either pSH322, pLK103 (chloramphenicol resistance gene inserted at the BglII site of *cls*) or pSH120 (*lacZ* inserted inside *cls*, see Table 4.1) grew normally on minimal media (Table 3.6). Growth inhibition due to a cloned *cls* was also observed in LR104 (*cls-1 srl::*Tn*10*) transformed with either pSH102 or pSH103.

DISCUSSION

As a prelude to creating *in vitro* gene fusions, between *cls* and *lacZ*, the *cls* gene was cloned. Initial efforts to clone *cls* focused on the proximity of *cls* to *narC* (approximately 7.0 Kb Figure 1.1). Plasmid pSR105 containing *narC* and an additional 10 Kb of chromosomal DNA, located on one side of *narC*, was obtained from DeMoss (60). Because of the small distance between *cls* and *narC* it seemed possible that pSR105 may also contain *cls*. Analysis of lipid extracts from QC120, transformed with DNA from SSR100/pSR105x, indicated the presence of a functioning *cls*. However, further investigation failed to confirm the presence of any plasmid DNA. Apparently some chromosomal rearrangement took place causing CL to be overproduced, possibly a chromosomal deletion fusing *cls* to an active promoter.

The next effort to clone *cls*, by selecting for *narC*, involved screening a λSE6 pool for phage able to complement *narC* and confer the ability to synthesize CL. Unfortunately these attempts were unsuccessful. A difficulty in using *narC*, or any neighboring gene, as a selectable marker is the decrease in transformation efficiency as the size of the transforming DNA increases (56). The closer the selectable gene is located to *cls* the better the odds of successfully cloning *cls*.

To maximize the possibility of cloning *cls*, a Tn*10*dCam was inserted about 0.5 Kb from *cls*. DNA fragments ranging in size from 10-18 Kb were isolated from SOH25 (*zch::*Tn*10*dCam) and ligated into pBR322. Plasmid pSH101 containing Tn*10*dCam was selected by transforming HB101 (*recA13*) and plating onto LB plates containing chloramphenicol. Plasmid pSH101, along with two smaller derivatives pSH102 and pSH103, enable *cls-1* bacteria to synthesize CL.

Physiological consequences of a cloned *cls* are not readily apparent. Bacteria containing pSH101 grow slowly on LB plates or in LB broth. Cells containing pSH102 or pSH103 grow normally on LB plates and in LB broth. Possibly, pSH101 contains a gene, other than *cls*, which has a harmful effect when present on a multicopy plasmid. The *cls* gene itself, when present on a multicopy plasmid inhibits growth of bacteria on minimal media (Table 3.6). This inhibition is not always complete. Occasionally strains harboring pSH102 or pSH103 grew to some degree on minimal medium plates. The partial growth may have been due to a high cell density, or carry over of nutrients from bacteria initially cultured on LB plates. Further examination of the growth inhibition of a cloned *cls* may prove interesting, particularly, in view of the inability of SOH53 or QC120/pSR105x to grow on minimal medium.

In addition to facilitating the study of *cls* at the genetic level, the cloned *cls* is being used to study CL synthase at the enzymatic level. By expressing *cls* in a high expression vector large quantities of the enzyme have been obtained (57,58). This readily available enzyme has been used to purify and characterize CL synthase (L. Ragolia, unpublished Data).

TABLE 3.1

Strain, Phage, and Plasmid List

Strain	Relevant properties	Source or reference
GE1033	supE42/Fzzf-1836::Tn10dCam	G. Weinstock
GE1806	HfrH KL16 recA56 srl::Tn10dTet	G. Weinstock
HB101	F' hsds20(r_B^-, m_B^-) recA13 supE44 ara14 galK2 lacY1 proA2 rpsL20(sm^r) xyl15 leu mtl1 λ^-	53
HW55	HfrC glpR cls-1	21
HW56	HfrC glpR	21
KA197	HfrKL16 thi-1 pheA97 relA1 spo77	B. Bachmann
KS1	HfrKL16 thi-1 pheA97 relA1 spo77 nal^r (spontaneous nal^r mutant of KA197)	This study
KS1A	MC4100 glpR glpD nal^r pheA97	a
KS2A	MC4100 glpR glpD nal^r pheA97 trp45 zch::Tn10	(P1) HW50 X KS1A
KS3A	MC4100 glpR glpD pss-1 trp45 zch::Tn10	(P1) OS2101 X KS2A
Lin8	λ glpR glpD ΔphoA	d
LK16	MC4100 glpR	(P1) HW56 x SOH2
LR104	glpR glpD recA56 srl::Tn10	(P1) GE1806 X QC104
LR120	glpR glpD *cls*-1 recA56 srl::Tn10	(P1) GE1806 X QC120
MEG9	MC4100 glpR glpD zch::Tn10dCam	c
MEG19	MC4100 glpR glpD zch::Tn10dCam2	c
OS2101	pss-1 strA118	4,b

Table 3.1

QC104	glpR glpD	21
QC120	glpR glpD cls-1	21
QC120/pSR105x	amp^r tet^r kan^r	c
QC30	MC4100 glpR glpD pss-1	(P1) Lin 8 X KS3A
QC300	MC4100 glpR glpD pss-1 cls-1	(P1) HW55 X KS3A
SOH2	MC4100 glpR glpD	d
SOH5	MC4100 glpR glpD narC	d
SOH9	MC4100 glpR glpD cls::Tn10dTet3	d
SOH14	narC cls::Tn10dTet3	d
SOH18	Lin8 narC cls::Tn10dTet3	d
SOH25	MC4100 glpR zch::Tn10dCam	(P1) MEG9 x LK16
Phage		
λSE6	kan	59
Plasmid		
pBR322	amp^r Tet^r	e
pSH322	amp^r	c
pSH101	amp^r Tn10dCam cls^+	c
pSH102	amp^r cls^+ part of Tn10dCam	c
pSH103	amp^r cls^+	c
pSR105	amp^r tet^r narG::Tn5	60
pSR201	amp^r tet^r narG::Tn5	60
pSR9	amp^r	60

Table 3.1

pLK103	amp^r cls::Tn10dCam	f
pSH120	amp^r Kan^r ϕ(cls-'lacZ)hyb	e

Table 3.1.

a- conjugation using KS1A as the donor and SOH2 as the recipient.

b- B. Bachmann, *E. coli* Genetic Stock Center, Yale University, New Haven, Conn.

c- See Material and Methods. The selected marker was nal^r.

d- See Table 2.1.

e- See Table 4.1.

f- The plasmid pLK103 was made by inserting a chloramphenicol resistance gene into pSH103 (Figure 3.1). The chloramphenicol resistance gene was obtained by digesting pSH101 with BamH1 and isolated as a 1.5 Kb fragment (in low melting point agarose). This fragment was inserted into the BglII site of pSH103.

TABLE 3.2

Phospholipid Distribution Of *narC* Plasmids

Strain	Mole % PE	PG	CL
QC120/pSR105x	71.0	16.3	12.7
QC120/pSR201	74.5	24.5	1.0
QC120/pSR9	72.2	26.4	1.4
QC120	75.5	23.6	0.9

Table 3.2. Plasmid bearing strains and QC120/pSR105x were grown shaking at 37° C in 5.0 mL of LB broth supplemented with 100 μg/mL ampicillin. At a cell density of 100 Klett units 1 μCi/mL of [^{14}C]acetate was added. Bacteria were incubated for another hour and then the phospholipids were isolated. Strain QC120 (*cls-1*) was cultured without ampicillin. Phospholipids were isolated, and separated by one dimensional chromatography (21).

TABLE 3.3

Phospholipid Distribution Of Cloned *cls*

	Mole %		
Strain	**PE**	**PG**	**CL**
LR120	70.2	27.4	0.6
LR120/pSH101	55.0	34.8	10.2

Table 3.3. Bacteria were grown shaking in 5.0 mL of LB broth at 37° C. Phospholipids were labelled with 5-10 μCi/mL of [^{32}P]phosphate. Radioactive phosphate was added to bacteria at a cell density of 1-3 Klett units. Phospholipids were isolated at mid log phase (100-200 Klett units). The plasmid inside LR120 (*cls-1 recA56 srl::*Tn*10*) was maintained by the addition of chloramphenicol to a concentration of 15 μg/mL. Phospholipids were separated by two dimensional chromatography.

TABLE 3.4

Phospholipid Distribution
Of subcloned *cls*

Mole %

Strain	PE	PG	CL
LR120	75.3	24.4	0.3
LR104	75.3	21.2	3.5
LR120/pSH322	76.4	23.3	0.3
LR120/pSH102	70.1	24.0	5.9
LR120/pSH103	77.3	17.2	5.5

Table 3.4. Strains LR104 (*cls*$^+$ *recA56 srl::*Tn*10*) and LR120 (*cls-1 recA56 srl::*Tn*10*) were cultured shaking in 5.0 mL of LB broth at 37° C. Phospholipids were labelled with 5-10 μCi/mL of [^{32}P]phosphate. Radioactive phosphate was added to bacteria at a cell density of 1-3 Klett units. Phospholipids were isolated at mid log phase (100-200 Klett units). The plasmid inside LR120 was maintained by the addition of ampicillin to a concentration of 100 μg/mL. Phospholipids were separated by two dimensional chromatography.

TABLE 3.5

Phospholipid Distribution Of Subcloned *cls* Inside QC300

	Mole %		
Strain	**PE**	**PG**	**CL**
QC300/pSH102	69.7	19.3	11.0
QC300/pSH103	70.7	17.5	11.8
QC300/pSH322	58.1	41.8	≤0.1

Table 3.5. Strain QC300 (*cls-1 pss-1*) containing pSH102, pSH103 or pSH322 was grown shaking in 5.0 mL of LB broth at 35° C. Phospholipids were labelled with 5-10 μCi/mL of [^{32}P]phosphate. Radioactive phosphate was added to bacteria at a cell density of 1-3 Klett units. Phospholipids were isolated at mid log phase (100-200 Klett units). The plasmids were maintained by addition of ampicillin to a concentration of 100 μg/mL. Phospholipids were separated by two dimensional chromatography.

TABLE 3.6

Effect of a cloned *cls* on growth of LR120

Strain	*cls*	Growth
LR120/pSH322	-	+
LR120/pSH102	+	-
LR120/pSH103	+	-
LR120/pSH120	-	+
LR120/pLK103	-	+

Table 3.6. Strain LR120 (*cls-1 recA56 srl::*Tn*10*) containing different plasmids was checked for growth on GL minimal medium plates supplemented with 0.4% potassium succinate and 100 μg/mL ampicillin. Bacteria were checked for growth after 24 hours. All the strains grew on LB plates containing ampicillin. *cls* + indicates the presence of a cloned *cls*. Growth + indicates growth. See Chapter 4 for description of pSH120.

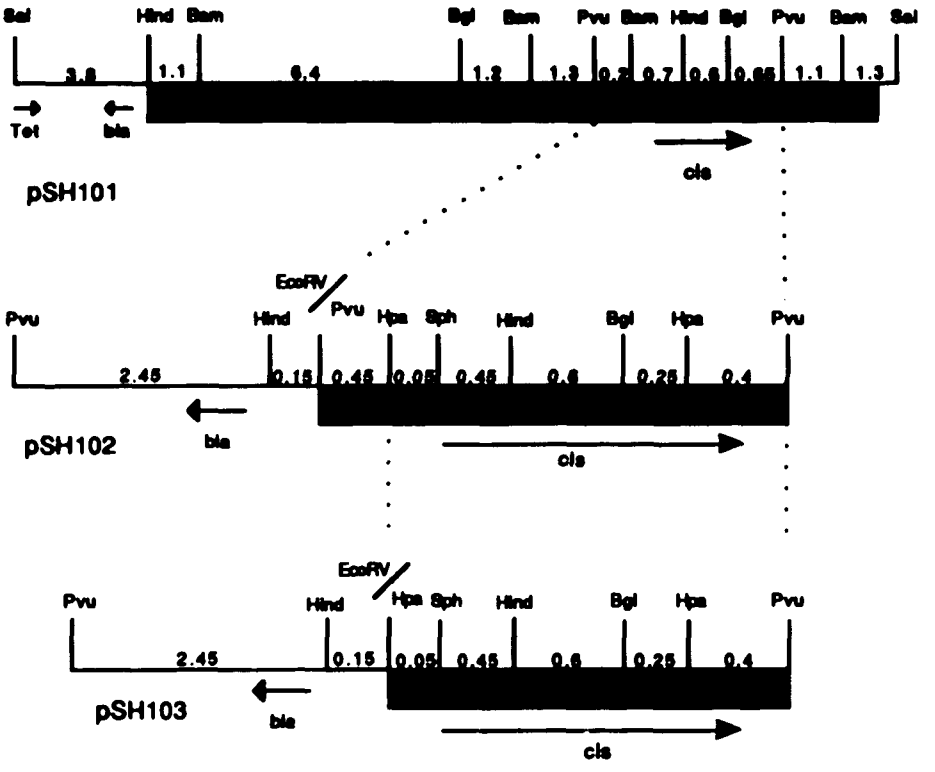

Figure 3.1. Restriction maps of pSH101, pSH102 and pSH103. Restriction enzymes used; Bam (BamH1), Bgl (BglII), EcoRV, Hind (HindIII), Hpa (HpaI), Pvu (PvuII), Sal (SalI), Sph (SphI). Solid line- pBR322 DNA. Black box- chromosomal DNA. Checked box- Tn*10*dCam DNA. The restriction map of pSH101 is incomplete (not all the PvuII or HindIII sites were determined). The plasmid pSH101 was obtained as described in the Material and Methods section. Plasmid pSH102 was subcloned from pSH101, by digesting pSH101 with PvuII, isolating a 2.15 Kb fragment in low melting point agarose, and ligating the fragment to pBR322, which had been cut with EcoRV and PvuII. Selection for pSH102 was based upon ampicillin resistance. Plasmid pSH103 was derived from pSH102 by first digesting pSH102 with an excess of PvuII and then different concentrations of HpaI. A 1.75 Kb fragment containing the *cls* gene was isolated in low melting point agarose. This fragment was ligated to pBR322 which had been cut with EcoRV and PvuII. All three plasmids contain a functioning *cls*. Distances refer to Kilobases.

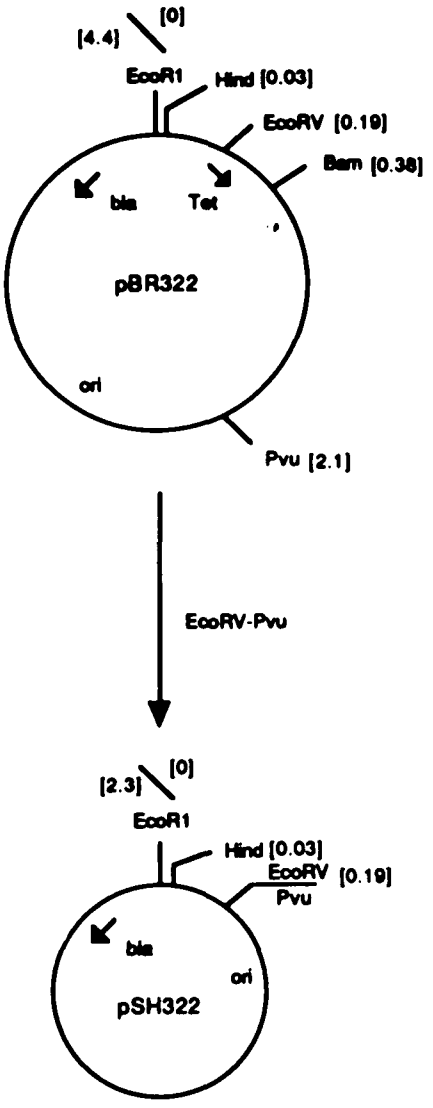

Figure 3.2. Restriction map of pSH322. Restriction enzymes; Bam (BamH1), EcoR1, EcoRV, Hind (HindIII), Pvu (PvuII). The plasmid pSH322 was created by digesting pBR322 with EcoRV and PvuII. Because EcoRV and PvuII are both blunt ends pSH322 could be formed by recirclization. Colonies containing pSH322 were selected on LB plates containing ampicillin. Distances refer to Kilobases.

CHAPTER 4

Expression of *cls*ϕ*lacZ*

INTRODUCTION

To examine the regulation of *cls*, it is essential to be able to measure the gene product. Unfortunately, CL synthase is a difficult enzyme to assay. The current assay system is poorly defined, consisting of a mixture of radioactive PG and a crude membrane fraction (24,30). CL formation is determined by isolating the phospholipids, separating them by two dimensional chromatography, detecting the labelled phospholipids using autoradiography, and measuring the amount of radioactive phospholipids formed. Aside from the technical difficulties in assaying CL synthase, are the problems in determining the significance of a specific activity obtained under different physiological conditions. The crude membrane preparation used to assay CL synthase contain a large quantity of the reactant, PG, and the product, CL. The interpretation of kinetic data is further complicated, because the reaction leading to the formation of CL is probably reversible (61).

A powerful technique which can be used to study gene regulation is the creation of gene fusions. Gene fusions involve the formation of hybrid operons or hybrid proteins (32,33,34,35). One part of the fusion contains the promoter of the gene of interest. The other part of the fusion contains a gene coding for a reporter protein. An extremely useful reporter protein is β-galactosidase. β-galactosidase activity can be assayed using a variety of substrates. These substrates facilitate the measurement of the fused gene product and the isolation of regulatory mutants.

Numerous efforts were made to fuse *cls* to *lacZ*. Initial attempts were performed using λ*plac*Mu9 and λ*plac*Mu53. As described in Chapter 3, these *in vivo* attempts were unsuccessful. Consequently, the *cls* gene was cloned and *in vitro* fusions between *lacZ* and *cls* were constructed. The *cls* gene provided the promoter enabling the *lacZ* gene to be turned on. To better study the regulation of *cls*, a single copy of the gene fusion was transferred into the λ attachment site.

Using SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls-'lacZ*)hyb], expression of *cls* was investigated. Physiological conditions causing a two to three fold increase in expression of the operon and protein fusion were observed. These were phase of growth, different growth mediums, and different electron acceptors. An increase in temperature resulted in an increase in expression of the operon fusion but not the protein fusion.

Under a variety of physiological conditions there was less than a 2 fold increase in expression of *cls*ϕ*lacZ*. Phosphate starvation caused a slight increase in β-galactosidase production, in strains bearing the protein and operon fusion. There was little or no change in β-galactosidase production, of strains bearing either an operon or a protein fusion, in response to the addition of phenethly alcohol or arbutin, or to a change in the osmolarity or pH of the medium.

Various genetic manipulations were performed on SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls-'lacZ*)hyb]. These included introduction of a cloned *cls*, a disrupted *cls* (*cls*::Tn*10*dTet3), a *pss-1* allele, and a *fnr-250* allele. None of these manipulations produced a significant change in expression of the gene fusions.

The gene fusions were also used to search for regulatory mutants. One stable insertion mutation, causing an increase in β-galactosidase activity, was isolated. The mutation was found to map near *lacZ*. A mutant with a decrease in β-galactosidase activity was also isolated. Further investigation of the up and down mutations failed to reveal any connection between regulation of *cls* and β-galactosidase activity.

MATERIAL AND METHODS

Chemicals: Ampicillin, tetracycline, chloramphenicol, bovine serum albumin (fraction V), Sephadex G-50 (G-50-300μ), thimerosal, triphenyltetrazolium chloride, Tween 40, o-onitrophenyl-β-D-galactoside (ONPG)[1], 5-bromo-4-chloro-3-indolyl-phosphate-p-toluidene (XP)[1], and p-nitrophenyl phosphate, were purchased from the Sigma Chemical Co., St. Louis, Mo. Bacto-antibiotic medium 2, Bacto-Tryptone, Bacto-yeast extract, Bacto-agar, Bacto-MacConkey agar base CS, and Bacto-MacConkey agar were products of Difco Laboratories, Detroit, Mich. Agarose, low melting point agarose, bacterial alkaline phosphatase (BAP)[1], BluGene detection kit, proteinase K, restriction enzymes, restriction enzyme buffers, streptavidin-alkaline phosphatase, T4 DNA ligase, ethidium bromide, sodium dodecyl sulfate (SDS)[1] and T4 ligase buffer were obtained from BRL., Gaithersburg, MD., or IBI., New Haven. CT. Calf intestine alkaline phosphatase (CIAP)[1] and 5-bromo-4-chloro-indolyl-β-D-galactose (XG) were obtained from Boehringer Mannheim Biochemicals, Indianapolis, IN. Nylon 66 plus hybridization transfer membrane and transphore unit are products of Hoefer Scientific Instruments, San Francisco, CA. Bromophenol blue was obtained from MCB, Norwood OH. Gene Screen Plus hybridization transfer membrane came form NEN

Research Products, Boston MA. Carnation natural nonfat dry milk, fortified with vitamins A and D, is a product of the Carnation company, Los Angeles, CA. Dextran sulfate was obtained from Pharmicia, Piscataway, NJ. Blotting paper was obtained from Schleicher and Schuell Inc., Keene, NH. Carrier free [^{32}P]phosphate was purchased from ICN., Irvine, CA. Polygram Sil G TLC plates were a product of Brinkmann Instruments, Inc., Westbury, NY. Anti-β-galactosidase purified mouse monoclonal antibody, anti-mouse IgG (H+L) alkaline phosphatase conjugated antibody, nitroblue tetrazolium (NBT)[1], and bromochloroindolyl phosphate (BCIP)[1] were purchased from Promega Corporation, Madison WI. All other chemicals were reagent grade or better.

Bacterial strains: Strains listed in Table 4.1 were created by standard genetic techniques (32,43). To move the *recA56* gene into different genetic backgrounds a Tn*10*dCam was inserted next to *recA56*. Strain SE5000 (*recA56*) was transformed with pNK972. An ampicillin resistant transformant was infected with a P1 lysate from GE1033 (*supE42/Fzzf*-1836::Tn*10*dCam) and chloramphenicol resistant colonies were selected. Approximately 10^3 chloramphenicol resistant transductants were pooled and infected with $P1_{vir}$. The resulting P1 lysate was used to transduce SOH121 [*srl::*Tn*10* ϕ(*cls-lacZ*$^+$)]. Transductants containing a functioning *srl* were selected by their red appearance on MacConkey-

sorbitol plates containing chloramphenicol (*srl* maps near *recA*, 36). A *recA* phenotype was determined by measuring UV sensitivity. The resulting strain, SOH127 [ϕ(*cls-lacZ*$^+$) *zeg::*Tn*10*dcam *recA56*], was used to transfer the *recA56* allele into different genetic backgrounds.

To facilitate transfer of *pss-1* into different genetic backgrounds, a Tn*10*dTet was inserted near *pss-1* (all strains containing *pss-1* were grown at 30° C unless otherwise stated). Strain QC30 (*pss-1*) was transformed with pNK972. An ampicillin resistant transformant was isolated and infected with a P1 lysate from GE1031 (*supE42/fzzf*-1831::Tn*10*dTet). Approximately 5 x 10^4 tetracycline resistant colonies were pooled and infected with $P1_{vir}$. The resulting P1 lysate was used to transduce KA197 (*pheA*). Transductants were selected on glucose minimal media plates containing tetracycline. Selection of tetracycline resistant *phe*$^+$ colonies enriched for inserts near both *pheA* and *pss* (*pheA* map near *pss*, 36). A *pss-1* phenotype was indicated by the inability to grow at 42° C on glucose M56/2 plates. The resulting strain, SOH142 (*pss-1 zee::*Tn*10*dTet), was used to transfer the *pss-1* allele into different genetic backgrounds.

Media and growth conditions: LB broth consisted of 1.0% tryptone, 0.5% yeast extract, and 0.5% sodium chloride. Unless otherwise stated, LB broth was adjusted to pH 7.4 using sodium hydroxide.

MacConkey-sorbitol plates were made by adding 40 g Bacto-MacConkey agar base to 950 mL water, autoclaving, then adding 50 mL 20% sorbitol. XP-plates consisted of MOPS medium lacking phosphate, containing 0.2% glucose, and 100 μL of 20 mg/mL XP (62). XP was spread onto the plates 30 minutes before the plates were to be used. Stock solutions of XP were prepared in N,N-dimethyl formamide.

MOPS medium made to a concentration of 10x consisted of the following (63); 400 ml of freshly prepared 1.0 M potassium morpholinopropane sulfonate (MOPS)[1], pH 7.4 with KOH; 40 ml of freshly prepared 1.0 M tricine, pH 7.4 with KOH; 10 mL of freshly prepared 0.01 M $FeSO_4$; 50 mL of 1.90 M NH_4Cl; 10 mL of 0.276 M K_2SO_4; 10 ml of 5 x 10^{-4} M $CaCl_2$; 10 mL of 0.528 M $MgCl_2$; 100 mL of 5.0 M NaCl; and 10 mL of micronutrients. The final volume was adjusted to 1.0 L using distilled water. Micronutrients consisted of 10 mL of the following; $(NH_4)_6(MO_7)_{24}$, 3 x 10^{-6} M; H_3BO_3, 4 x 10^{-4} M; $CoCl_2$, 3 x 10^{-5} M; $CuSO_4$-$5H_2O$, 1 x 10^{-5} M; $MnCl_2$, 8 x 10^{-5} M; and $ZnSO_4$, 1 x 10^{-5} M. MOPS medium was filter sterilized and stored at either -20° C or 5° C. Before use, MOPS medium was diluted with sterile distilled water and supplemented as needed with K_2HPO_4, carbon source, nucleotides, vitamins or amino acids.

Low osmolarity medium (LEM) was made as described by Kennedy (64). The medium contained; KH_2PO_4, 0.5 mM adjusted to pH 7.0 with Tris-base; $(NH_4)_2SO_4$, 15 mM; $MgSO_4$ 0.8 mM; Tris-HCl pH 7.0, 200 mM; glucose, 5 mg/mL; casein hydrolysate, 5 mg/mL; plus required amino acids and vitamins.

The minimal media of Spencer and Guest (SG)' (65), was made by preparing a 1.0 liter solution containing the following; KH_2PO_4, 5.44 g; K_2HPO_4, 10.49 g; $(NH_4)_2SO_4$, 2.0 g. In addition, 40 mM fumaric acid (pH 7.0 with 3.75 g NaOH) or 40 mM nitrate were added when cells were cultured under anaerobic conditions in medium containing glycerol. The solution was autoclaved and supplemented with the following (prepared separately); 1.0 mL metal ion mix; 5 mL 10% casamino acid; carbon source (40 mM glycerol or 0.4% of another carbon source); and any required vitamins or amino acids. The metal ion mix, which can be sterilized by autoclaving, consisted of the following; $MgSO_4$, 400 mM; $MnSO_4$-H_2O, 25 mM; $Fe_2(SO_4)_3$-nH_2O, 1 mM; and $CaCl_2$, 5 mM.

Solutions for Southern Analysis: Solutions used in performing the Southern analysis are listed in appendix 1.

Solutions for Western Analysis: Solution used in performing the Western analysis are listed in appendix 2.

Measuring β-galactosidase activity: Production of β-galactosidase was determined in solid medium using three different types of plates:

1- XG plates- A stock solution containing 100 µL of 20 mg/mL 5-bromo-4-chloro-indolyl-β-D-galactose (XG)[1] was spread onto LB plates, 30 minutes before using. The stock solution of XG was prepared in N,N-dimethyl formamide. A blue or green appearance indicates β-galactosidase activity.

2- MacConkey-lactose plates- These plates contained 50 g Bacto-MacConkey agar base CS, per liter of water. A red to purple color indicates β-galactosidase activity.

3- TTC-lactose plates- These plates contained 25.5 g Bacto-antibiotic medium 2 added to 950 mL of water. After autoclaving for 20 min, 50 mg TTC (5 mL of a 1% solution), and 50 mL of 20% lactose were added. At a low cell density, colonies having β-galactosidase activity appear white. At a high cell density colonies, having β-galactosidase activity appear red (32).

In liquid medium, β-galactosidase activity was assayed using ONPG. Cells which grew to an OD_{600} greater than 0.65 were diluted before assaying. Cells were disrupted using chloroform and SDS. Assay conditions were described by Miller (43). Units of β-galactosidase were

determined according to the following equation (43).

$$\frac{OD_{420} - 1.75 \times OD_{550}}{OD_{600}}$$

The OD_{550} term corrects for cell debris interfering with the OD_{420} reading (if cell debris is removed OD_{550} is equal to zero). The OD_{600} term corrects for the number of cells being assayed.

DNA isolation and manipulation: Restriction enzymes, T4 DNA ligase, bacterial alkaline phosphatase and calf intestine alkaline phosphatase were all used according to their manufacture's specifications. Chromosomal and plasmid DNA were isolated as described in the Material and Methods section of Chapter 2. DNA was stained with ethidium bromide as described in the Material and Methods section of Chapter 2. DNA fragments were isolated from low melting point agarose as described in the Material and Methods section of Chapter 3. Cells were made competent as described in the Methods and Material section of Chapter 3.

Creation of *cls* operon and protein fusions: Two sets of operon and protein fusions were constructed. One set, pSH113 and pSH115, was designed to be inserted into the λ attachment site (66). This set contains a kanamycin resistance gene and lacks any pBR322 DNA upstream from the *cls* promoter. The other set, pSH120 and pSH121, was constructed to be used in site directed insertion (67,68). This set of plasmids contain a *cls*

gene disrupted by the *lac* operon and a near by kanamycin resistance gene. A linear pSH120 or pSH121 should be able to replace *cls* with ϕ(*cls*-'*lacZ*)hyb or ϕ(*cls*-*lacZ*$^+$), when inserted into JC7623 (*recB recC sbcB*) (69,70).

Plasmid pSH112 was used in the construction of operon and protein fusions (except pSH120). Construction of pSH112 was accomplished by subcloning a 1.75 Kb fragment containing *cls*, from pSH103, into pRS415 (Figure 4.1). The 1.75 Kb fragment was excised from pSH103 by digesting completely with PvuII and partially with HpaI. The fragment was isolated in low melting point agarose and inserted into the SmaI site of pRS415 (the SmaI site was dephosphoralated using BAP). Because the *cls* gene is contained on a fragment containing two blunt ends, it can be inserted in two possible orientations. Plasmids with both orientations were obtained (Figure 4.1). Plasmid pSH111 has the T1 transcription terminator located downstream from *cls* (this plasmid was used in the subsequent transfer of *cls* to a high expression vector by L. Ragolia). While pSH112 has the terminator upstream from *cls*.

Protein fusions were made by inserting a series of cassettes into pSH103 (Figure 4.2). These cassettes contain a polylinker region, a promoterless *lacZY* and a kanamycin resistance gene. The polylinker region of each cassette differs, from that of the other, by one nucleotide.

Thus, one of these three cassettes should give an in frame protein fusion. A cassette was excised from either pLKC480, pLKC481 or pLKC482 by simultaneous digestion with SmaI and BamHI. The DNA was obtained as 6.3 Kb fragments in low melting point agarose. Each of the cassettes were ligated to the BglII-HpaI sites in pSH103. Ligation was performed for two hours at room temperature in 1x ligation buffer, using 1.0 unit of T4 ligase (BRL ligation buffer consisted of 66 mM Tris-HCl pH 7.6, 6.6 mM $MgCl_2$, 10 mM DTT, and 0.4 mM ATP). The ligation mix was used to transform highly competent HB101. Colonies containing a hybrid plasmid were selected on LB plates containing kanamycin. Plasmid DNA from 12 colonies were isolated (four colonies from each cassette). Each of these DNA preparations were simultaneously digested with EcoR1 and EcoRV. As expected, all of the hybrid plasmids contained a 2.2 Kb EcoR1-EcoRV fragment indicating the cassette was inserted into pSH103 (Figure 4.2).

One DNA preparation from each of the protein fusions was inserted into highly competent MC4100 (Δ*lac*). Transformants were selected on LB plates containing XG and either kanamycin or ampicillin. Approximately 10^4 colonies were obtained on each of these plates for each protein fusion. The only protein fusion to give a dark green color on XG after 24 hours was derived from pLKC480. Strain MC4100 containing the

protein fusion derived from pLKC481 had a very light green color after 48 hours. Strain MC4100 containing the protein fusion derived from pLKC482 had no green color after 48 hours.

The newly constructed hybrid plasmid pSH120 contains the *cls* promotor, part of *cls* joined to *lacZY*, and a 0.4 Kb fragment containing part of *cls* downstream from the *lac* operon (Figure 4.2). The possibility existed that *lacZY* activity was due to a promotor on pBR322 other than the *cls* promoter. To test this possibility, and move the fusions onto a pRS plasmid, pSH120 was digested completely with EcoRV and partially with HindIII, and a 1.7 Ko fragment was isolated in low melting point agarose. The fragment contains 0.5 Kb of *cls*, the polylinker region, and 1.2 Kb of *lacZ*. This fragment was inserted into the HindIII-EcoRV sites in pSH112 (Figure 4.3). The resulting plasmid, pSH114, when transferred into MC4100 was able to hydrolyze XG (Table 4.4). The presence of a T1 terminator in pSH114 just upstream from the *cls* promotor inhibits transcription of upstream promoters.

While pSH114 contains an in frame protein fusion, it lacks a gene for kanamycin resistance. Kanamycin resistance is useful for transferring the protein fusion into the bacterial chromosome. Plasmid pSH115, a kanamycin resistant derivative of pSH114, was constructed by moving the protein fusion along with part of *lacZ* into pRS552 (Figure 4.4).

An operon fusion between *cls* and *lac* was created by inserting the *cls* promoter along with part of the *cls* gene into pRS551 (Figure 4.3). A 1.1 Kb fragment containing the *cls* promoter was isolated in low melting point agarose, from pSH112 digested with EcoR1 and BglII. The recipient plasmid pRS551 was digested simultaneously with EcoR1 and BamH1. To prevent two pRS551 strands from ligating the digested pRS551 was treated with bacterial alkaline phosphatase. The treated pRS551 was ligated to the 1.1 Kb fragment containing *cls*. Highly competent HB101 was transformed with the ligation mix. Bacteria containing the recombinant DNA were selected on LB plates containing kanamycin. Plasmid DNA was isolated from the kanamycin resistant bacteria and analyzed with restriction enzymes (Figure 4.3). The newly constructed plasmid, pSH113, was transferred into MC4100 (Δ*lac*) and checked for the ability to hydrolyze XG (Table 4.4).

Two additional plasmids, pSH116 and pSH121, were constructed (Figures 4.5 and 4.6). Plasmid pSH116 is a kanamycin derivative of pSH112. It was constructed by transferring the *cls* gene from pSH112 into pRS551. Plasmid pSH121 is an operon fusion containing *lacZ* between two *cls* fragments. Construction of pSH121 was achieved by inserting an EcoR1-EcoRV fragment, obtained from pSH113 (isolated in

low melting point agarose), into the EcoR1-EcoRV sites of pSH120 (Figure 4.5).

Recombinant plasmids were tested for the ability to hydrolyze XG (Table 4.4) and produce CL. CL production was measured in QC300 (*cls-1 pss-1*) (Table 4.5). Because QC300 produces very little CL the ability of the plasmids to produce CL can be readily observed. As expected, only pSH116, which contains an intact *cls* gene, was able to increase CL production.

Insertion of fusions into the λ attachment site. The protein fusion, operon fusion, and corresponding protein and operon fusion vectors were transferred into the *E. coli* λ attachment site. Lysogens were selected according to Simons and Kleckner (66). Lysogens were induced, and amplified according to the procedures described by Silhavy *et. al.* (32).

Plasmid bearing strains MC4100/pSH113, MC4100/pSH115, MC4100/pRS551, MC4100/pRS552 were transferred to λ top agar, which was poured onto a LB plate containing kanamycin. Different concentrations of λRS45, in 50 μL aliquots, were applied to the plate. Lysogens were selected from zones of lysis and purified twice on LB plates. Confirmation of λ lysogens was accomplished by cross streaking against λ*NK330* and λ*imm441*. Lysogens SOH70 (MC4100/pRS551 λ*RS45*), SOH71 (MC4100/pRS552 λ*RS45*), SOH72 (MC4100/pSH113

λ*RS45*) and SOH73 (MC4100/pSH115 λ*RS45*), were resistant to λ*NK330* but sensitive to λ*imm441*. Each of these lysogens are ampicillin resistant, indicating the maintenance of their respective plasmid.

The next step was to transfer the fusion, from the plasmid bearing lysogen, into the λRS45 located at the λ attachment site. Lysates were obtained from SOH70, SOH71, SOH72 and SOH73 (32). Top agar, containing MC4100 (Δ*lac*), was poured onto a LB plate (32). Strains SOH70, SOH71, SOH72 and SOH73 were stabbed into the plate. Lysates were induced using UV light and obtained as agar plugs. These lysates were used to infect MC4100. Purified plaques from each lysate were amplified. The resulting λ's, λSOH80, λSOH81, λSOH82, and λSOH83, were used to transfer the fusion into MC4100 or P90C (Δ*lac*).

The presence of a single lysogen at the λ attachment site was tested by measuring the expression of β-galactosidase (Table 4.6). To determine the chromosomal location, lysogens were transduced with a P1 lysate from MS18087 (*nadA::*Tn*10*). The Tn*10* in MS18087 (*nadA::*Tn*10*) maps near the λ attachment site (36).

Southern Analysis: Retention of *cls*ϕ*lacZ* fusions inside the λ attachment site was checked by a Southern analysis. The Southern analysis consisted of four parts: preparation of probe, transfer of chromosomal DNA onto hybridization paper, hybridization of probe to

chromosomal DNA, and detection of probe hybridized to chromosomal DNA.

Probe was obtained by labelling pSH103 with biotinated-UTP. The labelled nucleotide was incorporated into pSH103 using DNA polymerase I as described by the BRL Bluegene detection kit as follows: pipet into a 1.5 mL microfuge tube (sitting on ice) 5 μL of a nucleotide solutions (0.1 mM dCTP, 0.1 mM dGTP, 0.1 mM dATP, 500 mM Tris-HCl (pH 7.8), 50 mM $MgCl_2$, 100 mM 2-mercaptoethanol, and 100 μg/mL BSA), 2 μL pSH103 DNA (about 1 μg), 2.5 μL 0.4 mM biotin-UTP, and 35.5 μL water. Mix briefly. Add 5 μL of enzyme solution; 0.4 units/μL BRL DNA polymerase I, 40 pg/μL DNA Pol I/DNase (nick translation grade), 50 mM Tris-HCl (pH 7.5), 5 mM Mg-acetate, 1 mM 2-mercaptoethanol, 0.1 mM phenylmethylsulfonyl fluoride, 50% glycerol and 100 μg/mL nuclease-free BSA. Mix briefly. Centrifuge briefly to bring the liquid to the bottom of the microfuge tube. Incubate at 15° C for 90 minutes. Add 5 μL 300 mM Na_2EDTA (pH 8.0) and 1.25 μL of 5% (w/v) SDS.

Labelled probe was purified by gel filtration on a Sephadex G-50 column (G-50-300, particle size 100-300 μ) as described by BRL Blugene detection kit. Southern transfer was preformed on a trough (baking dish) with a piece of plexi-glass on top (Figure 4.7). The glass ran laterally across the trough but did not completely cover it. A sheet of Whatman

3MM paper was placed on top of the glass so that both ends of the paper were immersed in blotting solution (0.4 N NaOH and 0.6 M NaCl) (Figure 4.7).

Chromosomal DNA was digested with restriction enzymes and separated by overnight electrophoresis in 1.0% agarose (using TAE buffer). Chromosomal DNA was transferred onto a nitrocellulose membrane in the following manner: the gel was prepared by washing twice in 500 mL of 0.25 N HCl with constant agitation for 10 minutes, to break down large fragments enabling them to be transferred to the membrane (this step is omitted when looking at small fragments). It was then rinsed with water and washed in blotting solution with constant agitation for 30 minutes (shaking gently because rapid shaking may cause gel to break).

The hybridization membrane was cut to match the gel. The A and B sides of the membrane were labelled (B side of membrane curls up). The date and any other pertinent information were recorded on the B side. The membrane was fully submerged in water and agitated for 15 min. The membrane was transferred to blotting solution (just enough solution to cover the membrane).

Blotting solution was used to wet the Whatman 3MM paper on top of the plexiglass. A piece of uncut thin blotting paper (GB002) was placed onto the Whatman paper and air bubbles were removed (this was

done by rolling a disposable pipet up and down over the thin blotting paper). The thin blotting paper was thoroughly wet with blotting solution. The gel was turned upside down and placed onto the thin blotting paper. Air bubbles were carefully removed. The gel was wet with blotting solution. The B side of the membrane was placed onto the gel (air bubbles were removed). The following were stacked onto the membrane, in the following order: 4 sheets of thin blotting paper, 4-8 sheets of thick blotting paper (GB004), and 2-3 inches of paper towels (all the papers were cut to the same dimensions as the gel). A weight was placed on top of the paper towels.

The next day, the upper layers of paper were removed until only the nitrocellulose membrane, and gel sandwiched between thin blotting paper, were left. The "sandwich" was turned upside down so the gel was on top of the membrane. The top sheet of thin blotting paper was peeled off. The wells on the membrane were marked by indenting, with a pencil or a pen through the gel onto the membrane, and darkened with a marker. The membrane was placed in a tray containing a solution of 0.5 M Tris-HCl (pH 7.5) and 1.0 M NaCl and agitated for 15 minutes. The membrane was removed, with the B side down, and blotted dry on blotting paper. It was then allowed to air dry until the membrane started to curl. Before proceeding further, the gel was stained with ethidium bromide and any

remaining DNA was visualized using UV light. If the DNA was efficiently transferred, there should be very little DNA remaining (high molecule weight DNA was not transferred efficiently).

Probe was hybridized to bound DNA in the following manner: fresh prehybridization buffer was prepared (7 mL H_2O, 2 mL 20% filter sterilized dextran sulfate and 1 mL 10% SDS), mixed by inversion, and heated at 65° C for 10-15 minutes. Sodium Chloride (0.58 g NaCl) was added to the solution, which was mixed by inversion and heated at 65° C for 10-15 minutes. The blotted membrane was inserted into a hybridization bag. Heated prehybridization mix was poured onto the membrane. Air bubbles were gently removed. The bag was sealed and incubated in a 65° C water bath, shaking at about 50 RPM for at least 15 minutes. The probe mix (100 µL of 10 mg/mL salmon sperm DNA, 100 µL probe DNA, and 50 µL probe for the ladder, amounts of probe can be adjusted depending upon how dilute they are), was heated at 90-100° C for 10 minutes. A small hole was cut in the bag and the probe mix added. The bag was then resealed and incubated overnight at 65° C shaking at about 50 RPM.

Bound probe was detected in the following manner: the membrane was removed from the hybridization bag, by cutting three sides of the hybridization bag, and carefully transferred to a clean tray. It was washed

twice, each time with constant agitation, in 2x SSC (20x SSC consists of 3.0 M NaCl, 0.3 M sodium citrate pH 7.0), at room temperature for 5 min. The solution was poured off and washed twice, each time with constant agitation, using 2x SSC containing 1% SDS, at 65° C for 30 min. The solution was poured off and washed twice, each time shaking at room temperature for 30 minutes, with 0.1 SSC. The membrane was washed in buffer 1 (0.1 M Tris-HCl pH 7.5, 0.15 M NaCl) shaking for 1 minute. The solution was poured off and 50 mL of buffer 2 (3% w/v bovine serum albumin -fraction V- in buffer 1) was added. The membrane inside buffer 2 was agitated at 65° C for 1 hour. The solution was drained off. Streptavidin-alkaline phosphatase mix (5 ml buffer 1 and 5 µl streptavidin-alkaline phosphatase) was poured onto the membrane, and rocked back and forth over the membrane for 10 minutes. The solution was poured off and the membrane was washed twice with 400 mL of buffer 1 (agitating gently for 15 minutes at room temperature). Enough buffer 3 (0.1 M Tris-HCl pH 9.5, 0.1 M NaCl, 50 mM $MgCl_2$) was added to just cover the membrane, and the membrane and buffer were agitated for 10 minutes. The membrane was placed in a hybridization bag, which was then sealed on three sides within a few millimeters of the membrane. Developer (7.5 mL buffer 3, 33 µL NBT, and 25 µL BCIP, mix by swirling) was added. The forth side of the bag was sealed and the membrane plus

developer agitated at 50 RPM at room temperature. After bands were visualized, but before bands were obscured by background, the reaction was stopped by placing the membrane in TE buffer for at least 5 minutes.

Western analysis: Western analysis was carried out as described by Harlow and Lane (71). Proteins were isolated via a mini prep procedure. Cells were grown in 6.0 mL of LB broth to 50 Klett units (100 μg/mL ampicillin was used to maintain plasmids). Bacteria were pelleted then dissolved by repipetting in 500 μL of 2x loading buffer. The resuspended cells were transferred to a microfuge tube, which was placed inside boiling water for 1 minute, followed by 15 minute centrifugation. Cell debris was removed with a tooth pick and cleared lysates were stored at -20° C. Strain SOH141 (P90C *recA56 up::*Tn*10*dTet *zeg::*Tn*10*dCam) were prepared as above, except it was grown to 250 Klett units and 4 mL were used.

Isolated proteins were loaded, in 20 μL portions, onto two different 10% polyacrylamide gels. Different size proteins were separated by electrophoresis, which was carried out for about 90 minutes at a current of 40 mA. The proteins were visualized on one of gels by staining for 5 minutes with coomassie blue stain (25 mL isopropanol, 12.5 mL 80% acetic acid, 62.5 mL water and 0.25 gm coomassie brilliant blue, solution can be reused), followed by destaining with 1x destaining solution (2x

destaining; 25 mL methanol, 43.75 mL 80% acetic acid, adjust volume to 250 mL with water). The stained gel was used for MW estimation and verification of loaded protein (Figure 4.10A). The other gel was used to visualize β-galactosidase.

Transfer of proteins from the polyacrylamide gel onto a nylon membrane was achieved using a transphore unit in the following manner: a piece of nylon membrane, two sponges, and three pieces of 3MM Whatman chromatography paper were soaked in RB. While submerged in RB the following was placed onto one side of the transphore unit cassette, in the following order, one sponge, one piece of 3MM Whatman chromatography paper, the nylon membrane and the gel (the gel was kept wet at all times). The gel was covered with the remaining two pieces of 3MM Whatman chromatography paper. Air bubbles between the gel and the nylon membrane were removed. The other sponge was placed on top of the Whatman chromatography paper and the cassette was closed. The cassette was transferred to the transphore unit. The unit was filled to the indicated line with RB. The cassette was orientated so that the nylon membrane was between the positive electrode and the gel. The unit run on for 2 hours at 70 volts (transfer was performed at 5° C). The nylon membrane was carefully removed (may be air dried and stored at 4° C

before continuing). Transfer of protein from the gel onto the membrane was checked by staining the gel with coomassie blue.

The nylon membrane was placed in blocking solution, for at least 2 hours, constantly rocking at room temperature. The membrane was rinsed twice with TBST for 5 minutes at room temperature and transferred into a hybridization bag. Blocking solution, 10 mL, containing 2 μL of anti-β-galactosidase purified mouse monoclonal antibody was added. Air bubbles were removed, the bag was completely sealed, and rocked overnight at room temperature. The next morning the nylon membrane was transferred to a clean tray and washed 4 times with TBST at room temperature (5-10 minutes of agitation per wash). Blocking solution, 50 mL, containing 7.5 μL anti-mouse IgG (H+L) alkaline phosphatase conjugated antibody was added. The membrane plus the blocking solution was incubated for 1 hour with constant agitation at room temperature. The membrane was then rinsed 4 times, for 5-10 minutes each time, with TBST, followed by the addition of 10 mL of ABP, which contained 33 μL BCIP and 66 μL NBT (the solution was prepared just prior to use). The membrane plus the APB solution was incubated with constant agitation at room temperature until bands were visualized, but before background obscured the bands (about 20 minutes). The reaction was stopped with TE buffer.

Lipid analysis: Phospholipids were isolated as described in Chapters 2 and 3 Material and Methods.

Localization of β-galactosidase activity: The cellular location of the β-galactosidase activity in SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls-'lacZ*)hyb] was determined according to Silhavy *et al.* (72).

Determination of cardiolipin synthase activity: The activity of cardiolipin synthase was determined at early and late log phases. Strain 8 was cultured in five 2 L flasks, each containing 200 mL of LB broth. The cells were grown shaking at 37° C. At a cell density of 25-35 Klett units, the bacteria from four of the flasks (800 mL) were combined. The bacteria were centrifuged. Cells were resuspended in 10 mM Tris-HCl pH 7.5, containing 2 mM β-mercaptoethanol (10 mL of buffer were used per gram of cells, 25). The cells were disrupted using five 30 seconds bursts from a Bronson model W140D Sonifier, at a setting of 8 (25). Cell debris was removed by centrifuging at 8000 x g for 15 minutes. The cell envelope was pellet by centrifuging at 100,000 x g for 60 minutes.

The bacteria in the remaining 2.0 L flask were allowed to continue growing. When the cells reached a density of 300-325 Klett units, 80.0 mL of bacteria were harvested and the cell envelope isolated in the same way as described above.

Cardiolipin synthase assay conditions were those described by Tunitas and Cronan (24). The reaction was terminated by the addition of 3.6 mL of a chloroform:methanol mixture (1.2 mL chloroform and 2.4 mL methanol that was 0.1 N HCl). The phospholipids were isolated by a modified Bligh and Dyer (21). Phospholipids were separated in two dimensions and visualized by exposure to X-ray film. Radioactive spots were cut out and quantitated in an ISOCAP 300. Protein determination was done using Folin reagent (73).

Effect of KCN on *cls* expression: To measure the effect of KCN on *cls*ϕ*lacZ* expression, a sublethal concentration was used (74). Bacteria were grown shaking in LB broth at 37° C. At a cell density of 24-29 Klett units, KCN was added to a concentration of 150 μM. The cells were allowed to continue growing. At various time points aliquots were removed and assayed for β-galactosidase activity.

Effect of different electron acceptors: The expression of *cls* was measured in LB broth and GS medium using different electron acceptors. Anaerobic growth conditions were achieved by filling screw cap tubes to the top with approximately 15 mL of growth medium.

Measurement of *cls* expression in low phosphate medium: Strains SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls-'lacZ*)hyb] were grown overnight in MOPS medium supplemented with proline, thiamine, 0.4% glucose and

2.0 mM sodium phosphate. The next morning, the cells were harvested and washed twice in MOPS medium (lacking phosphate). Bacteria were resuspended in MOPS medium containing proline, thiamine, 0.4% glucose and either 2.0 mM phosphate or 0.2 mM phosphate. Cells were grown shaking at 37° C. The production of β-galactosidase and alkaline phosphatase were determined at early and late log phases (75).

Measurement of *cls* expression in media of varying osmolarity: To investigate the effect of osmolarity on *cls* expression, SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls-'lacZ*)hyb] were grown in medium containing 1.0% Bacto-tryptone, 0.5% Bacto-yeast extract, or the same medium supplemented with either 0.85 mM or 3.0 mM sodium chloride. Production of β-galactosidase was determined when the cells reached early log phase (25-35 Klett units). Because different osmolarities will affect cell size and consequently cell absorbance, β-galactosidase is expressed in Units x 10^7/cell number (Units were not divided by OD_{600}). The number of cells was estimated by plating out several dilutions of cells onto LB plates and counting the colonies.

Effect of arbutin on *cls* expression: The effect of arbutin on *cls* expression was determined in LEM medium using 17 mM arbutin (63).

Effect of phenethyl alcohol (PEA)[1] on *cls* expression: Strains SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls-'lacZ*)hyb] were grown in 10 mL

of LB broth to early log phase (25-35 Klett units). PEA was added to a concentration of 0.15% (v/v) and the incubation continued. Aliquots of cells were removed at various times and assayed for β-galactosidase activity.

Search for regulatory mutants: Random Tn*10*dTet inserts were created inside P90C/pNK972 by transducing with P1 lysate from GE1031 (*supE42/Fzzf*-1831::Tn*10*dTet), and selecting for tetracycline resistance. Approximately 5 x 10^5 tetracycline resistant colonies were pooled and infected with $P1_{vir}$. The resulting P1 lysate was used in subsequent Tn*10*dTet mutagenesis.

To search for *cls* down mutations, SOH92 [ϕ(*cls-lacZ*$^+$)] was transduced with a P1 lysate from the Tn*10*dTet pool. Transductants were selected on TTC-lactose plates containing tetracycline. A total of about 10^4 colonies were isolated. One of these colonies, SOH92-1 (SOH92 *dm::*Tn*10*dTet) was dark red, indicating a decrease in β-galactosidase activity. Strain SOH92-1 was infected with $P1_{vir}$. The subsequent P1 lysate was used to transduce SOH92. Each transductant of SOH92 showed a decrease in β-galactosidase activity on TTC-lactose plates and MacConkey-lactose plates.

To look for *cls* up mutations, SOH93 [ϕ(*cls-'lacZ*)hyb] was transduced with a P1 lysate from the Tn*10*dTet pool. In this case,

transductants were selected on MacConkey-lactose plates supplemented with tetracycline. A total of about 5 x 10^3 tetracycline resistant colonies were isolated. Four of these were dark purple on MacConkey-lactose plate. Three of the colonies were light red on a TTC-lactose plate. The fourth colony was white on a TTC-lactose plate. The dark purple color on MacConkey-lactose plates and the white appearance on TTC-lactose plates are both indicative of an elevated β-galactosidase activity.

Each of the colonies containing a putative up mutation was infected with $P1_{vir}$. The resulting P1 lysates were used to infect SOH93. The P1 lysates from the three light red putative up mutations gave less than 80% cotransduction of β-galactosidase activity and tetracycline resistance. The P1 lysate from the fourth colony, SOH93-1 [*up*::Tn10dTet ϕ(*cls*-'*lacZ*)hyb], gave a 100% cotransduction of these traits.

Analysis of up mutation: The Tn*10*dTet from SOH93-1 [*up*::Tn*10*dTet ϕ(*cls*-'*lacZ*)hyb] was transferred into SOH92 [ϕ(*cls*-*lacZ*$^+$)] and P90C (Δ*lac*), creating SOH110 [*up*::Tn*10*dTet ϕ(cls-*lacZ*$^+$) and SOH112 (Δ*lac up*::Tn*10*dTet) respectively. All strains containing the up mutation appeared as dark purple on MacConkey-lactose plates, white on TTC-lactose plates, and grew on minimal medium lactose plates. The ability of SOH112 (Δlac *up*::Tn*10*dTet) to hydrolyze lactose indicates the β-galactosidase activity is not due to the ϕ(*cls*-'*lacZ*)hyb fusion.

The up mutation was further analyzed and its chromosomal location determined. The up mutation is very unstable. In the absence of tetracycline, tetracycline resistance along with the ability to hydrolyze lactose is lost. The degree of instability was determined by growing SOH93-1, SOH105 (Lin8 *up::*Tn*10*dTet), SOH118 (Δ*lac trp::*Tn*10*dTet), SOH130 (SOH93 *recA56 up::*Tn*10*dTet *zeg::*Tn*10*dCam), SOH112/pGE226, SOH112/pSH322, SOH141/pGE226, SOH141/pSH322 overnight in LB broth (ampicillin was added to maintain plasmids). The next day 100 μL of overnight was used to inoculate 10 mL of LB broth. Bacteria were grown to 25 Klett units, diluted, and plated onto LB plates. Colonies growing on LB plates were tested for tetracycline resistance and β-galactosidase activity.

RESULTS

Characterization of cloned gene fusions: Gene fusions were created as described in the Material and Methods section. The β-galactosidase activity of the cloned gene fusions was observed using LB plates containing XG. Plasmids pSH113, pSH114, pSH115, pSH120 and pSH121, all of which contain a gene fusion between *cls* and *lacZ*, are dark green on XG plates. Control plasmids pRS551 and pRS552, containing a promoterless *lacZ'* or *lac* operon were light green on XG plates. The β-galactosidase activity of these plasmids was due to their high copy number (66).

The plasmids pRS551, pRS552, pSH113, pSH115, pSH116, were tested for a functioning *cls* by placing them inside QC300 (*pss-1 cls-1*) (Table 4.5). Neither pRS551 or pRS552 contain any part of *cls*. As expected, QC300 containing these plasmids produce very little CL. Plasmids pSH113 and pSH115 were designed to contain a *cls* gene disrupted with *lacZ*. The lack of a functioning *cls* gene in these plasmids confirms the placement of *lacZ* to be inside *cls*. The presence of a functioning *cls* inside pSH116 enabled QC300 to produce CL, indicating that a functioning *cls* was present prior to insertion of *lacZ*.

Transfer of gene fusions to the chromosome: Homology between the gene fusions and the λ attachment site was created by inserting λRS45 into the λ attachment site. Phage λRS45 has a 5 Kb insert containing part of the *bla* gene at one side of the insert, and part of the *lac* operon on the other side of the insert. Gene fusions inside pSH113 and pSH115 are constructed to be *bla-kan*-T1-*cls*ϕ*lacZ*. The gene fusions via the *bla* and *lac* homology can integrate into λRS45 (Figure 4.8).

Integration was performed as described in the Materials and Methods section. To determine whether a monolysogen or a dilysogen had been formed, lysogens were scored for their ability to hydrolyze lactose (Table 4.6). The β-galactosidase activity from lysogens derived from pRS551 and pRS552 was very small and indistinguishable from each other. Lysogens derived from both pSH113 (SOH92 strains) and pSH115 (SOH93 strains) differed in their level of β-galactosidase activity. All SOH92 [ϕ(*cls-lacZ*$^+$)] lysogens appeared dark green on XG plates and purple on MacConkey-lactose plates. However, two levels of β-galactosidase were observed on TTC-lactose plates (Table 4.6). Monolysogens had a redder appearance on TTC-lactose plates than dilysogens. The different levels of β-galactosidase were confirmed by growing the cells in liquid medium, lysing the cells with SDS and chloroform, and assaying using ONPG as a substrate.

Two levels of β-galactosidase activity were also observed in SOH93 [ϕ(*cls*-'*lacZ*)hyb] lysogens. Monolysogens were less purple on MacConkey-lactose plates and redder on TTC-lactose plates, than dilysogens (Table 4.6). The different levels of β-galactosidase activity were verified in liquid medium using ONPG.

To establish that the gene fusions were located at the λ attachment, strains appearing as monolysogens and dilysogens were transduced with a P1 lysate from MS18087 (*nadA*::Tn*10*) (Table 4.6). The insertion inside MS18087 maps close to the λ attachment site (36). Tetracycline resistant transductants were scored for kanamycin resistance, the ability to hydrolyze lactose, and resistance to λ*NK330*. All of the colonies which became to kanamycin sensitive were unable to hydrolyze lactose and were λ*NK330* sensitive. Kanamycin sensitive colonies were scored as having their lysogens, at the λ attachment site, replaced with the λ attachment site from MS18087. The ability of a P1 lysate from MS18087 to knock out kanamycin resistance indicates that the gene fusions are indeed located at the λ attachment site.

Southern analysis: To confirm the retention of gene fusions at the λ attachment site, SOH90 (*lacZ*), SOH91 ('*lacZ*), SOH92 [ϕ(*cls*-*lacZ*$^+$)] and SOH93 [ϕ(*cls*-'*lacZ*)hyb] were subjected to a Southern analysis. Biotin-labelled probe was prepared by nick translating pSH103.

Chromosomal DNA was digested with EcoR1, HindIII, or EcoR1 + HindIII, transferred to nitrocellulose membrane paper, and incubated with nick translated pSH103. Bands containing homology to pSH103 were visualized using a sterptavidin conjugate (Figure 4.9). The DNA fragments visualized by Southern analysis were compared to restriction maps of pRS551, pRS552, pSH113, and pSH115.

Strains SOH90 and SOH91 have probe homology at *cls* (located at min 27) and *bla* (integrated at the λ attachment site). Strains SOH92 and SOH93, in addition to having *cls* at its chromosomal location, have a *cls*ϕ*lacZ* at the λ attachment site. A band of approximately 7.7 Kb was observed with an EcoR1 digest of SOH92 and SOH93 (Figure 4.8). This band corresponds to the *cls*ϕ*lacZ* fusion digested just before *cls*' and after *lacA*. Strains SOH90 and SOH91 contain part of the *lac* operon but do not have *cls* fused to *lacZ*. Consequently no corresponding 7.7 Kb band is observed.

Of particular interest in verifying the maintenance of the *cls*ϕ*lacZ* fusion at the λ attachment site were the low molecular weight bands. Southern analysis of SOH92 shows a low molecular weight band for the HindIII + EcoR1 digest of 0.5 Kb (Figure 4.9). This band is absent from the Southern analysis of SOH90 and is observed in the restriction mapping of pSH113 (Figure 4.3). Southern analysis of SOH93 reveals the

following low molecular weight bands: HindIII- 0.6 Kb, HindIII + EcoR1- 0.6 Kb and 0.5 Kb (Figure 4.9). These bands are not present in SOH91 and correspond to bands observed during the restriction mapping of pSH115 (Figure 4.4).

Western analysis: Strains P90C (Δ*lac*), SOH92 [ϕ(*cls-lacZ*$^+$)], SOH93 [ϕ(*cls-'lacZ*)hyb], P90C/pSH113, P90C/pRS551, P90C/pSH115, P90C/pRS552 and SOH141 (P90C *recA56 up::*Tn*10*dTet *zeg::*Tn*10*dCam), were subjected to Western analysis (Figure 4.10). Strains SOH92 [ϕ(*cls-lacZ*$^+$)], and P90C/pSH113 have a dark band with a MW of 118,000 corresponding to standard β-galactosidase. Strain P90C/pSH115 has a dark band with a MW of about 136,000 and a light band with a MW of 118,000. Strain SOH93 has a faint band, which has a MW of about 136,000. The production of a hybrid protein is consistent with the 136,000 band seen with SOH93 and pSH115. No β-galactosidase was observed in P90C/pRS551 or P90C/pRS552.

Localization of β-galactosidase: The cellular distribution of β-galactosidase produced by SOH92 [ϕ(*cls-lacZ*$^+$] and SOH93 [ϕ(*cls-'lacZ*)hyb] was determined (Table 4.7). The operon fusion produced soluble β-galactosidase, while β-galactosidase activity produced from the protein fusion was insoluble. The localization of hybrid β-galactosidase to

the membrane is consistent with CL synthase being an integral membrane protein (L. Ragolia, unpublished data).

Expression of *cls* increases with increasing cell density: Phase of growth can affect the proteins produced in the bacteria cell envelope (76,77). The CL content in *E. coli* increases at stationary phase (78). The protein and operon fusions were used to investigate whether there is also an increase in *cls* expression at increasing cell density. Strains SOH90 (*lacZ*), SOH91 ('*lacZ*), SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls*-'*lacZ*)hyb] were grown in LB broth. Samples of the cells were removed at different points and assayed for production of β-galactosidase. Less than two units of β-galactosidase activity was observed, at any time point, for SOH90 and SOH91. Expression of *cls*ϕ*lacZ* increased as SOH92 or SOH93 grew to early stationary phase (Figure 4.11). There is approximately a 2.5 fold increase in β-galactosidase activity, of the protein and operon fusion, as the cell density increases from 30 to 300 Klett units.

Strain Lin8 was used to determine whether the increase in expression of *cls*ϕ*lacZ* is accompanied by an increase in CL synthase. CL synthase activity was measured at early log phase (30 Klett units), and late log phase (300 Klett units) (Table 4.8). Consistent with the increase in gene expression, the specific activity of CL synthase of cells at late log phase was 2.1 fold greater than cells at early log phase.

Anaerobic expression of *cls*. The increase in *cls*, expression as the cells grow from exponential to stationary phase, may be related to respiration. This possibility was examined by adding KCN, a respiratory inhibitor, to growing cultures of operon and protein fusion bearing strains. In both cases, the addition of KCN causes an initial increase in β-galactosidase activity per cell density (Figure 4.12).

To further study anaerobic regulation of *cls*, SH92 [ϕ(*cls-lacZ*$^+$)] and SH93 [ϕ(*cls-'lacZ*)hyb] were grown in screw cap test tubes filled with LB broth (Table 4.9). Cells growing anaerobically had about twice the β-galactosidase activity of cells growing aerobically (Table 4.9). This increase was observed with both the protein and operon fusions. Despite the increase in expression, there was no difference in CL content, at 25 Klett units, between cells grown anaerobically or aerobically (Table 4.10).

The *fnr* gene is an anaerobic global regulator (79). It activates numerous anaerobic genes such as *narC* (80), *glpA* (81), and *frdABCD* (82). To test whether *fnr* is involved in anaerobic *cls* activation, the *fnr-250* allele was inserted into SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls-'lacZ*)hyb]. Colonies containing a *fnr250* allele were identified by their red appearance on TTC-nitrate plates. As indicated in Table 4.11, the *fnr-250* has no effect on β-galactosidase activity in cells grown either anaerobically or aerobically in LB broth. Cells containing *fnr-250* were

able to grow to a cell density of only 25 Klett units in LB broth under anaerobic condition, but grew normally under aerobic conditions.

To further investigate anaerobic induction, the β-galactosidase activity was measured in cells grown with different electron acceptors. Strains SOH92 and SOH93 were grown in GS medium with glycerol as the carbon source and either oxygen, nitrate, or fumarate as the electron acceptor. Expression of ϕ(*cls-lacZ*$^+$) and ϕ(*cls-'lacZ*)hyb varied depending upon the electron acceptor (Table 4.12). The better the electron acceptor the lower the expression of *clsϕlacZ* fusions. Fumarate induction of *clsϕlacZ* was inhibited by potassium nitrate and oxygen (Table 4.13). The indicated differences in *cls* expression was not accompanied by an increase in CL content (Table 4.14). The introduction of a *fnr-250* allele to bacteria grown anaerobically in GS with glucose as the carbon source, caused a slight increase in expression of the operon fusion (Table 4.15).

Effect of medium: Bacteria grow more slowly under anaerobic conditions than they do under aerobic conditions. To investigate whether differences in growth rate can affect *clsϕlacZ* expression, SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls-'lacZ*)hyb] were grown in different media and the β-galactosidase activity was assayed. Bacteria grown in minimal medium had about a 1.75 fold increase in ϕ(*cls-lacZ*$^+$) and ϕ(*cls-'lacZ*)hyb expression, over bacteria grown in LB broth (Table 4.16). Stains SOH92

and SOH93 grown in different minimal medium with either potassium succinate or glucose as the carbon source, had the same level of β-galactosidase activity, despite different growth rates. Neither catabolite repression nor different growth rates in minimal medium affected *cls*ϕ*lacZ* expression. The differences between bacteria grown in rich medium versus minimal medium affected the CL content. Strain SOH93 grown in minimal medium has a 2-3 fold increase in CL as compared to SOH93 grown in LB broth (Tables 4.10 and 4.14).

Investigation of the effect of phosphate starvation: The half life of CL turnover increases from 25 minutes to 60 minutes during phosphate starvation (83). To investigate the effect of phosphate starvation on *cls* expression, SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls*-'*lacZ*)hyb] were grown in limiting phosphate (0.1 mM) and excess phosphate (20 mM). As the bacteria grow in limiting phosphate they use up the available phosphate and induce alkaline phosphatase (84). As expected, cells grown in limiting phosphate grew worse and produced 200-300 times more alkaline phosphatase than bacteria grown in excess phosphate (Tables 4.13 and 4.17). Under conditions where phosphate concentration limits cell growth there is a 30-50% increase in ϕ(*cls*-'*lacZ*)hyb and ϕ(*cls-lacZ*$^+$) expression (Figure 4.13 and Table 4.17).

Investigation of the effects of osmolarity and arbutin: Turnover of PG, CL and to a lesser extent PE is stimulated by the production of membrane derived oligosaccharides (MDO)[1] (85,86). MDO production increases at low osmotic pressure (87). To investigate whether the expression of *cls* changes at different osmolarities, SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls-'lacZ*)hyb] were grown in medium containing 1.0% Bacto-tryptone, 0.5% Bacto-yeast extract, or the same medium supplemented with either 0.85 mM, or 3.0 mM sodium chloride (Table 4.18). Expression of ϕ(*cls-'lacZ*)hyb and ϕ(*cls-lacZ*$^+$) were unaffected by the difference in salt concentration.

The enzyme phosphoglycerol transferase I catalyzes the transfer of *sn*-1-phosphoglycerol groups to MDO and certain β-glucosides (88). Under conditions of high osmolarity, where MDO is not produced, phosphoglycerol transferase I is still active (89). Consequently, if a model substrate such as arbutin is present, turnover of PG and possibly CL can occur. The effect of 17 mM arbutin on *clsϕlacZ* expression was investigated in LEM medium. As indicated in Table 4.19, arbutin had no affect on *clsϕlacZ* expression.

Effect of PEA: The addition of PEA to bacteria results in an increase in CL content (24). The affect of PEA on *cls* expression was determined by adding PEA to SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls-*

'*lacZ*)hyb]. Addition of PEA decreased cell growth rate (Figure 4.14). Bacteria treated with PEA for 45 minutes had a 10-30% increase in β-galactosidase activity (Table 4.20). After incubation with PEA for 90 minutes there was no stimulation in *cls*ϕ*lacZ* expression (Table 4.20).

Change in pH: One of the phenotypes associated with a CL deficiency is difficulty growing at an elevated pH (25). The effect of alkaline pH on SOH93 [ϕ(*cls*-'*lacZ*)hyb] and SOH97 [*cls*::Tn*10*dTet3 ϕ(*cls*-'*lacZ*)hyb] was therefore determined. As expected, SOH97 grew less rapidly than SOH93 at an elevated pH (Tables 4.21, 4.22). While there was an increase in *cls*ϕ*lacZ* expression per cell density in both SOH93 and SOH97, no overall increase in *cls*ϕ*lacZ* expression was observed.

Effect of *pss-1*: Strains containing a *pss-1* mutation have an elevated CL content (4). To determine whether this increase in CL is due to enzymatic or genetic regulation, a *pss-1* mutation was introduced into SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls*-'*lacZ*)hyb]. Strains SOH143 [*pss-1* ϕ(*cls-lacZ*$^+$)] and SOH145 [*pss-1* ϕ(*cls*-'*lacZ*)hyb] produced two fold more CL than SOH144 [ϕ(*cls-lacZ*$^+$)] and SOH146 [ϕ(*cls*-'*lacZ*)hyb], while having only a 10% increase in *cls*ϕ*lacZ* expression (Table 4.23). Apparently the increase in CL is due to enzymatic regulation, rather than genetic regulation.

Autoregulation: The possibility that the *cls* gene is autoregulated was examined in strains either deficient in CL synthase or containing a cloned *cls*. Strains SOH96 [*cls*::Tn*10*dTet3 ϕ(*cls-lacZ*$^+$)] and SOH97 [*cls*::Tn*10*dTet3 ϕ(*cls*-'*lacZ*)hyb] have the same level of *cls*ϕ*lacZ* expression as SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls*-'*lacZ*)hyb], respectively (Figure 4.15). To measure the effect of CL synthase overproduction, SOH92 and SOH93 were transformed with either pSH322 or pSH103. The presence of a cloned *cls* had no effect on β-galactosidase activity (Table 4.24).

Effect of temperature: Shifting *E. coli* from 30° C to 42° C results in a slight stimulation in PG and CL content (90). When SOH92 [ϕ(*cls-lacZ*$^+$)] or SOH93 [ϕ(*cls*-'*lacZ*)hyb] were shifted from 30° C to 42° C no increase in β-galactosidase activity was observed. However, SOH92 grown at 30° C had a higher than expected level of β-galactosidase activity. The effect of temperature on ϕ(*cls-lacZ*$^+$) and ϕ(*cls*-'*lacZ*)hyb expression was further examined. As the temperature decreased ϕ(*cls-lacZ*$^+$) expression increased (Figure 4.16). An increase in β-galactosidase activity per cell density was also observed during a temperature shift from 42° C to 25° C (Figure 4.17). Paradoxically, the expression of the protein fusion did not change under the same conditions.

Search for regulatory mutants: An up mutation causing an increase in β-galactosidase activity was isolated by transposon mutagenesis of SOH93 [ϕ(*cls*-'*lacZ*)hyb]. The up mutation caused SOH93-1 [ϕ(*cls*-'*lacZ*)hyb *up*::Tn*10*dTet) to appear white on TTC-lactose plates containing tetracycline, and dark purple on MacConkey-lactose plates containing tetracycline. Despite the difference in expression between SOH93 and SOH93-1 on solid medium, there was no difference in β-galactosidase activity between SOH93 and SOH93-1 grown in liquid medium. The possibility that the differences in expression observed in liquid and solid medium were due to anaerobic induction was examined by growing SOH93 and SOH93-1 overnight in screw cap test tubes filled with LB-broth. The next morning production of β-galactosidase was determined. Strain SOH93-1 had a 2.2 fold increase in β-galactosidase activity, compared to SOH93 (Table 4.25).

Further examination of the up mutation revealed the increase in β-galactosidase activity to be independent of *clsϕlacZ* expression. Strain SOH112 (P90C *up*::Tn*10*dTet) contains the up mutation inside a P90C (Δ*lac*) background. As evidenced on TTC-lactose plates containing tetracycline, MacConkey-lactose plates containing tetracycline, and in liquid medium, SOH112 contains β-galactosidase activity. The β-galactosidase

activity in liquid medium was induced under anaerobic conditions (Table 4.25).

When either SOH93-1 or SOH112 were restreaked onto TTC-lactose plates or MacConkey lactose plates, lacking tetracycline, two types of colonies appeared. One type of colony had the same color as its parent, indicating a high level of β-galactosidase activity. The other type of colony had little, in the case of SOH93-1, or none, in the case of SOH112, β-galactosidase activity. All colonies showing a decrease in β-galactosidase became sensitive to tetracycline. A better measure of the instability of the up mutation was achieved by growing the bacteria containing the up mutation, in LB broth lacking tetracycline. Bacteria grown to early log phase were spread onto LB plates lacking tetracycline. Approximately 40% of the bacteria grown on LB plates were tetracycline sensitive and lost their increase in β-galactosidase activity (Table 4.26). Apparently the up mutation is unstable and needs the presence of tetracycline to be maintained.

The high frequency of instability is indicative of RecA dependent recombination. In an attempt to render the mutation stable, a *recA56* allele was introduced into SOH93-1 and SOH112. The *recA56* allele succeeded in stabilizing the up mutation in SOH130 (SOH93 *up::*Tn*10*dTet *zeg::*Tn*10*dCam *recA56*) and SOH141 (P90C *up::*Tn*10*dTet

*zeg::*Tn*10*dCam) (4.27). To confirm the importance of *recA56* in stabilizing the up mutation, SOH112 and SOH141 were transformed with either pGE226 or pSH322. The presence of a functioning *recA* gene in pGE226 resulted in the up mutation becoming unstable (Table 4.26).

While the up mutation is unrelated to *cls* expression the presence of β-galactosidase activity in a strain supposed to be Δ*lac* poses an interesting problem. To better understand the up mutation, its chromosomal location was determined. Conjugation of SOH100 (HfrH3000 *up::*Tn*10*dTet) with CSH57A placed the up mutation between minutes 8 and 12 on the *E. coli* genetic map. To more accurately define the location of the up mutation, three factor analysis was performed (Tables 4.27 and 4.28). Reciprocal crosses between SOH105 (Δ*phoA up::*Tn*10*dTet) and SOH106 (*lacZU lacI::kan*) established a gene order of *lacZ-lacI::kan-up*::Tn*10*dTet-*phoA* (Figure 4.18).

DISCUSSION

Using a cloned *cls* and *lacZ* fusion vectors, *in vitro* gene fusions between *cls* and *lacZ* were created. The constructed operon and protein fusions were transferred from multicopy plasmids to λRS45, which was inserted into the λ attachment site. The establishment of a gene fusion, between *cls* and *lacZ*, were thoroughly examined. Monolysogens containing the fusion were identified by their β-galactosidase activity. Insertion of the fusion into the λ attachment site was confirmed by transduction with MS18087 (*nadA*::Tn*10*). The proper integration of the fusion was confirmed by Southern analysis (Figure 4.9). Production of standard β-galactosidase, in the case of the operon fusion, and hybrid β-galactosidase, in the case of the protein fusion, was visualized using Western analysis (Figure 4.10).

Expression of β-galactosidase activity in SOH92 [ϕ(*cls-lacZ*$^+$)] was about 20 fold greater than in SOH93 [ϕ(*cls-'lacZ*)hyb]. There are several possible explanations which can account for the differences in expression. The protein fusion may have a weaker translational region than the operon fusion. Expression of the protein fusion may be decreased when the hybrid protein is inserted into the membrane. Alternately, the hybrid protein may be unstable. The degree of instability, of the protein fusion,

may obscure interpretation of data obtained with the protein fusion. Western analysis suggests that high concentrations of the hybrid protein are degraded. This degradation may depend upon both the CL synthase region and the β-galactosidase region of the hybrid protein.

Using SOH92 and SOH93 several aspect of *cls* regulation were examined. Changes in physiological conditions had either a 2-3 fold increase, a 10%-50% increase, or no effect on *cls*ϕ*lacZ* expression. Conditions which caused a 2-3 fold increase in *cls*ϕ*lacZ* expression were: increase in cell density, the presence of different electron acceptors, growth in different media, and changes in temperature. To confirm an increase in *cls* expression as bacteria approach stationary phase, the specific activity of CL synthase was measured in cells grown to 25 and 250 Klett units. While a reproducible increase in specific activity was observed, the assay had two complications (Table 4.8): (*i*) components other than CL synthase, which were present in the crude membranes used in the assay, might have influenced the results, (*ii*) enzyme activity is extremely low.

The increase in *cls*ϕ*lacZ* expression of cells grown under anaerobic conditions was further explored. Anaerobic stimulation was examined by growing SOH92 and SOH93 in medium containing different electron acceptors. The lower the reduction potential of the electron acceptor the

higher the level of expression of *cls*ϕ*lacZ* (Tables 4.12). The increase in expression was not offset by the *fnr250* allele (Tables 4.11 and 4.15).

No correlation between growth rate and *cls*ϕ*lacZ* expression was observed when SOH92 and SOH93 were cultured in different minimal media (Table 4.16). However, differences in expression between cells grown in rich medium and cells grown in minimal medium were observed. Consistent with an increase in *cls* expression, bacteria grown in minimal medium had a 2-3 fold higher content of CL.

Those conditions causing a slight increase in *cls*ϕ*lacZ* expression were phosphate starvation and the addition of PEA. Addition of arbutin, alteration of the osmolarity, and changes in pH, had no effect on *cls*ϕ*lacZ* expression.

Using SOH92 and SOH93, a mutant with a decrease and a mutant with an increase in β-galactosidase activity were isolated. Unfortunately, neither of these mutations is related to the expression of *cls*. The proximity of the up mutation to the *lac* operon is disturbing. Possibly the *lac* operon is not deleted in P90C but only inverted.

Insights into regulation of *cls* have been obtained using the operon and protein fusion. As illustrated by the *pss-1* mutation, regulation of CL synthesis is occurring at the enzymatic level (Table 4.23). However, the level of expression of *cls*ϕ*lacZ* can also be regulated at the genetic level.

Under conditions of increased phase of growth and growth in minimal medium versus rich medium an increase in both *cls*ϕ*lacZ* and CL content are observed. The increase in *cls*ϕ*lacZ* expression during growth with different electron acceptors is not accompanied by an increase an CL content. Apparently some compensatory mechanism is at work. Possibly regulation at the enzymatic level. Alternately, the expression of some of the other genes involved in phospholipid synthesis may be increasing.

While extensive studies with the operon and protein have been performed, the usefulness of the fusions have not been exhausted. In particular, the fusions should be used to search further for regulatory mutants. Mutagenesis should not only be done using transposons but should be performed using chemical mutagens. The isolation of regulatory mutants, and construction of like fusions in other genes involved in phospholipid synthesis, can lead to a better understanding of the genetic regulation of phospholipid synthesis.

TABLE 4.1

Strain List

Strain	Relevant properties	Source or Reference
CAG18420	lacZU118 lacI3098::Tn10kan	91
CSH57A	ara leu lacY purE gal trp his argG malA strA xyl mtl ilv metA or B thi	43
GA18-11	MC4100 glpR glpD trp::Tn10dTet	c
GE1031	supE42/Fzzf-1831::Tn10dTet	G. Weinstock
GE1033	supE42/Fzzf-1836::Tn10dCam	G. Weinstock
GE1150	MC4100 fnr250 zcj::Tn10dTet	G. Weinstock
GE1806	HfrH KL16 recA56 srl::Tn10dTet	G. Weinstock
HB101	F' hsds20(r_B^-, m_B^-) recA13 supE44 ara14 galK2 lacY1 proA2 rpsL20(sm^r)	a
JC7623	recB recC sbcB	67
KA197	HfrKL16 thi-1 pheA97 relA1 spo77	B. Bachmann
Lin 8	λ glpR glpD ΔphoA	a
MC4100	F^- araD139 Δ(argF-lac)U169 rpsL150 relA1 deoC1 ptsF25 rbsR flbB5301	G. Weinstock
MS18087	MG1655 rpsL galK⁻ nadA::Tn10	M. Singer
P90C	ara Δ(lac-proAB) thi	92
SE5000	MC4100 recA56	34
SOH5	MC4100 glpR glpD narC	a

Table 4.1

SOH9	MC4100 glpR glpD cls::Tn10dTet3	a
SOH70	MC4100 pRS551 λRS45	d
SOH71	MC4100 pRS552 λRS45	d
SOH72	MC4100 pSH113 λRS45	d
SOH73	MC4100 pSH115 λRS45	d
SOH90	P90C lacZ (λSOH80)	d
SOH91	P90C 'lacZ (λSOH81)	d
SOH92	P90C ϕ(cls-lacZ$^+$) (λSOH82)	d
SOH92-1	SOH92 dm::Tn10dTet	d,e
SOH93	P90C ϕ(cls-'lacZ)hyb (λSOH83)	d
SOH93-1	SOH93 up::Tn10dTet	d,f
SOH96	SOH92 cls::Tn10dTet3	(P1) SOH9 x SOH92
SOH97	SOH93 cls::Tn10dTet3	(P1) SOH9 x SOH93
SOH100	Hfr3000 up::Tn10dTet	(P1) SOH93-1 x Hfr3000
SOH105	glpR glpD ΔphoA up::Tn10dTet	(P1) SOH100 x Lin8
SOH106	glpR glpD lacZU118 lacI3098::Tn10kan	(P1) CAG18420 x Lin8
SOH110	S0H92 up::Tn10dTet	(P1) SOH93-1 x SOH92
SOH112	P90C up::Tn10dTet	(P1) SOH93-1 x P90C
SOH118	P90C trp::Tn10dTet	(P1) GA18-11 x P90C
SOH121	SOH92 srl::Tn10	(P1) GE1806 x SOH92
SOH127	SOH92 zeg::Tn10dCam recA56	d
SOH130	SOH93 up::Tn10dTet zeg::Tn10dCam recA56	(P1) SOH123 x SOH93-1

Table 4.1

SOH132	SOH92 fnr250 zcj::Tn10dTet	(P1) GE1150 x SOH92
SOH133	SOH92 zcj::Tn10dTet	(P1) GE1150 x SOH92
SOH134	SOH93 fnr250 zcj::Tn10dTet	(P1) GE1150 x SOH93
SOH135	SOH93 zcj::Tn10dTet	(P1) GE1150 x SOH93
SOH141	P90C recA56 up::Tn10dTet zeg::Tn10dCam	(P1) SOH127 x SOH112
SOH142	KA197 phe$^+$ pss-1 zee::Tn10dTet	d
SOH143	SOH92 pss-1 zee::Tn10dTet	(P1) SOH142 x SOH92
SOH144	SOH92 zee::Tn10dTet	(P1) SOH142 x SOH92
SOH145	SOH93 pss-1 zee::Tn10dTet	(P1) SOH142 x SOH93
SOH146	SOH93 zee::Tn10dTet	(P1) SOH142 x SOH93
QC30	MC4100 glpR glpD pss-1	b
QC300	MC4100 glpR glpD cls-1 pss-1	b

Table 4.1.

a- See Table 2.1.

b- See Table 3.1.

c- Created by transducing SOH5 (*narC*) with a P1 lysate made from a random Tn*10*dTet pool.

d- See Material and Methods.

e- *dn* is the down mutation causing a decrease in β-galactosidase.

f- *up* is the up mutation causing an increase in β-galactosidase.

TABLE 4.2

Phage List

Phage	Relevant properties	Source or Reference
λimm441	λimm441	G. Weinstock
λNK330	λimm21 cI^-	65
λRS45	bla'-$lacZ_{\omega}$ $lacY^+$ $lacA^+$ imm21 ind^+	65,a
λSOH80	bla kan $T1_4$ lacZ $lacY^+$ $lacA^+$ imm21	b,c
λSOH81	bla kan $T1_4$ 'lacZ $lacY^+$ $lacA^+$ imm21	b,d
λSOH82	bla kan $T1_4$ ϕ(cIs-$lacZ^+$) $lacY^+$ $lacA^+$ imm21	b,e
λSOH83	bla kan $T1_4$ ϕ(cIs-'lacZ)hyb $lacY^+$ $lacA^+$ imm21	b,f

Table 4.2.
- a- SC one third of *lacZ*.
- b- See Material and Methods.
- c- Derived from pRS551.
- d- Derived from pRS552.
- e- Derived from pSH113.
- f- Derived from pSH115.

TABLE 4.3 Plasmid List

Plasmids	Relevant properties	Source or Reference
pBR322	bla tet^r	93
pGE226	pBR327 Δ(HindIII-AvaI) $recA^+$	94
pLKC480	bla kan^r 'lacZ $lacY^+$	66
pLKC481	bla kan^r 'lacZ $lacY^+$	66
pLKC482	bla kan^r 'lacZ $lacY^+$	66
pNK972	bla transposase	a
pRS414	bla $T1_4$ 'lacZ $lacY^+$ $lacA^+$	65,b
pRS415	bla $T1_4$ lacZ $lacY^+$ $lacA^+$	65,b
pRS551	bla kan^r $T1_4$ lacZ $lacY^+$ $lacA^+$	65,b
pRS552	bla kan^r $T1_4$ 'lacZ $lacY^+$ $lacA^+$	65,b
pSH111	bla $T1_4$ cls^+ 'lacZ $lacY^+$ $lacA^+$	c
pSH112	bla $T1_4$ cls^+ 'lacZ $lacY^+$ $lacA^+$	c
pSH113	bla kan ϕ(cls-$lacZ^+$) $lacY^+$ $lacA^+$	c
pSH114	bla ϕ(cls-lacZ)hyb $lacY^+$ $lacA^+$	c
pSH115	bla kan^r ϕ(cls-lacZ)hyb $lacY^+$ $lacA^+$	c
pSH116	bla kan $T1_4$ cls^+ 'lacZ $lacY^+$ $lacA^+$	c
pSH120	kan^r bla ϕ(cls-lacZ)hyb $lacY^+$	c
pSH121	kan^r bla ϕ(cls-$lacZ^+$) $lacY^+$	c

Table 4.3.

a- See Table 2.1.

b- Plasmids pRS415 and pRS552 are used to create operon fusions to *lacZ*. These plasmids lack the *lacZ* promoter but contain the *lacZ* translation initiation sites. Plasmids pRS415 and pRS552 are used to create protein fusions to '*lacZ*. These plasmids lack the *lacZ* promoter and translation initiation sites.

c-See Material and Methods.

TABLE 4.4

Ability Of *lacZ* Plasmids To Hydrolyze XG

Strain	Color
MC4100/pRS551	Light Green
MC4100/pRS552	Green
MC4100/pSH112	Light Green
MC4100/pSH113	Dark Green
MC4100/pSH114	Dark Green
MC4100/pSH115	Dark Green
MC4100/pSH120	Dark Green
MC4100/pSH121	Dark Green

Table 4.4. Colonies were streaked onto LB plates containing 100 μg/mL ampicillin and 100 μL XG (20 mg/mL). Colors were observed after 24 hours.

TABLE 4.5

Phospholipid Distribution
Of Gene Fusions Inside QC300

	Mole %		
Strain	**PE**	**PG**	**CL**
QC300/pSH113	56.3	43.7	N
QC300/pSH115	55.8	44.2	N
QC300/pSH116	69.7	17.2	13.1
QC300/pRS551	59.3	40.7	N
QC300/pRS552	59.8	40.2	N

Table 4.5. QC300 (*pss-1 cls-1*), containing different plasmids, was grown shaking in 5.0 mL of LB broth at 35° C. Phospholipids were labelled with 5-10 μCi/mL of [^{32}P]phosphate. Radioactive phosphate was added at a cell density of 2-5 Klett units. Phospholipids were isolated at about 100 Klett units. Phospholipids were separated by two dimensional chromatography. N- No spot observed on X-ray paper (after 16 hours of exposure).

TABLE 4.6

Testing for dilysogens

Strain	XG	MacConkey	TTC	Kan^s/Tet^r
SOH90	+/-	-	-	3/9
SOH90d	+/-	-	-	1/11
SOH91	+/-	-	-	11/18
SOH92	+	+++++	++++	8/19
SOH92d	+	+++++	++++++	3/20
SOH93	+	++	++	9/25
SOH93d	+	+++	+++	1/19

Table 4.6. Lysogens SOH90 (*lacZ*), SOH91 ('*lacZ*), SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls-'lacZ*)hyb] were tested for on LB plates containing XG, MacConkey-lactose plates and TTC-lactose plates. A + indicates the ability to hydrolyze lactose, with respect to other colonies tested. A green color on XG was scored as +. On MacConkey-lactose plates a + indicates a purple color. On TTC-lactose plates colonies able to hydrolyze lactose appear white. Kan^s/Tet^r colonies are the number of cells in which tetracycline resistance, from MS18087 (*nadA::*Tn*10*), knocked out kanamycin resistance at the λ attachment site. A "d" signifies a dilysogen.

TABLE 4.7

Localization Of β-galactosidase Activity

Strain		Total	Soluble	Insoluble
SOH92	ϕ(*cls-lacZ*$^+$)	100	71.0	24.0
SOH93	ϕ(*cls-'lacZ*)hyb	100	20.1	75.2

Table 4.7. Bacteria were grown in LB broth shaking at 37° C. The cell envelope was isolated as described by Silhavy *et al.* (72). The β-galactosidase activity in the sonicate, after cell removal was taken to be 100%. The activity in the soluble and insoluble fractions are expressed as the % of sonicate activity. Soluble and insoluble fractions were obtained after centrifugation at 100,000 x g for 1 hr (72).

TABLE 4.8

Cardiolipin Synthase Activity Measured At Early and Late Log Phase

	Specific activity	
	Early	**Late**
Lin 8	0.83	1.74

Table 4.8. Bacteria were grown in LB broth shaking at 37° C. The cell envelope was isolate at early and late log phase. Bacteria were centrifuged and resuspended in 10 mM Tris-HCl pH 7.5 containing 2 mM β-mercaptoethanol (1 gram of cells was resuspended in 10 mL of buffer). The cells were disrupted using five 30 seconds bursts from a Bronson model W140D Sonifier, at a setting of 8 (25). Cell debris was removed by centrifuging at 8000 x g. The cell envelope was pelleted by centrifuging at 100,000 x g. CL synthase was assayed by incubation with [^{32}P]PG (24). Phospholipids were separated in two dimensions. Specific activity is expressed as pmole/(min-mg protein) of [^{32}P]cardiolipin formed after 10 minutes. Assay conditions were a described by Tunaitis and Cronan (24).

TABLE 4.9

Anaerobic Expression in LB Broth

Strain		Klett units:	β-galactosidase activity 24-25	34-35	50-55
SOH92	ϕ(*cls-lacZ*$^+$)	Anaerobic	773	939	738
SOH92	ϕ(*cls-lacZ*$^+$)	Aerobic	390	410	---
SOH93	ϕ(*cls-'lacZ*)hyb	Anaerobic	48.6	52.5	42.9
SOH93	ϕ(*cls-'lacZ*)hyb	Aerobic	21.0	22.0	---

Table 4.9. Bacteria were grown anaerobically in screw cap culture tubes filled with LB broth at 37° C. Bacteria were grown aerobically in side arm flasks shaking at 37° C. The doubling time for SOH92 and SOH93 grown anaerobically and aerobically was 60 and 23 minutes, respectively. The β-galactosidase activity was determined at the indicated Klett units.

TABLE 4.10

Phospholipid Distribution
Of SOH93 Grown Aerobically And Anaerobically

	Mole %		
	PE	**PG**	**CL**
Aerobically	85.1%	12.4%	2.5%
Anaerobically	88.2%	9.3%	2.5%

Table 4.10. Strain SOH93 [ϕ(*cls*-'*lacZ*)hyb] was grown in 5.0 mL of LB broth in the presence of 5-10 μCi/mL of [^{32}P]phosphate until early log phase (about 25 Klett units). Radioactive phosphate was added at a cell density of 1-3 Klett units. Aerobic growth was achieved by growing the bacteria shaking at 37° C. Cells were grown anaerobically at 37° C in test tubes filled with LB broth. Isolated phospholipids were separated in two dimensions.

TABLE 4.11

Effect Of *fnr* On Gene Fusions

		Aerobic		Anaerobic
Strain	**Klett units**	**25-30**	**250-270**	**22-28**
SOH132	ϕ(*cls-lacZ*$^+$) *fnr250*	421	928	818
SOH133	ϕ(*cls-lacZ*$^+$)	411	858	795
SOH134	ϕ(*cls-'lacZ*)hyb *fnr250*	24.0	56.0	44.4
SOH135	ϕ(*cls-'lacZ*)hyb	23.2	54.0	48.8

Table 4.11. At the indicated Klett units aliquots were removed and β-galactosidase activity determined. Aerobic cultures were grown in LB broth shaking at 37° C. Anaerobic cultures were grown in screw cap test tubes filled with LB broth at 37° C. Anaerobic cultures containing a *fnr250* allele stopped growing at about 25 Klett units.

TABLE 4.12

Different Electron Acceptors

	Units β-galactosidase		
Strain:	**SOH92**	**SOH93**	**Doubling Time**
Glycerol + Oxygen	759	24.2	45
Glycerol + Nitrate	1179	52.8	105
Glycerol + Fumarate	2003	75.8	120

Table 4.12. Bacteria were grown in GS medium. Bacteria grown in glycerol + nitrate or glycerol + fumarate were cultured at 37° C in test tubes filled with the respective medium. Bacteria grown in glycerol + oxygen were cultured shaking at 37 ° C. Units of β-galactosidase was determined when the cells had reached a density 25-33 Klett units.

TABLE 4.13

Inhibition Of Fumarate Induction

	Units β-galactosidase	
Strain:	**SOH92**	**SOH93**
Glycerol + Fumarate	2003	75.8
Glycerol + Fumarate + Oxygen	820	38.5
Glycerol + Fumarate + Nitrate	1230	61.1

Table 4.13. Bacteria were grown at 37° C in GS medium supplemented with glycerol + fumarate, or glycerol + fumarate + potassium nitrate. Anaerobic growth was performed in a screw cap test tube filled with growth medium. Cells were grown aerobically by shaking on a floor shaker at 37° C.

TABLE 4.14

Phospholipid Distribution
Of Cells Grown With Glycerol And Different Electrons Acceptors

	Mole %		
	PE	**PG**	**CL**
Glycerol + Oxygen	82.7	11.3	6.0
Glycerol + Nitrate	80.1	12.6	7.2
Glycerol + Fumarate	81.2	13.6	5.2

Table 4.14. Strain SOH93 [ϕ(*cls*-'*lacZ*)hyb] was grown in 5.0 mL of LB broth and labelled with 5-10 μCi/mL of [^{32}P]phosphate. Radioactive phosphate was added at a cell density of 1-3 Klett units. At about 25 Klett units the phospholipids were isolated and identified as described in material and methods. Bacteria grown in glycerol + potassium nitrate or glycerol + fumarate, were cultured at 37° C in screw cap test tubes filled with their respective medium. Cells were grown aerobically in GS + glycerol, shaking at 37° C.

TABLE 4.15

Effect Of *fnr* On Gene Fusions With Glucose As The Carbon Source

	Units β-galactosidase
SOH132 ϕ(*cls-lacZ*$^+$) *fnr250*	1676
SOH133 ϕ(*cls-lacZ*$^+$)	1279
SOH134 ϕ(*cls-'lacZ*)hyb *fnr250*	63.3
SOH135 ϕ(*cls-'lacZ*)hyb	61.3

Table 4.15. Bacteria were grown in GS medium containing 0.4% glucose. Cells were cultured in screw cap test tubes at 37° C. At 25-30 Klett units the β-galactosidase activity was assayed.

TABLE 4.16

Effects Of Different Growth Media On Expression Of Gene Fusions

	Units β-galactosidase				
	M9		GS		
Strain	**Glu**	**Succ**	**Glu**	**Succ**	**LB**
SOH92	666	600	757	649	400
SOH93	34.8	30.6	39.9	39.1	23.0
Doubling Time (min)	60	118	37	45	23
Final Klett units	120	46	250	145	360

Table 4.16. Strains SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls-'lacZ*)hyb] were grown shaking at 37° C, in either M9, GS medium, or LB broth. Glu- 0.4% glucose. Succ- 0.4% potassium succinate. Units of β-galactosidase were determined at early log phase (24-30 Klett units). Doubling time was measured in minutes.

TABLE 4.17

Effect Of Phosphate Starvation

Strain	Phosphate (mM): 0.1	2.0	0.1	2.0
SOH92	Klett units	24	24	44	71
	β-galactosidase	553	570	924	698
	Phosphatase	164	9.7	1868	7.5
SOH93	Klett units	21	23	41	66
	β-galactosidase	32.4	35.0	63.2	46.3
	Phosphatase	127	7.1	2247	7.4

Table 4.17. Bacteria were grown shaking at 37° C in MOPS medium containing 0.4% glucose and either 0.1 mM potassium phosphate or 2.0 mM potassium phosphate. At the indicated Klett units aliquots of SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls-'lacZ*)hyb] were removed. Production of β-galactosidase and alkaline phosphatase were determined as described in Materials and Methods. Cells grew to a final density of 50 Klett units in 0.1 mM phosphate and 85 Klett units in 2.0 mM phosphate.

TABLE 4.18

Effect Of Osmolarity

β-galactosidase; Units x 10^7/cell number

Strain	NaCl (mM):	0.0	0.85	3.0
SOH92	ϕ(*cls-lacZ*$^+$)	151	189	178
SOH93	ϕ(*cls-'lacZ*)hyb	11.4	12.3	15.6

Table 4.16. Cells were grown shaking at 37° C to early log phase (25-35 Klett units) in 1.0% Bacto-tryptone, 0.5% Bacto-yeast extract, or the same medium supplemented with either 0.85 mM or 3.0 mM of sodium chloride. Production of β-galactosidase was determined as described in Materials and Methods. Units x 10^7/cell number was calculated by substituting number of cells for OD_{600}. The number of cells was estimated by plating out several dilutions of cells on to LB plates.

TABLE 4.19

Effect Of Arbutin

Strain	Units β-galactosidase 0 mM	17 mM
SOH92 ϕ(*cls-lacZ*$^+$)	457	472
SOH93 ϕ(*cls-'lacZ*)hyb	35.0	30.4

Table 4.19. Cells were grown shaking at 37° C in either LEM medium, or LEM medium supplemented with 17 mM arbutin. The presence of arbutin had no affect on cell growth. At a cell density of about 25 Klett units aliquots of cells were removed and the β-galactosidase activity determined.

TABLE 4.20

Effect Of PEA

			Units β-galactosidase			
	Klett Units:		**25-30**	**67-88**	**104-114**	**182-187**
SOH92	ϕ(*cls-lacZ*$^+$)	-PEA	368	494	---	654
		+PEA	368	674	654	---
SOH93	ϕ(*cls-'lacZ*)hyb	-PEA	25.3	30.3	----	39.1
		+PEA	25.3	34.8	34.6	----

Table 4.20. Cells were grown shaking in LB broth at 37° C. At 25-30 Klett units bacteria were divided into two portions. One portion was treated with 0.15% (v/v) PEA. The other portion was untreated. Aliquots of cells were removed and assayed for β-galactosidase after 45 minutes and 90 minutes.

TABLE 4.21

Effect Of A pH Shift On β-galactosidase Activity In SOH93

pH:	5.5	6.5	7.5	8.5	9.3	9.6
0 minutes shaking after pH shift						
Klett	30	30	30	26	28	33
Units	24.9	24.9	24.9	24.9	24.9	24.9
30 minutes shaking after pH shift						
Klett	50	65	67	57	41	41
Units	28.8	30.8	30.0	29.8	31.8	25
60 minutes shaking after pH shift						
Klett	92	126	137	128	65	44
Units	32.9	28.9	30.7	34.8	35.4	33.8
Final pH	6.71	7.11	7.53	7.94	8.53	8.86

Table 4.21. Strain SOH93 [ϕ(*cls*-'*lacZ*)hyb] was grown shaking at 37° C in 70 mL LB broth pH 7.35. At about 25 Klett units the cells were at a pH of 7.10. Cells at 25 Klett units were divided into seven 9 mL portions and centrifuged. The cells were washed once and resuspended in filter sterilized LB broth, pH 5.5, 6.5, 7.5, 8.5, 9.3, and 9.6. Cells were cultured shaking for 30 and 60 minutes, at which time aliquots were removed and the β-galactosidase activity was determined. Units refer to β-galactosidase activity.

TABLE 4.22

Effect Of A pH Shift
On β-galactosidase Activity In SOH97

pH:	5.5	6.5	7.5	8.5	9.3	9.6
0 minutes shaking after pH shift						
Klett	24	30	30	35	28	26
Units	25.6	25.6	25.6	25.6	25.6	25.6
30 minutes shaking after pH shift						
Klett	45	62	67	72	39	41
Units	37.8	30.6	28.0	29.5	32.6	25.5
60 minutes shaking after pH shift						
Klett	85	122	142	145	56	34
Units	33.5	32.1	30.5	34.7	38.7	25.4
Final pH	6.56	7.21	7.47	7.95	8.55	9.14

Table 4.22. Strain SOH97 [ϕ(*cls*-'*lacZ*)hyb *cls*::Tn10dTet3] was grown shaking at 37° C in 70 mL LB broth pH 7.35. At about 25 Klett units the cells were at a pH of 7.10. Cells at 25 Klett units were divided into seven 9 mL portions and harvested. The cells were washed once and resuspended in filter sterilized LB broth, pH 5.5, 6.5, 7.5, 8.5, 9.3, and 9.6. Cells were cultured shaking for 30 and 60 minutes, at which time aliquots were removed and the β-galactosidase activity was determined. Units refer to β-galactosidase activity.

TABLE 4.23

Effect Of *pss-1*

Strain	β-galactosidase Units	Phospholipid distribution mole % PE	PG	CL
SOH143 [*pss-1* ϕ(*cls-lacZ*$^+$)]	522	73.8	21.3	4.9
SOH144 [ϕ(*cls-lacZ*$^+$)]	471	83.1	14.8	2.1
SOH145 [*pss-1* ϕ(*cls-'lacZ*)hyb]	26.8	74.7	20.6	4.7
SOH146 [ϕ(*cls-'lacZ*)hyb]	24.5	82.8	14.8	2.4

Table 4.23. Bacteria were cultured overnight night at 30° C. The next day cells were diluted 1:100 in 5-10 mL of fresh LB broth and grown shaking at 35° C. Units of β-galactosidase was determined when the cells reached a density of 26-35 Klett units. Phospholipid distribution was determined by labelling with 5 μCi/mL of [^{32}P]phosphate. Radioactive phosphate was added at a cell density of 3-5 Klett units. Phospholipids were isolated at a cell density of 26-35 Klett units. Phospholipids were separated by two dimensional chromatography.

TABLE 4.24

Effect Of pSH103 On Expression of Gene Fusions

Strain	Units: β-galactosidase
SOH92/pSH322	394
SOH92/pSH103	359
SOH93/pSH322	21.0
SOH93/pSH103	20.8

Table 4.24. Strains SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls-'lacZ*)hyb] containing either pSH322 or pSH103 were grown at 37° C shaking in LB broth. To maintain the plasmid, LB broth contained 100 μg/mL ampicillin. At a cell density of 26-32 Klett units β-galactosidase activity was determined.

TABLE 4.25

β-galactosidase Activity Of *up::*Tn*10*dTet

Strain		Units: β-galactosidase
SOH93	[ϕ(*cls-'lacZ*)hyb]	38.0
S0H93-1	[*up::*Tn*10*dTet ϕ(*cls-'lacZ*)hyb]	85.2
SOH93-1	[*up::*Tn*10*dTet ϕ(*cls-'lacZ*)hyb] + Tet	91.4
P90C	[Δ*lac*]	0.0
SOH141	[P90C *up::*Tn*10*dTet *zeg::*Tn*10*dCam *recA56*]	21.0

Table 4.25. Strains SOH93 and SOH93-1 were grown in screw cap test tubes filled with LB broth. Strains P90C and SOH141 were grown shaking at 37° C in LB broth. β-galactosidase activity was determined when the cells reached a cell density of about 28 Klett units. + Tet signifies the addition of 20 μg/mL to the LB broth.

TABLE 4.26

Stability Of *up*::Tn*10*dTet

Strain	Total	Tet^r	Tet^s	Stability
SOH112/pGE226	63	40	23	63.5%
SOH112/pSH322	63	41	22	65.1%
SOH141/pGE226	63	42	20	66.7%
SOH141/pSH322	63	0	64	100%
SOH93-1	49	29	20	59.2%
SOH105	49	0	29	100%
SOH118	49	0	49	100%
SOH130	49	0	49	100%

Table 4.26. Strains SOH112 (P90C *up*::Tn*10*dTet) and SOH141 (P90C *up*::Tn*10*dTet *zeg*::Tn*10*dCam *recA56*) containing either pSH322 or pGE226 were subcultured overnight in LB broth containing 100 μg/mL ampicillin. The next morning 100 μL of cell culture was added to 5 mL LB broth. Cells were grown to early log phase then spread on to LB plates containing ampicillin. Colonies were tested for growth on LB plates containing tetracycline and on TTC-lactose plates. All tetracycline resistant colonies had an elevated level of β-galactosidase activity. Strains SOH93-1 [ϕ(*cls*-'*lacZ*)hyb *up*::Tn*10*dTet], SOH105 (*glpR glpD* Δ*phoA up*::Tn*10*dTet), SOH118 (Δ*lac trp*::Tn*10*dTet), and SOH130 [ϕ(*cls*-'*lacZ*)hyb *up*::Tn*10*dTet *zeg*::Tn*10*dCam *recA56*) were grown in LB broth lacking ampicillin, and tested as described above. Plasmid pGE226 contains a functioning *recA*. Stability refers to percentage of colonies retaining tetracycline resistance. Bacteria were cultured at 37° C.

TABLE 4.27

Mapping of *up*::Tn*10*dTet

Donor	Recipient	Selected Marker	Unselected Marker	Number of Transductants
SOH105	SOH106	Tet	kan^r, pho^+	15
			kan^r, phoA	6
			kan^s, pho^+	63
			kan^s, phoA	20
SOH105	SOH106	Tet	kan^r, lac^+	1
			kan^r, lacZ	20
			kan^s, lac^+	75
			kan^s, lacZ	8
SOH105	SOH106	Tet	pho^+, lac^+	57
			pho^+, lacZ	21
			phoA, lac^+	19
			phoA, lacZ	7

Indicated gene order: lacZ-lacI::kan-Tn10dTet

Table 4.27. Transduction of SOH105 (Δ*phoA up*::Tn*10*dTet *lac*$^+$) with a P1 lysate from SOH106 (*lacZU118 lacI3098*::Tn*10kan pho*$^+$). Transductants were selected for on LB plates containing tetracycline and scored for the following: kan^r- ability to grow on LB plates containing kanamycin, pho^+- a blue appearance on phosphatase indicator plates (XP plates), lac^+- a red appearance on MacConkey-lactose plates. The indicated gene order is based upon three factor analysis. The overall gene order in shown in Figure 4.18. The location of Δ*phoA* could not be determined unambiguously from the data in Table 4.28.

TABLE 4.28

Mapping of *up*::Tn*10*dTet

Donor	Recipient	Selected Marker	Unselected Marker		Number of Transductants
SOH106	SOH105	kan	tet^r,	lacZ	27
			tet^r,	lac^+	3
			tet^s,	lacZ	68
			tet^s,	lac^+	5
SOH106	SOH105	kan	tet^r,	phoA	30
			tet^r,	pho^+	0
			tet^s,	phoA	52
			tet^s,	pho^+	21
SOH106	SOH105	kan	lac^+,	phoA	6
			lac^+,	pho^+	2
			lacZ,	phoA	75
			lacZ,	pho^+	20

Indicated gene order: lacI::kan-Tn10dTet-phoA

Table 4.28. Transduction of SOH106 (*lacZU118 lacI3098*::Tn*10kan* pho^+) with a P1 lysate from SOH105 (Δ*phoA up*::Tn*10*dTet lac^+). Transductants were selected for on LB plates containing kanamycin and scored for the following: tet^r- ability to grow on LB plates containing tetracycline, pho^+- a blue appearance on phosphatase indicator plates (XP plates), lac^+- a red appearance on MacConkey-lactose plates. The indicated gene order is based upon three factor analysis. The overall gene order in shown in Figure 4.18. The location of *lacZ* with respect to neighboring genes could not be determined from the data in Table 4.29.

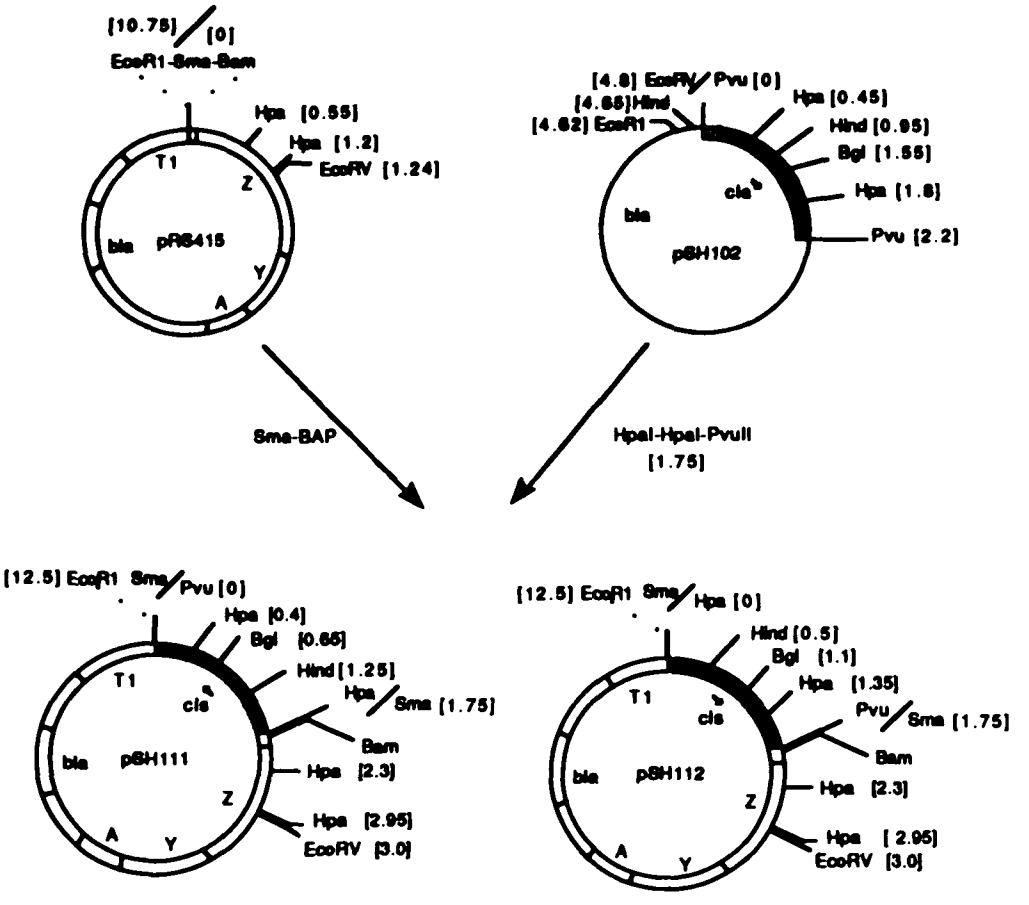

Figure 4.1. Construction of pSH111 and pSH112. A HpaI-HpaI-PvuII (1.75 kb) fragment from pSH102, was inserted into the Sma site of pRS415 (pRS415 was treated with bacterial alkaline phosphatase in order to prevent reannealing). Because Both of the ends of the recipient DNA and of the donor DNA were blunt ends. Two different insertion orientations were possible. Both types of recombinant plasmids, SH111 and pSH112 were obtained. Not all PvuII sites are listed in pSH111 or pSH112. Solid line, pBR322; DNA; Open box, pRS DNA; Black box, chromosomal DNA; Dotted box, Tn*10*dCam DNA. Restriction enzymes: Bam (BamH1), Bgl (BglII), EcoR1, EcoRV, Hind (HindIII), Hpa (HpaI), Pvu (PvuII), Sma (SmaI). Distances are given in Kilobases.

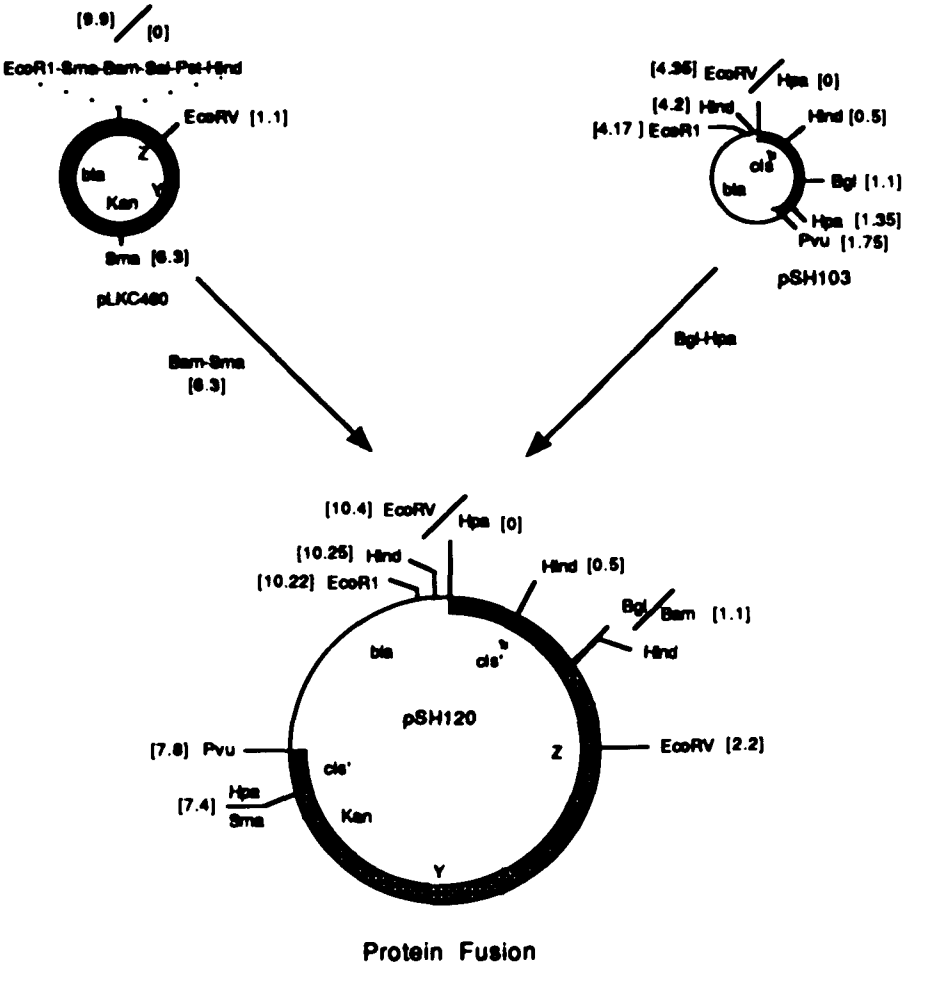

Figure 4.2. Construction of pSH120. A series of 6.3 Kb BamHI-SmaI fragments were obtained from pLKC480, pLKC481 and pLKC482. These fragments were inserted into the BglII-HpaI sites of pSH103. Recombinant plasmids were selected for on LB plates containing kanamycin. Of the resulting plasmids only the one derived from pLKC480 (pSH120) was dark green on LB plates containing XG. Not all PvuII or HpaI sites are listed in pLKC480 or pSH120. Restriction enzymes: Bam (BamHI), Bgl (BglII), EcoR1, EcoRV, Hind (HindIII), Hpa (HpaI), Pst (PstI), Pvu (PvuII), Sma (SmaI). Checkered box; pLK480 DNA. Solid line; pBR322 DNA. Black box; chromosomal DNA. Distances are given in Kilobases.

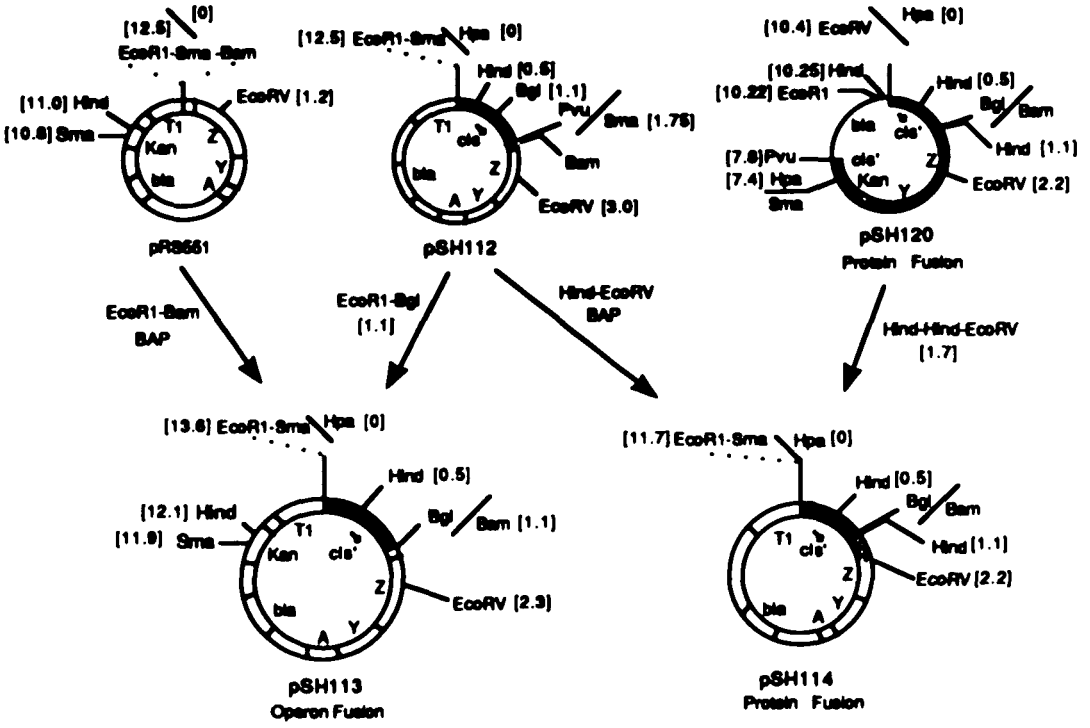

Figure 4.3. Construction of pSH113 and pSH114. Plasmid pSH113 was constructed by inserting a 1.1 Kb EcoR1-BglII, fragment from pSH112, into EcoR1-BamHI sites of pRS551. Two pRS551 plasmids were prevented from ligating to each other by treatment with bacterial alkaline phosphatase. Selection for pSH113 was based on kanamycin resistance. Plasmid pSH114 was constructed by inserting a 1.7 Kb HindIII-HindIII-EcoRV, from pSH120, into the HindIII-EcoRV site of pSH112. To prevent two pSH112 plasmids from combining bacterial alkaline phosphatase was used. Selection of pSH114 was based upon ampicillin resistance. Restriction enzymes: Bam (BamH1), Bgl (BglII), EcoR1, EcoRV, Hind (HindIII), Hpa (HpaI), Pvu (PvuII), Sma (SmaI). Not all Pvu or HpaI sites are listed. Checkered box, pLK480 DNA; Solid line, pBR322 DNA; Black box, chromosomal DNA; Open box, pRS DNA. Distances are given in Kilobases.

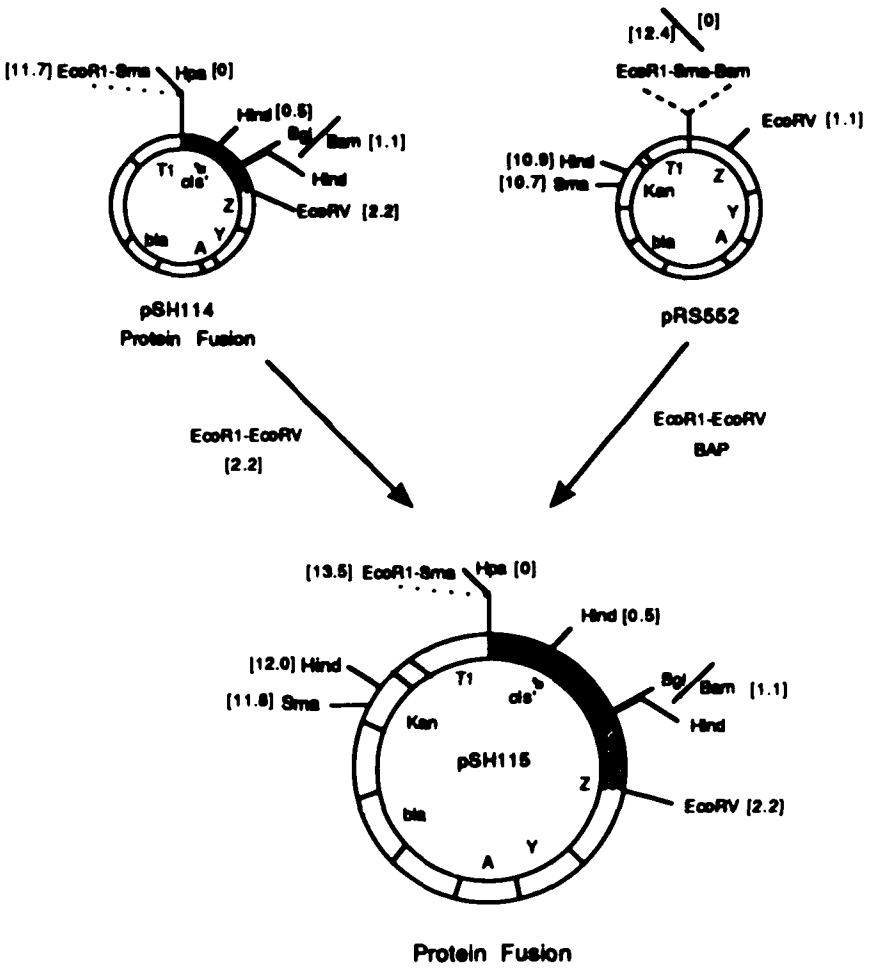

Figure 4.4. Construction of pSH115. A 2.2 Kb EcoR1-EcoRV fragment from pSH114, containing part of *cls* joined to part of *lacZ*, was inserted into the EcoR1-EcoRV sites of pRS552. Selection was based on kanamycin resistance. Ligation of pRS552 to another pRS552 was prevented by treatment with bacterial alkaline phosphatase. Restriction enzymes: Bam (BamH1), Bgl (BglII), EcoR1, EcoRV, Hind (HindIII), Hpa (HpaI), Sma (SmaI). Not all HpaI sites are listed. Checkered box, pLKC480 DNA; Black box, chromosomal DNA; Open box, pRS DNA. Distances are given in Kilobases.

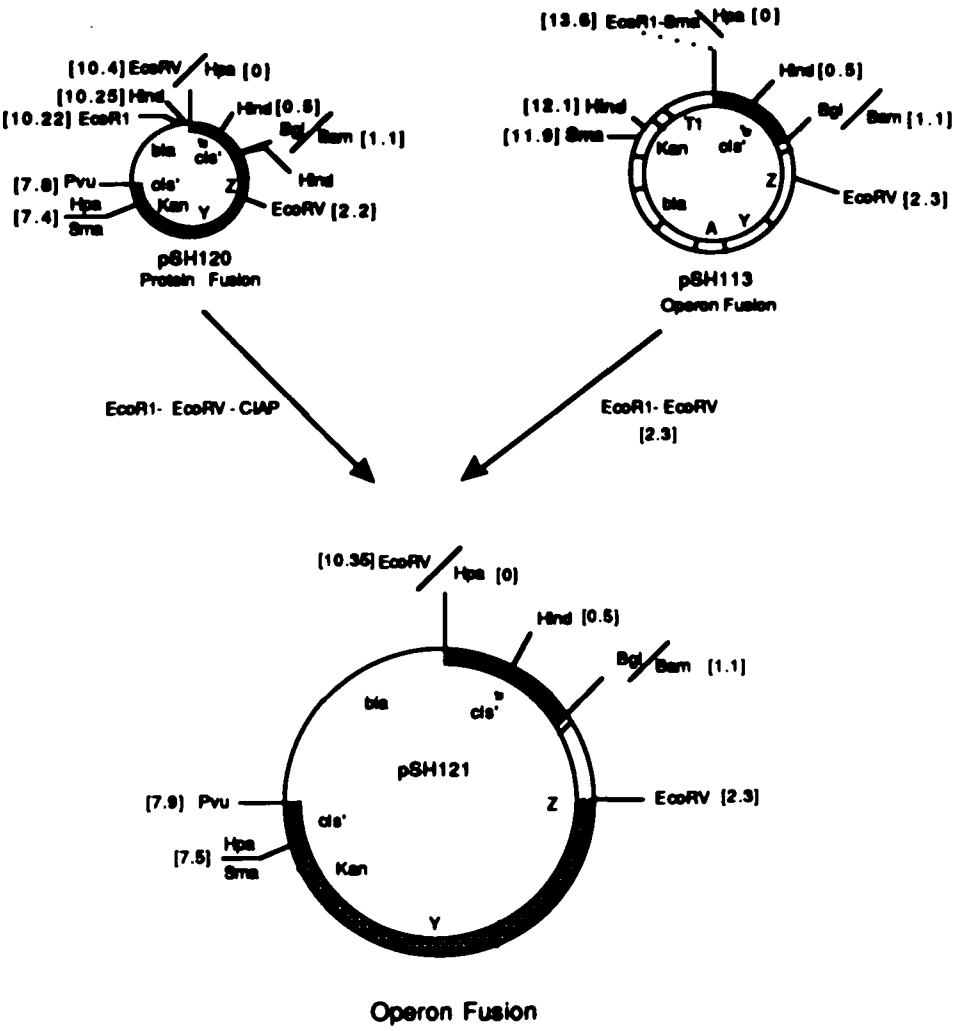

Figure 4.5. Construction of pSH121. Plasmid pSH120 was digested with EcoR1-EcoRV and treated with calf intestine alkaline phosphatase. A 2.3 Kb EcoR1-EcoRV fragment from pSH113 was inserted into the EcoR1-EcoRV sites of pSH120 creating pSH121. Restriction enzymes: Bam (BamH1), Bgl (BglII), EcoR1, EcoRV, Hind (HindIII), Hpa (HpaI), Pvu (PvuII), Sma (SmaI). Not all Pvu or HpaI sites are listed. Checkered box; pLK480, DNA; Solid line, pBR322 DNA; Black box, chromosomal DNA; Open box, pRS DNA. Distances are given in Kilobases.

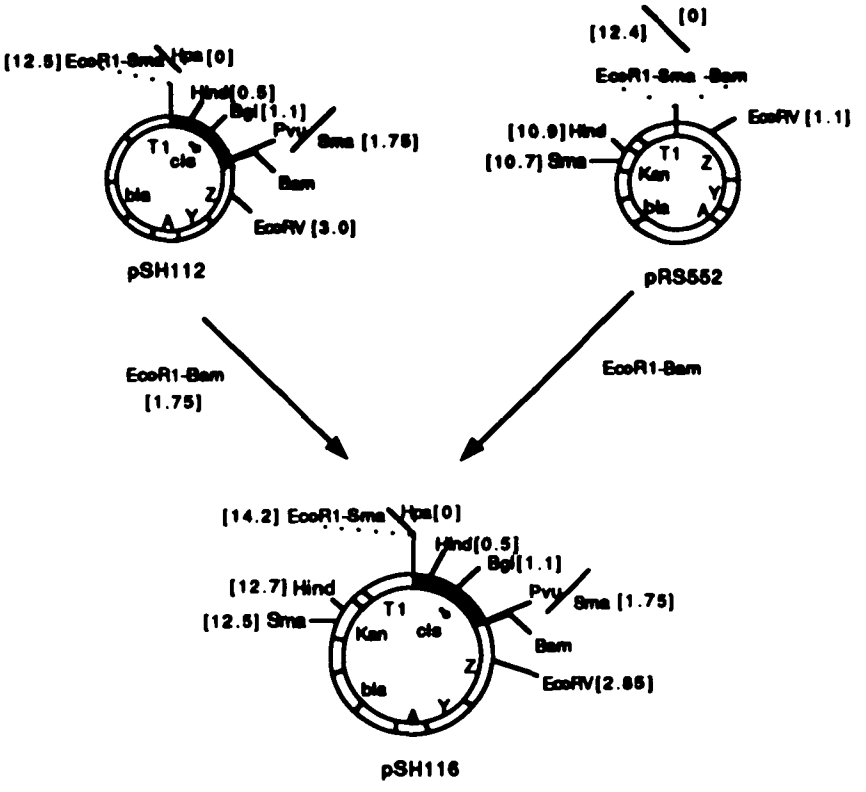

Figure 4.6. Construction of pSH116. A 1.75 EcoR1-BamHI fragment from pSH112 was inserted into the EcoR1-BamHI sites of pRS552. CIAP was used to prevent two pRS552 plasmids from religating. Restriction enzymes: Bam (BamH1), Bgl (BglII), EcoR1, EcoRV, Hind (HindIII), Hpa (HpaI), Pvu (PvuII), Sma (SmaI). Not all Pvu or HpaI sites are listed. Black box, chromosomal DNA; Open box, pRS DNA.

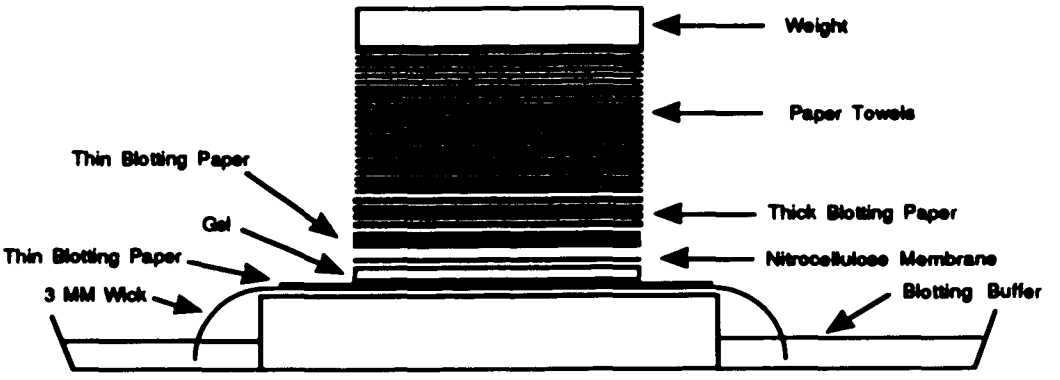

Figure 4.7. Southern apparatus.

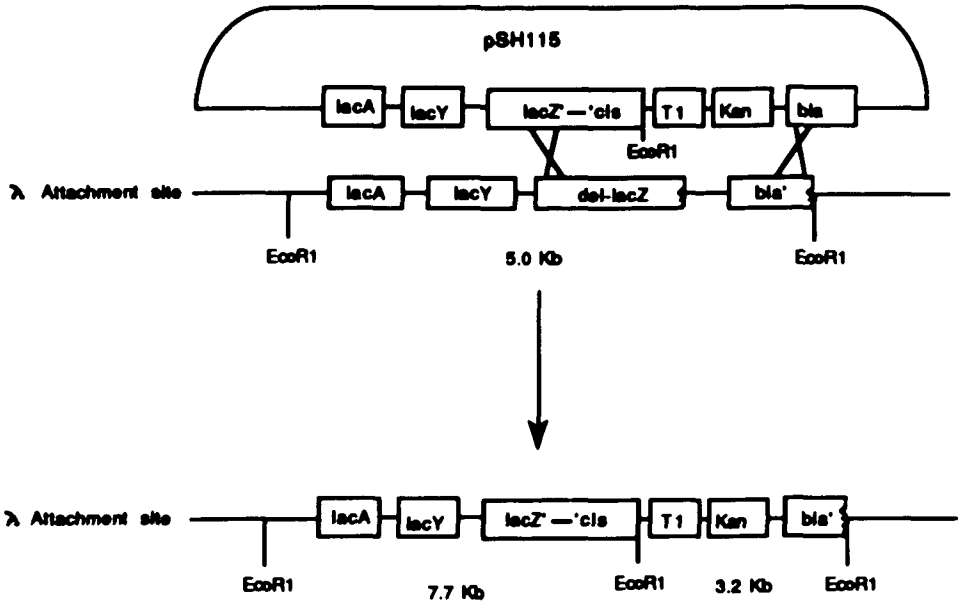

Figure 4.8. Recombination of ϕ(*cls*-'*lacZ*)hyb into the λ attachment site. Possible points of crossover are shown. Lysogens containing ϕ(*cls*-'*lacZ*)hyb were selected by their resistance to kanamycin.

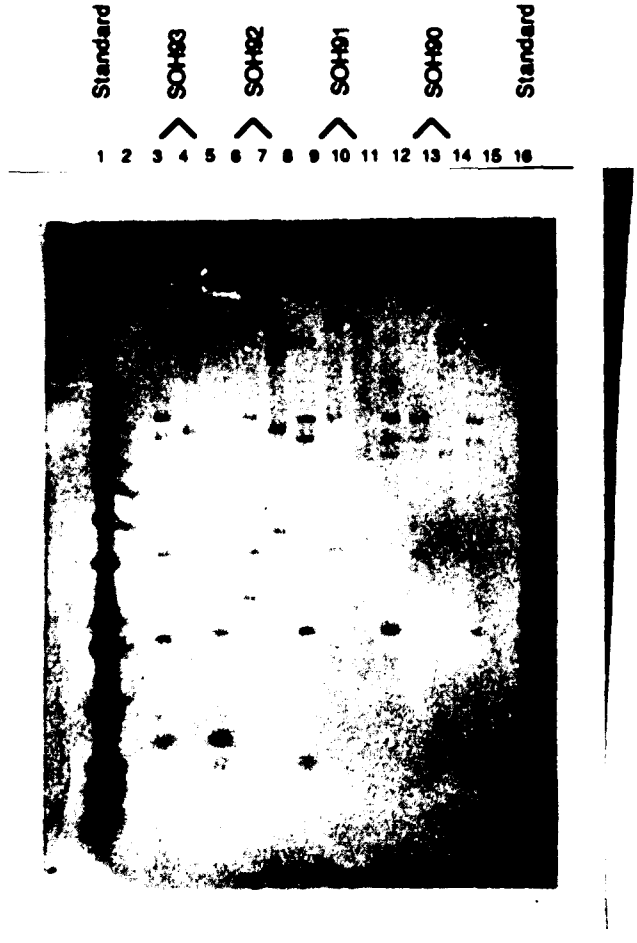

Figure 4.9. Southern analysis. Lanes 3-5, SOH93 [ϕ(*cls*-'*lacZ*)hyb]; Lanes 6-8, SOH92 [ϕ(*cls*-*lacZ*$^+$)]; Lanes 9-11, SOH91 ('*lac*); Lanes 12-14, SOH90 (*lacZ*). Lanes 1 and 16 contain a BRL Kilobase ladder consisting of the following Kb fragments: 12,216; 11,198; 10,180; 9,162; 8,144; 7,126; 6,108; 5,090; 4,072; 3,054; 2,036; 1,636; 1,018; 517; 396; 344; 298. HindIII digest, lanes 3, 6, 9, 12; EcoR1 digest, lanes 4, 7, 10, 13; HindIII + EcoR1 digest, lanes 5, 8, 11, 14. Identification of DNA was based upon homology with biotin-labelled pSH103.

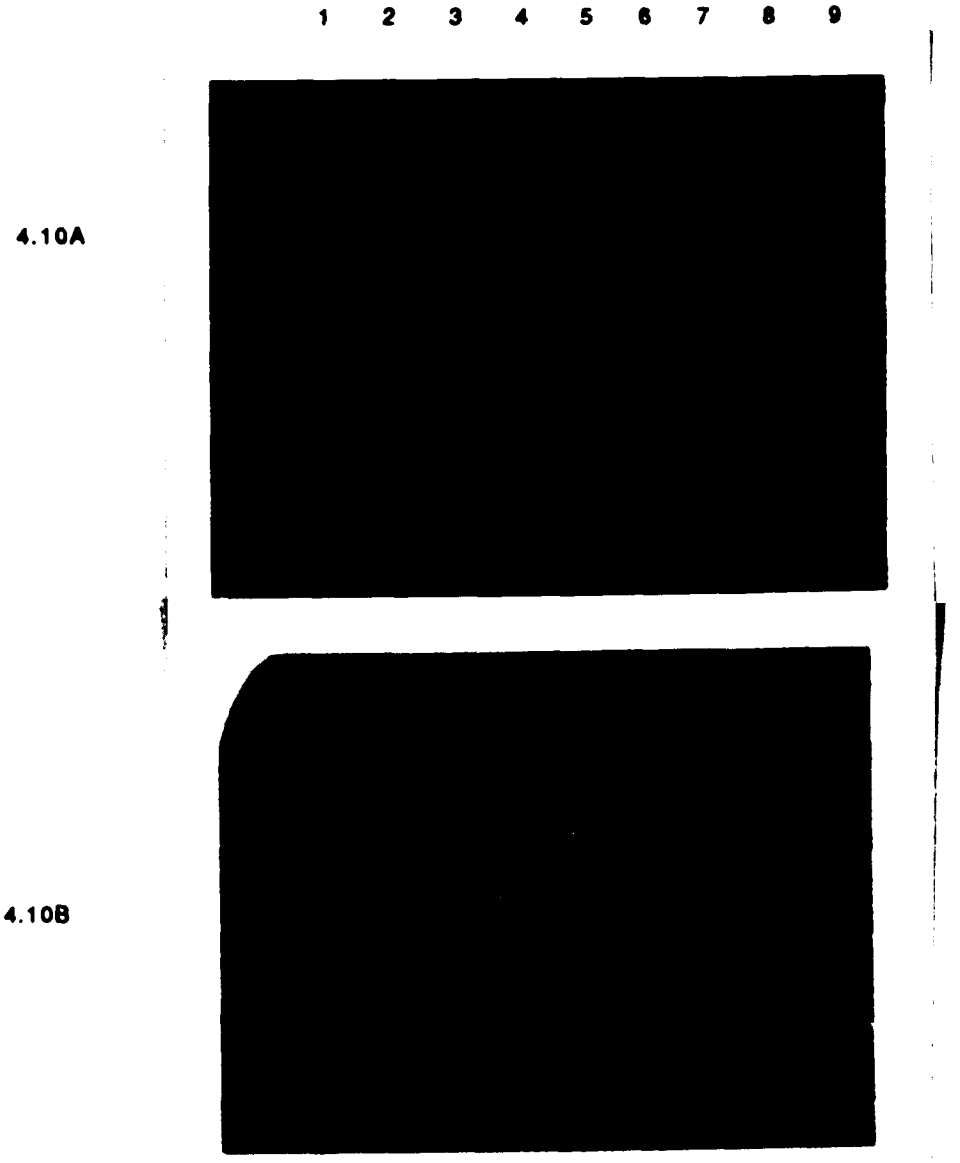

Figure 4.10A and 4.10B. Western analysis. Lane 1, SOH141; Lane 2, P90C/pSH115; Lane 3, P90C/pRS552; Lane 4, P90C/pSH113; Lane 5, P90C/pRS551; Lane 6, SOH92; Lane 7, SOH93; Lane 8, P90C; Lane 9 molecular weight standards. Proteins were identified in Figure A by staining with coomasie blue. Proteins in Figure B were identified using mouse anti β-galactosidase antibody followed by anti mouse alkaline phosphatase conjugate. Molecular weight standards: rabbit myosin, 205 Kda; β-galactosidase, 116 Kda; rabbit muscle phosphorylase B, 97.4 Kda; Bovine albumin, 66 Kda; egg albumin 45 Kda; and bovine erythrocytes carbonic anhydrase, 29 Kda.

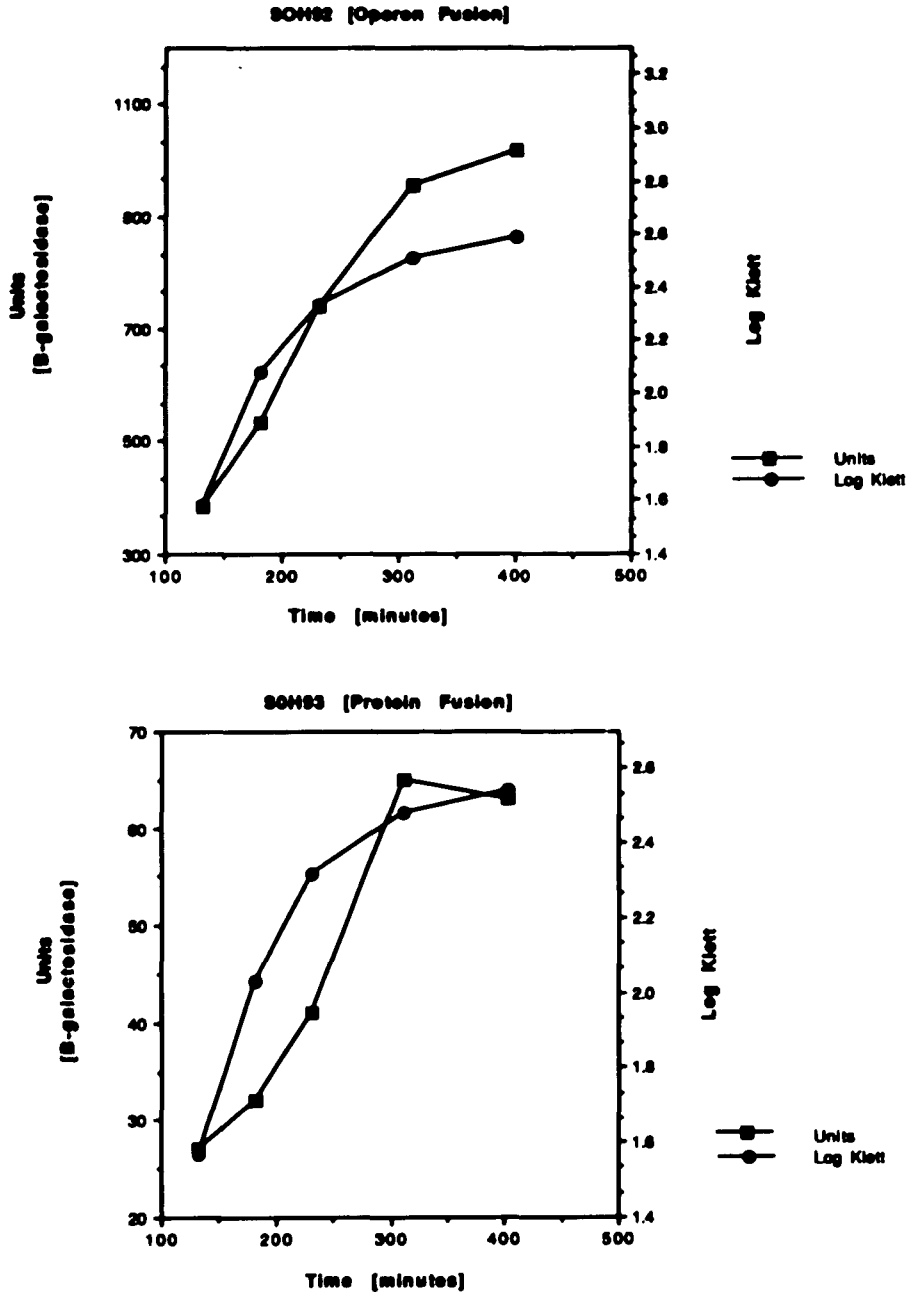

Figure 4.11. Effect of cell density. Bacteria were grown in LB broth shaking at 37° C. Expression of SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls-'lacZ*)hyb] was determined as the cells grew to stationary phase. Aliquots of cells were removed at the indicated times and the β-galactosidase activity determined.

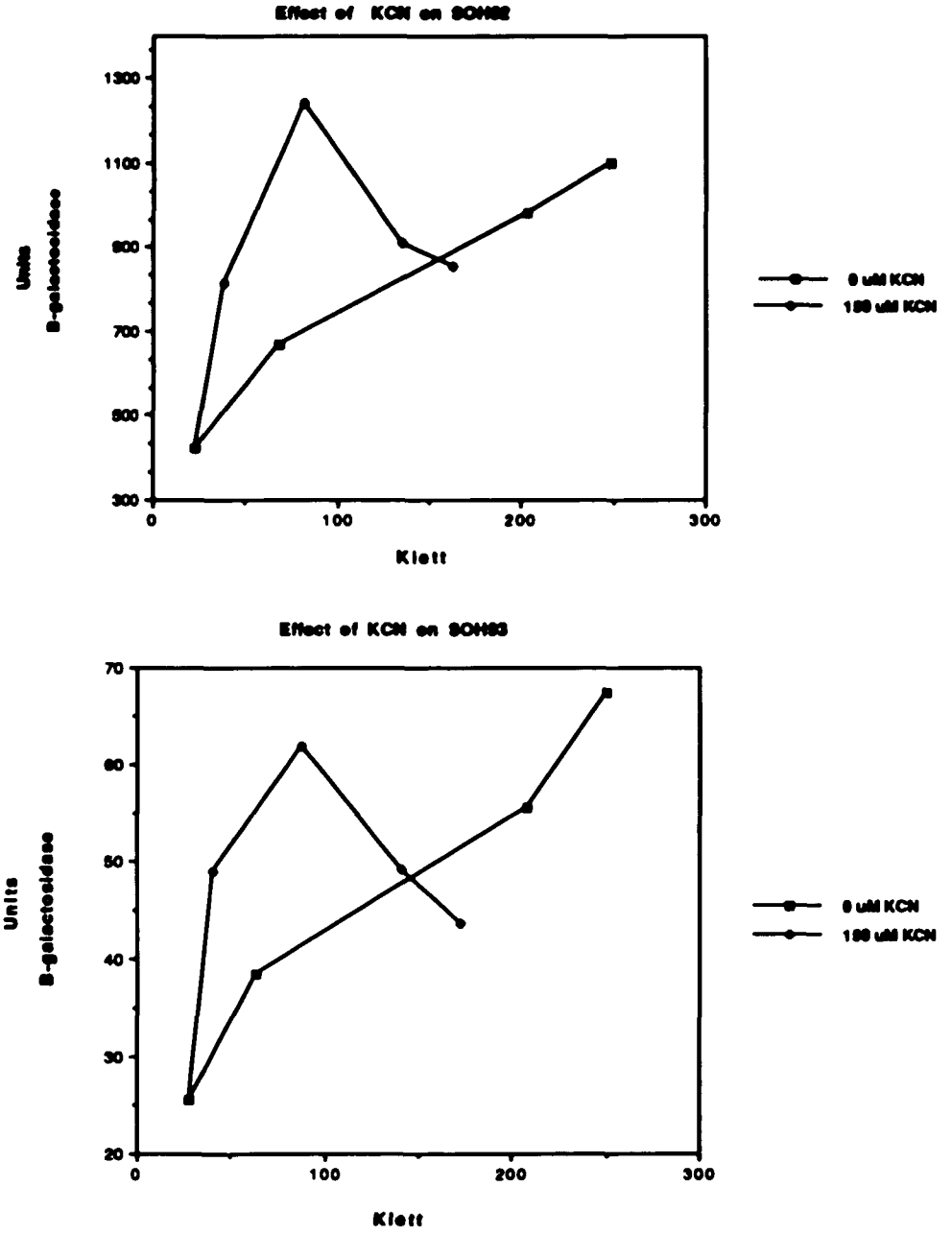

Figure 4.12. Effect of KCN. Bacteria were grown in LB broth shaking at 37° C. When SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls-'lacZ*)hyb] reached a density of 21-28 Klett units KCN was added to a concentration of 150 μM. Aliquots of cells were removed and assayed for β-galactosidase after 35, 105 and 145 min.

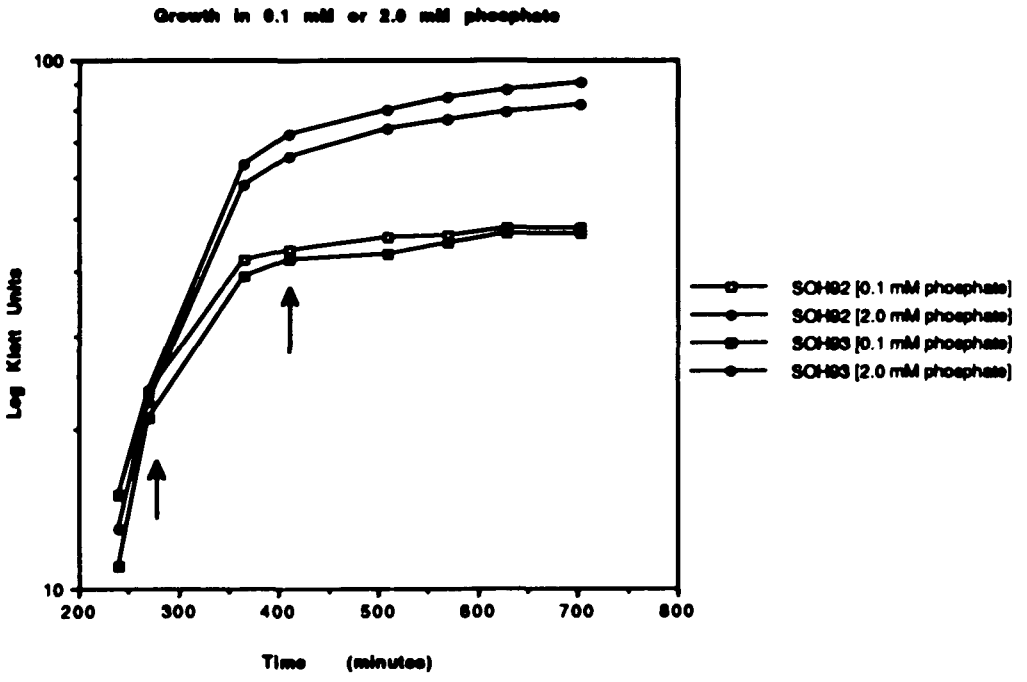

Figure 4.13. Growth in 0.1 mM or 2.0 mM phosphate. Strains SOH92 [ϕ(*cls-lacZ*$^+$)], and SOH93 [ϕ(*cls-'lacZ*)hyb] were grown in MOPS medium, shaking at 37° C, containing either 0.1 mM or 2.0 mM phosphate (K_2HPO_4). The arrows indicates points at which β-galactosidase activity was determined (Table 4.17).

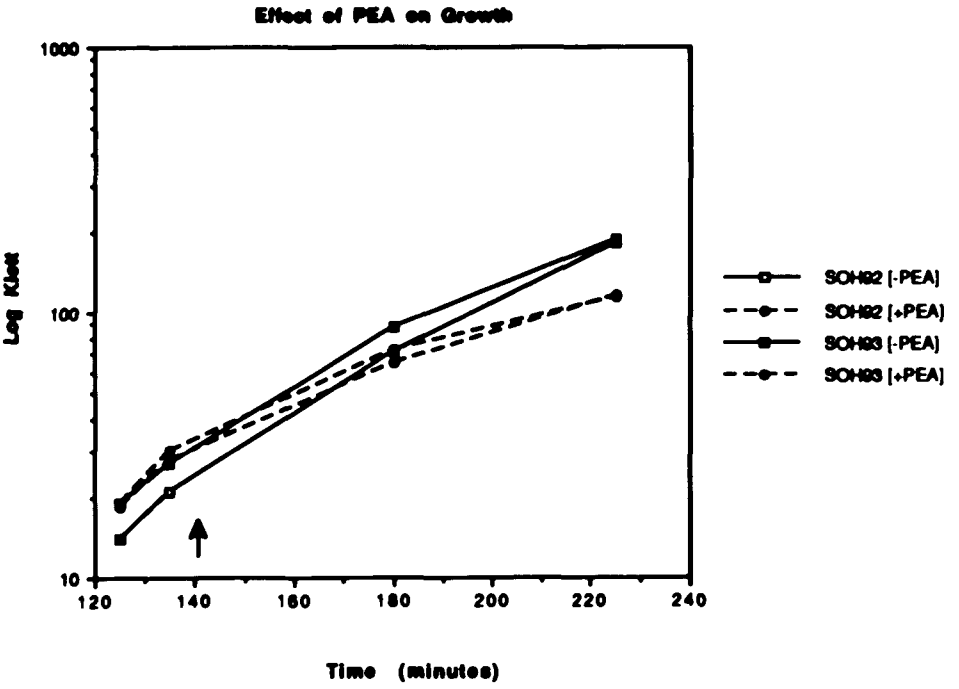

Figure 4.14. Effect of PEA on Growth. Strains SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls-'lacZ*)hyb were grown in LB broth shaking at 37° C. The arrow denotes when the cells were treated with PEA (0.15% v/v final concentration). Samples were removed and assayed for β-galactosidase activity 45 and 90 minutes after treatment (Table 4.20).

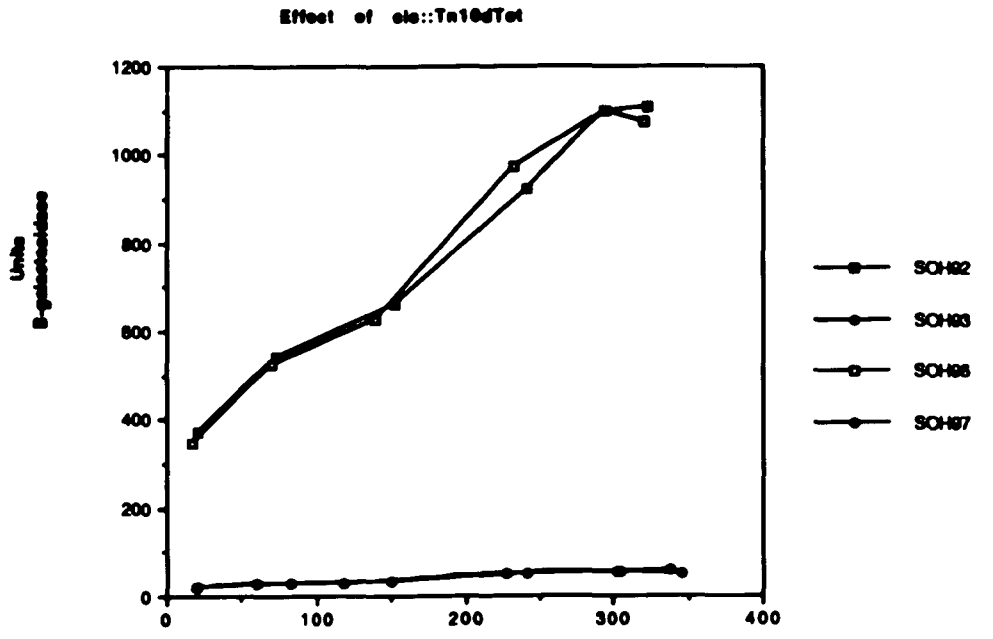

Figure 4.15. Effect of *cls*::Tn*10*dTet. Strains SOH92 [ϕ(*cls-lacZ*$^+$)], SOH93 [ϕ(*cls*-'*lacZ*)hyb], SOH96 [ϕ(*cls-lacZ*$^+$) *cls*::Tn*10*dTet3] and SOH97 [ϕ(*cls*-'*lacZ*)hyb *cls*::Tn*10*dTet3] were grown in LB broth shaking at 37° C. At the indicated points aliquots of cells were removed and the β-galactosidase activity determined.

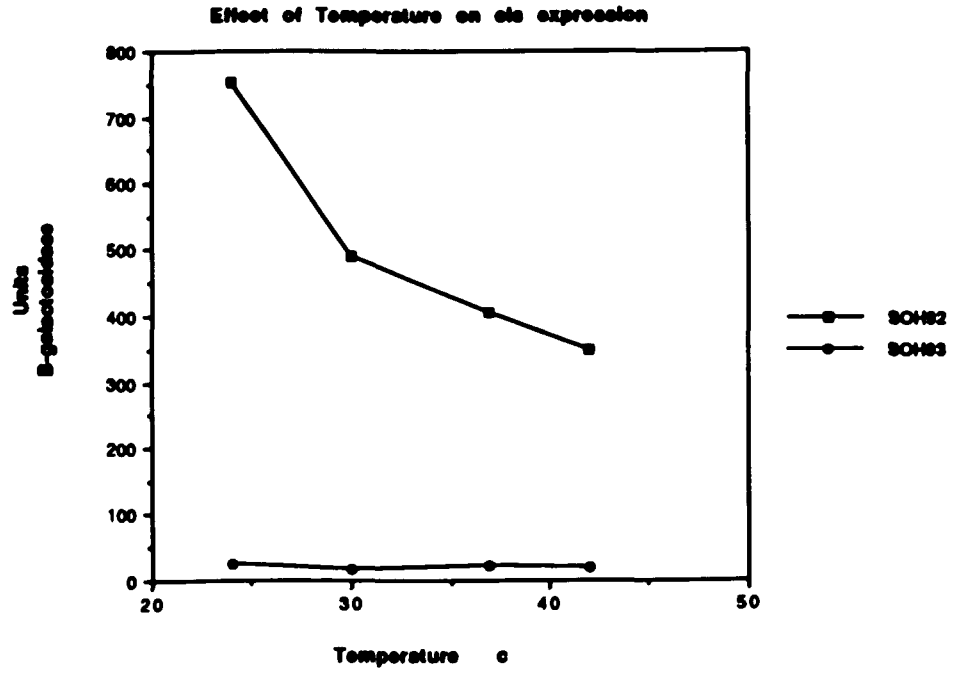

Figure 4.16. Effect of temperature. Strains SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls-'lacZ*)hyb] were grown in shaking LB broth at 25° C, 30° C, 37° C, or 42° C. β-galactosidase activity was determined at 25-35 Klett units.

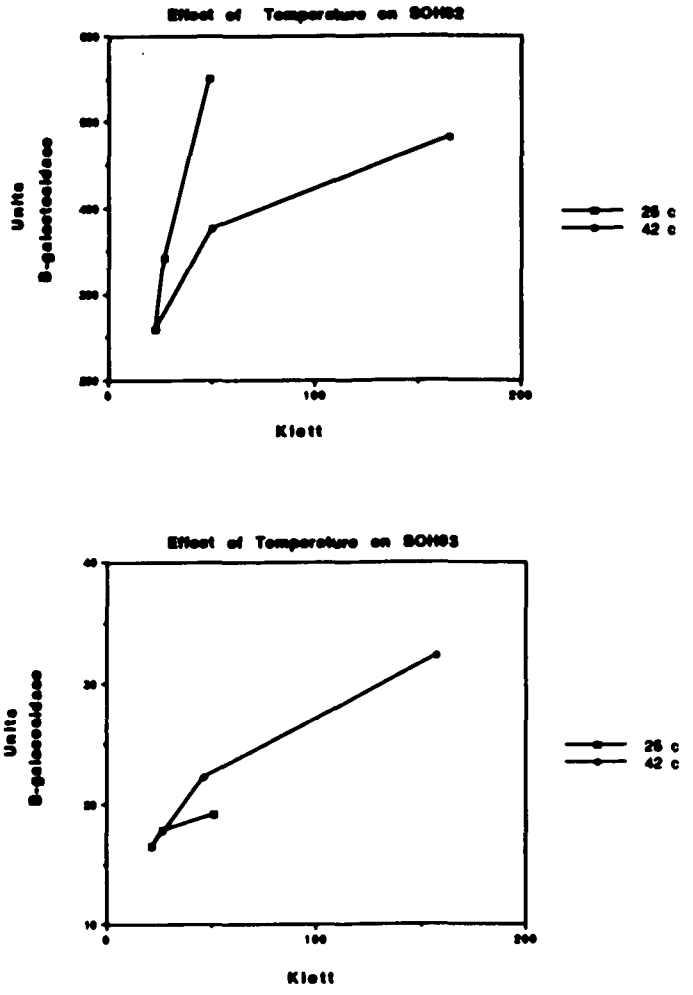

Figure 4.17. Temperature Shift. Strains SOH92 [ϕ(*cls-lacZ*$^+$)] and SOH93 [ϕ(*cls-'lacZ*)hyb] were grown shaking in LB broth 42° C. At a cell density of 20-25 Klett units the bacteria were shifted to 25° C. β-galactosidase activity was determined after 0 min, 20 min, and 75 min.

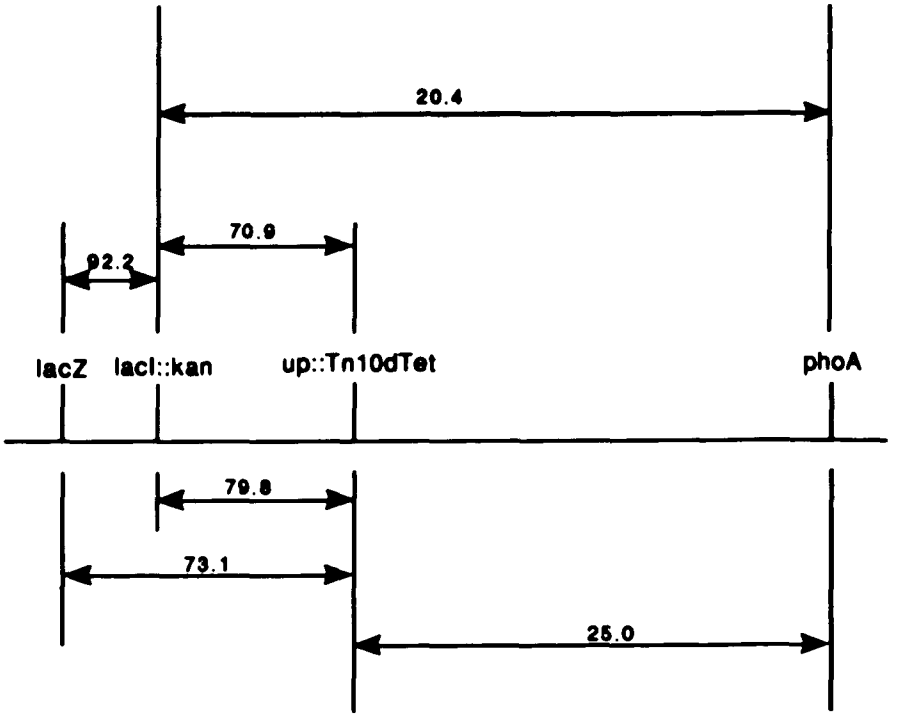

Figure 4.18. Chromosomal location of *up*::Tn*10*dTet. Numbers refer to cotransduction frequency.

Appendix 1

Solutions for Southern Analysis

Blotting Buffer:
0.4 N NaOH
0.6 M NaCl

Prehybridization Buffer:
2 mL 20% dextran sulfate (filter sterilized)
1 mL 10% SDS
7 mL water

20x SSC:
3.0 M NaCl
0.3 M sodium citrate pH 7.0

Buffer 1:
0.1 M Tris-HCl pH 7.5
0.15 M NaCl

Buffer 2:
Buffer 1
3% w/v bovine serum albumin -fraction V-

Buffer 3:
0.1 M Tris-HCl pH 9.5
0.1 M NaCl
50 mM $MgCl_2$

Streptavidin-Alkaline Phosphatase Mix:
5 ml buffer 1
5 ul streptavidin-alkaline phosphatase

Developer:
7.5 mL buffer 3
33 µL NBT
25 µL BCIP
mix by swirling

Appendix 2

Solutions for Western Analysis

Bromphenol blue (BpB)[1] :
10 mg bromophenol blue
10 mL water

4x Upper Buffer (UP)[1] :
0.5 M Tris-HCl pH 6.8
0.4% SDS

2x Sample Buffer (SB)[1] :
12.5 mL 4x UP
20.0 mL glycerol
adjust volume to 60 mL with water

2x Loading Buffer (LB)[1] :
0.5 mL β-mercaptoethanol
0.25 mL 0.1% BpB
4.0 mL 10% SDS
5.3 mL 2x SB

10x Reservoir Buffer Stock (RBS)[1] :
30 gm. Tris-base
40 gm glycine
adjust volume to 1.0 L with water

Running Buffer (RB)[1] :
100 mL 10x RBS
10 mL 10% SDS
890 mL water

TBST:
50 mL 1 M Tris-HCl pH 7.4
30 mL 5 M NaCl
0.5 mL Tween 40
0.2 gm Thimerosal
Adjust volume to 1.0 L with water

Blocking Solution:
25 mL Tris-HCl pH 7.4
15 mL 5 M NaCl
25 g powdered milk
0.250 mL Tween 40
0.05 gm thimerosal
Adjust volume to 500 mL with water

Alkaline Phosphatase Buffer (APB):
12.1 gm Tris-HCl pH 9.4
20 mL 5 M NaCl
5 mL 1 M $MgCl_2$
Adjust volume to 1.0 L with water

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