Escherichia coli mutants unable to use a combination of L-serine, glycine and L-leucine as carbon source

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Abstract

Escherichia coli Mutants Unable to Use a Combination of L-serine, glycine and L-leucine as a Carbon Source

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Previously, medium containing a combination of L-serine, glycine and L-leucine (SGL medium) has been used to study the characteristics, including the activation system, of L-serine deaminase (L-SD), an interesting enzyme from *Escherichia coli* that is first synthesized in an inactive form. In this thesis, I isolated two new *E. coli* SGL- mutants through λplacMu insertion. All these mutants MEW20b and MEWb1 are quite likely involved in the activation of L-SD enzyme. Applying inverse PCR technique, I identified the mutated gene in these two mutant strains to be *nuoM* and *torA* respectively. A formerly isolated SGL- mutant MEW84 was also identified as a λplacMu insertion in the *glpC* gene. Further experiments proved that mutations in these three genes actually were responsible for the SGL- phenotype of the mutant strains. A comparison of the functions of the three mutated genes suggests that electron donation may be part of the process necessary to activate L-SD. Physiological studies were carried out to investigate further these three mutants.

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Introduction

L-serine deaminase (L-SD) is an *Escherichia coli K-12* enzyme that can break down L-serine to pyruvate and ammonia. This enzyme has been drawing attention since it is synthesized in *E. coli K-12* in a considerable amount even when cells are grown in glucose-minimal medium while it does not seem to have any clear functions (Newman *et al.*, 1963; Newman *et al.*, 1985b). It is also interesting that the activity of L-SD can be induced by glycine and L-leucine but not by its own substrate L-serine (Pardee *et al.*, 1955; Newman *et al.*, 1982).

Other than those odd features of L-SD, the activity of this enzyme has been found to be highly regulated. At least two global regulators, the products of the *ssd* and *lrp* genes, have been reported to be involved in the regulation of the expression of L-SD (Newman *et al.*, 1981; Lin *et al.*, 1990). The amount of L-SD is also affected by a number of environmental factors: it has been shown that L-SD was induced by DNA damaging agents like UV irradiation and mitomycin, by anaerobic growth, by growth at 42°C and by addition of ethanol to the growth medium (Newman, personal communication). Moreover, in *E. coli K-12*, both *in vitro* and *in vivo*, L-SD can be isolated in an inactive but activatable form. At least three mutant strains: MEW128, MEW191 and MEW84 have been found that lack the *in vivo* L-SD activity yet have an activatable enzyme *in vitro*. This *in vitro* activation can be achieved through incubation of the cell extract with iron and dithiothreithol (DTT) (Newman *et al.*, 1985a, b; Feng,

1990). This *in vitro* activation by iron and DTT is probably a reflection of the *in vivo* enzymatic activation mechanism. Thus the activity of L-SD is seemingly also subjected to post-translational regulation.

Wild type *E. coli K-12* can use a wide range of carbon sources, but L-serine is not one of them, unless glycine and L-leucine are also supplied in the medium. This is probably due to the induction by these two amino acids of L-serine deaminase (Newman and Walker, 1982; Pardee and Prestidge, 1955). In fact the combination of glycine and L-leucine was found to elevate L-SD activity dramatically in glucose-minimal medium (Newman, personal communication). It seems that this induced high amount of L-SD allows *E. coli* to degrade enough serine to pyruvate. Pyruvate can then be used as a carbon and energy source in *E. coli*. In practice, selection of most of the mutants with altered L-serine deaminase was based on screening for the inability to grow on L-serine as carbon source with the addition of glycine and L-leucine.

Studies in Dr.Newman's lab revealed that there are two L-serine deaminases in *E. coli*, coded by the *sdaA* and *sdaB* genes (Su *et al.*, 1991). These two genes have been cloned and sequenced (Su *et al.*, 1989; Shao and Newman, 1993). Among them, *sdaA* was the structural gene for L-SD1, which was synthesized in both glucose-minimal and LB medium, and *sdaB*, which codes for L-SD2, is only expressed in LB medium (Su *et al.*, 1989; Su *et al.*, 1991).

The mechanism of the activation of L-SD has also drawn a lot of attention in Dr. Newman's lab. Some hypotheses have been presented. Yet the true activation mechanism still remains unclear. To provide the necessary background on this research, the following sections review some experimental evidence related to the topic.

Part 1. Serine Synthesis and Degradation in *E. coli*

1.1. The synthesis of L-serine

The synthesis of L-serine in *E. coli* through the Embden-Meyerhof pathway is illustrated in Fig.1. From 3-phosphoglycerate, this intermediate is converted to L-serine through oxidation, transamination and dephosphorylation step by step by the products of *serA*, *serC* and *serB* genes respectively.

1.2. The degradation and metabolic fate of L-serine

L-serine can be degraded to pyruvate and ammonia by L-serine deaminase, which is constitutively synthesized in *E. coli K-12* (Pardee *et al.*, 1955). Although pyruvate can be used as carbon and nitrogen source by *E. coli*, conversion of serine to pyruvate does not yield energy. In fact, using L-SD to degrade L-serine is energetically wasteful in glucose-grown cells (Ramotar and Newman, 1986), which may indicate that L-SD has other functions for *E. coli*. The high Km of L-serine deaminase raises some doubt as to whether L-serine is indeed the primary substrate of L-SD (Moniakis, 1992). On the other

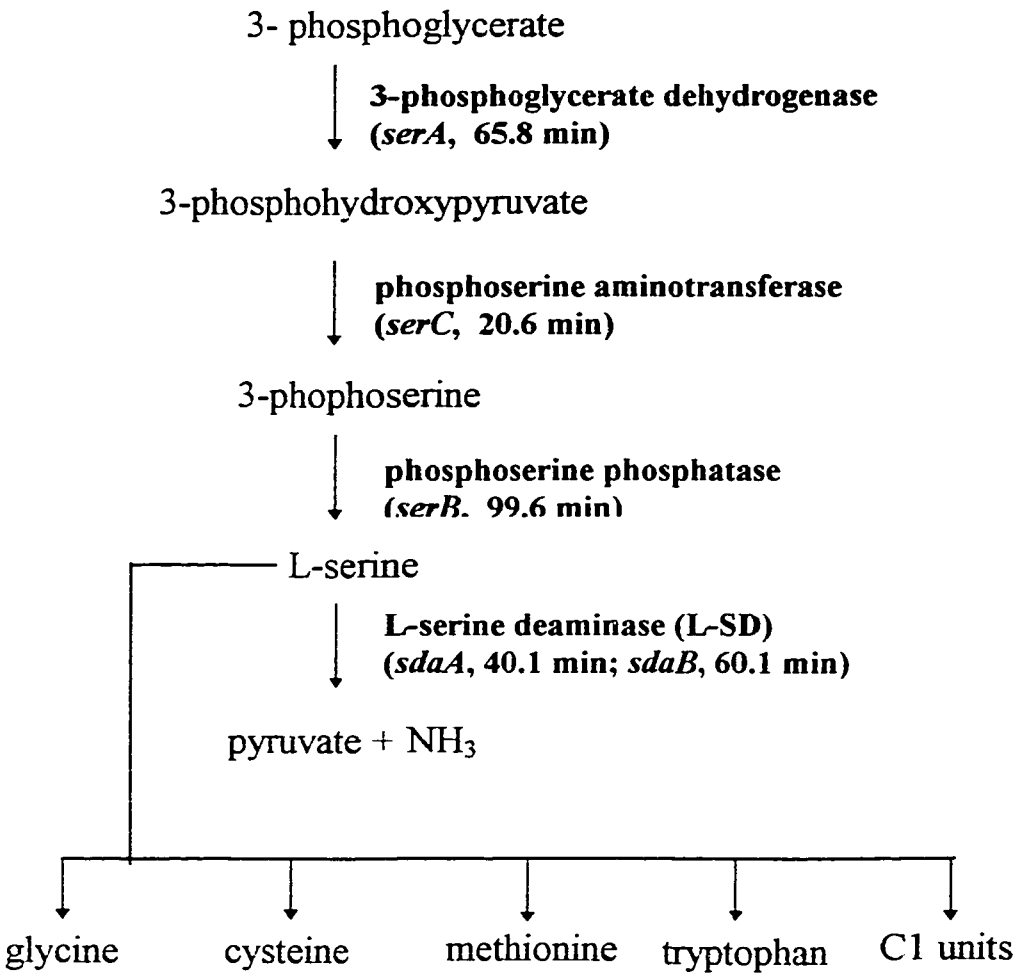


Fig. 1. L-serine metabolism in *E. coli*

hand, L-serine deaminase may either function to regulate the L-serine pool in the cell (Su, 1991; Su and Newman, 1989), since high concentration of L-serine is toxic to *E. coli* (Cosloy and McFall, 1970; Hama *et al.*, 1990; Uzan and Danchin, 1978), or to convert excess L-serine to carbon and nitrogen sources.

The L-serine biosynthetic pathway is one of the important metabolic pathways in *E. coli*: about 15% of the carbon from glucose passes through it (Pizer and Potochny, 1964). Other than L-serine, this path is the main biosynthetic source for glycine, and a major source of one-carbon units. L-serine, glycine and C1 units, products from this pathway, are themselves precursors for a number of metabolites. L-serine itself has been found to be required for a large number of biosynthetic reactions. It serves as a precursor for the synthesis of L-tryptophan (Yanofsky, 1960), cysteine (Kredich and Tomikins, 1966) and methionine (Tran *et al.*, 1983).

1.3. Regulation of L-serine synthesis

Instead of having any feedback effect on L-serine deaminase, the end-product L-serine regulates the flow of carbon through the L-serine synthesis pathway by inhibiting the first enzyme in the pathway, phosphoglyceric acid dehydrogenase (Stauffer, 1987). The transcription of *serA*, the gene coding for phosphoglyceric acid dehydrogenase, was also found to be inhibited by glycine (Pizer, 1963), L-leucine and several other amino acids that are not directly related to serine biosynthesis. Two global regulators, products of the

lrp and *ssd* genes, have been shown to control the transcription of *serA* (Lin *et al.*, 1990; Newman *et al.*, 1981).

1.4. Other enzymes that can deaminate L-serine

It has already been found that L-serine deaminase is not the only enzyme that can deaminate L-serine. At least the product of *ilvA* and *tdcB* genes, the biosynthetic L-threonine deaminase and biodegradative L-threonine deaminase respectively, possess this function (Umbarger, 1957; Goss and Datta, 1984).

The biosynthetic L-threonine deaminase (biosynthetic L-TD) is the enzyme that catalyses the first step in the L-isoleucine synthesis pathway, through which L-threonine is deaminated to form α-ketobutyrate for the synthesis of L-isoleucine (Calhoun *et al.*, 1973). At least *in vitro*, this protein was found to also degrade L-serine (Umbarger, 1957).

The product of the *tdcB* gene, the biodegradative L-threonine deaminase (biodegradative L-TD) also deaminates L-threonine, but this protein is induced in anaerobic conditions in both rich medium and minimal medium supplied with threonine, serine, valine and isoleucine together with cyclic AMP and fumarate (Datta *et al.*, 1987). Meanwhile the other gene in this operon, *tdcC* gene codes for a membrane-associated threonine-serine permease (Sumantran *et al.*, 1990).

Part 2. Regulation of L-SD in *E. coli*

2.1. Environmental factors that affect L-SD activity

L-SD can be induced by some enviromental stresses such as: exposure to DNA damaging agents like UV irradiation, nalidixic acid and mitomycin; growth at high temperature; growth in anaerobic conditions and alcohol shock (Newman *et al.*, 1982a; Newman, personal communication).

When grown in glucose-minimal medium, a low but significant amount of L-SD is produced. This enzyme activity can be even induced to a much higher level with the addition of glycine and L-leucine (Pardee and Prestidge, 1955). L-SD activity is also increased by growth in LB medium (Newman et al., 1982a). However, the substrate of L-SD, L-serine does not induce (Pardee and Prestidge, 1955; Isenberg and Newman, 1974).

2.2. Regulation of L-SD by *lrp*

As a global regulator, Lrp (leucine-responsive regulatory protein), the product of the *lrp* gene, has been found to regulate the expression of many genes in *E. coli*. L-SD activity is repressed by Lrp, since the activity of L-SD is greatly increased in *lrp* mutant strains (Lin *et al.*, 1990). In this case, Lrp likely serves as a repressor. When exogenous

L-leucine is added, the repression is released by the interaction of L-leucine and Lrp (Lin *et al.*, 1990; Lin, 1992).

In fact the *lrp* mutant strain was first isolated through the selection for *E. coli K-12* able to grow on L-serine minimal agar medium, i.e. *E. coli* able to use L-serine as sole carbon source (Shao, personal communication). This phenomenon is probably due to the increased L-SD activity in the *lrp* mutant which allows *E. coli* to break down L-serine to produce enough pyruvate to serve as its carbon and energy sources.

The *lrp* mutation increases expression of genes for degrading L-serine and decreases expression of genes for L-serine synthesis.

2.3. Regulation of L-SD by *ssd*

Although the nature of the *ssd* gene and its product is still quite obscure, it is known that a mutation in this gene also affects the expression of several genes including the ones coding for L-serine deaminase. With the L-SD activity being induced 7-fold higher than normal wild-type cells, the *ssd* mutant strain was also able to grow on L-serine as sole carbon and energy source (Newman *et al.*, 1981). At least two other enzyme activities, the ones of L-threonine dehydrogenase (TDH) and 3-phosphoglycerate dehydrogenase were also found to be altered dramatically in the *ssd* mutant (Lin *et al.*,1990; Newman *et al.*, 1981).

2.4. L-SD is subjected to post-translational modification

L-SD has been proved to be very unstable, for its activity could not be detected in crude wild-type cell extracts. However, this activity could be restored with the addition of iron and DTT (Newman *et al.*, 1985b).

The isolation of mutants MEW128, MEW191 and MEW84 in Dr. Newman's lab is another strong indication that L-SD may be subjected to post-translational modification. All these mutants have a very low level of L-SD activity *in vivo*. Therefore none of them can grow on L-serine even with the addition of glycine and L-leucine. However, L-SD activity in the crude extract from all these mutants could be activated when incubated with iron and DTT (Newman *et al.* 1985a; Feng, 1990). This suggests that an *in vivo* activation system is needed to post-translationally modify the inactive form of L-SD to turn it into a functional enzyme.

Part 3. Genes Coding for L-SD

3.1. L-SD1 is coded by *sdaA* gene

The L-SD coding gene was first mapped at 40.1 min in a λplacMu insertion mutant with the characteristic that the L-SD activity in it when grown in glucose-minimal medium could neither be detected *in vivo* nor be activated *in vitro* by incubation with iron and DTT (Su and Newman, 1991). The experimental fact that the cloned wild type *sdaA*

complemented the phenotype of the *sdaA* mutation and showed high L-SD activity both *in vivo* and *in vitro* clearly indicated that *sdaA* was the structural gene for L-SD. Furthermore, Su's work of hybridization with the mutant's genomic DNA by using the cloned wild type *sdaA* as a probe proved that the *sdaA* gene was indeed interrupted by the insertion of λplacMu (Su *et al.*, 1989).

Following that, the whole *sdaA* gene which codes for a 454 amino acid protein was sequenced, and this sequence was found to be consistant with the Edman degradation of the N-terminal sequence of the purified L-SD (Su et al., 1993). An *in vitro sdaA-lacZ* in-frame fusion was constructed and the β-galactosidase was used to purify the *sdaA-lacZ* fusion protein from plasmid through affinity chromatography. Proteins purified through this method showed both β-galactosidase and L-serine deaminase activity. This left no doubt that *sdaA* is the gene coding for L-SD (Su *et al.*, 1993).

The *sdaA*::λplacMu fusion or *sdaA::lacZ* fusion constructed *in vitro* provided a approach to investigate the regulation of the expression of *sdaA* in detail. β-galactosidase activity assays revealed that the products of both the *ssd* and *lrp* genes play roles in regulation of the transcription of *sdaA* (Su *et al.*, 1989). Of all the environmental factors that affect the L-SD activity, growth in anaerobic conditions and UV irradiation have been shown to actually change the transcription of *sdaA* (Su, 1991). Lin's elaborate work further exhibited that Lrp represses the transcription of *sdaA* by binding to two of its upstream sites, while extra L-leucine seems to activate *sdaA* by releasing the binding of Lrp (Lin, 1992; Lin *et al.*, 1992).

The fact that the *sdaA* null mutation strain still produces considerable amount of L-SD in LB medium hinted that there might be another L-serine deaminase that is expressed in different conditions (Su and Newman, 1991). Later on, it was discovered that this was indeed the case.

3.2. L-SD2 is coded by the *sdaB* gene

A strain carrying an insertion in *sdaA* showed no L-SD in minimal medium. However, expression of L-SD in minimal medium was established in that strain by a further mutation mapped at 60.1 min (Su and Newman, 1991). It then seemed reasonable that the gene in which the second mutation occurs codes for L-SD2. This is usually only expressed in LB medium, but in the mutant it was turned on even in minimal medium. Immediately after that, *sdaB* was cloned and sequenced and was proved to be the exact coding gene for L-SD2 (Shao,1993; Shao and Newman, 1993). The point mutation at 60.1 min which allowed the expression in glucose-minimal medium was revealed to be a single base-pair change in the Shine-Dalgarno sequence of *sdaB*, through which the translation was improved (Shao, 1993; Shine and Dalgarno, 1974). Consequently it seems the mRNA from *sdaB* may exist in glucose-minimal medium. If so why it is not translated in minimal medium, and when it is translated in LB medium remains a puzzle (Shao, 1993).

From the study of *sdaB::lacZ* in frame fusion, it was discovered that unlike the case of *sdaA*, no amino acids had any effect on the *sdaB* expression, nor did UV

irradiation, anaerobic growth condition and elevated temperature induce *sdaB*. Similarly, neither the *ssd* nor *lrp* gene product was involved in regulation of the expression of *sdaB*. However *sdaB* was shown to be under catabolite repression. Failure to synthesize the cyclic AMP receptor protein (Crp), the mediator of catabolite repression in *E. coli*, had a dramatic effect on the expression of *sdaB* (Shao, 1993; Shao and Newman, 1993).

Comparison of the nucleotide sequence of *sdaA* and *sdaB* showed about 73% identity, while the amino acid sequence deduced from these two genes shared even higher similarity (76.5%) (Shao, 1993; Shao and Newman, 1993). The high similarity of the *sdaA* and *sdaB* hinted that they are probably descended from the same ancestor. The fact that both *sdaA* and *sdaB* are involved in such a complicated regulation system indicates that their function must be very important in *E. coli* metabolism.

Shao's intensive study also led to the discovery of an *sdaC* gene which is located right upstream of *sdaB* and was translated from the same promoter. The product of this gene might be a membrane bound protein for L-serine transport (Shao, 1993).

Part 4. Possible Mechanism for Activation of L-SD

4.1. *In vitro* activation of L-SD by iron and DTT

4.1.a. Possible function of iron and DTT in activating L-SD

L-serine deaminase obtained from wild type *E. coli K-12* is in an inactive form. This inactive form of L-SD in crude extract can be activated *in vitro* by incubation with iron, either in ferric (Fe^{3+}) or ferrous ion (Fe^{2+}) form, and DTT in aerobic conditions (Newman *et al.*, 1985b; Newman *et al.*, 1990). From this it seems neither Fe^{2+} nor Fe^{3+} alone is the activating species. Furthermore, chelators stabilizing the oxidized form of iron could inhibit L-SD activation. Therefore the activation might involve the cycling of iron between Fe^{2+} and Fe^{3+}. Similar to *E. coli* L-SD, the L-serine dehydratase from *Clostridium acidiurici* was also reported to require ferrous ion and a thiol reducing agent to activate it (Carter and Sagers, 1972).

It has long been known that reactive oxygen species like superoxide radical anion, hydrogen peroxide and hydroxyl radical can react with various biomolecules and change the state of molecules (Basaga, 1990). Iron cycling between the ferric and ferrous state can generate these oxidants, while DTT, as a reducing agent, may facilitate this cycling reaction by reducing Fe^{3+} to Fe^{2+} (Walling, 1975). β-mercaptoethanol (ME) can substitute for DTT. The use of ME in a much higher amount, to activate L-SD also supports the idea that cycling of iron is functioning in activation (Moniakis, 1992). Most likely, the efficiency of DTT is due to its being a much stronger reducing agent than ME (Burns and Whitesides, 1990). In fact, it is quite likely that hydroxyl radical is the active species for activating L-SD, since hydroxyl radical scavengers such as mannitol, α-naphthol, 8-hydroxyquinoline and L-histidine inhibit L-SD activation when added during the activation period (Newman *et al.*, 1990).

Iron and DTT are known to cleave proteins in the case of the modification of glutamine synthetase of the yeast (Kim *et al.*, 1985). A possible activation of L-SD might also involve the cleavage by iron and DTT generated hydroxyl radicals, after which an active form of L-SD would be released (Newman *et al.*, 1990). The cleavage site might be the serine-serine bond between amino acids 243 and 244 of the protein, as suggested by Su *et al.* (Su *et al.*, 1989). As an example, histidine decarboxylase is also found to be activated by serinolysis at the serine-serine bond (Rasci *et al.*, 1983).

Apart from the formation of hydroxyl radicals that might cleave and activate L-SD, iron and DTT may also function separately on this protein. The inactive form of L-SD was also activatable with β-mercaptoethanol (ME) and DTT. Both of these are commonly used disulfide reducing agents (Jocelyn, 1987). DTT can completely reduce non-cyclic disulfides to thiols, and intermediate mixed disulfides are not present in a significant concentration (Singh and Whitesides, 1991). Therefore the activation process might involve DTT, or possibly both DTT and ME, to reduce disulfide bonds in L-SD (Moniakis, 1992).

4.1.b. General effect of hydroxyl and other free radicals to proteins

Among all the free radicals, the hydroxyl radical ($OH^{\cdot}$) is regarded as the most reactive, with a half-life of less than 1 nanosecond (Slater, 1984). Oxidation of metal ions like Fe^{2+} can generate hydroxyl radicals together with other free radicals:

$$Fe^{2+} + O_2 \longrightarrow Fe^{3+} + O_2^-$$

$$2\,O_2^- + 2\,H^+ \longrightarrow H_2O_2 + O_2$$

$$Fe^{2+} + H_2O_2 \longrightarrow Fe^{3+} + OH^- + OH^-$$

As mentioned before, DTT is used to recycle the oxidized metal ion. When the metal ion bound to an enzyme is involved in these oxidation-reduction reactions, the locally generated radical species can then react with the enzyme (Kim *et al.*, 1985). For example: glutamine-dependent carbamyl phosphate synthetase from *E. coli* (Trotta *et al.*, 1974), erythrocyte superoxide dismutase (Hodgson and Fridovich, 1975), *E. coli* glutamine synthetase (Levine, 1983) and many other enzymes can be impaired through site specific oxidation. The highly reactive hydroxyl radicals were found to abstract carbon-bound hydrogen atoms non-selectively (Schuchmann and Von Sonntag, 1977) and to add to carbon-carbon double bonds (Steenken and O'Neill, 1978). As for yeast glutamine synthetase, the level of cleavage by hydroxyl radical species was noticeable in a time-dependent manner after incubation with iron and DTT (Kim *et al.*, 1985). The oxidative inactivation of carbamoyl phosphate synthetase was also considered to involve cleavage by a hydroxyl radical species (Alonso *et al.*, 1992).

Another feature of hydroxyl radical reaction is the ability to cross-link polypeptides. Both the Penzes and Kim groups reported the formation of higher molecular weight protein complexes during incubation with iron and DTT (Penzes *et al.*, 1984; Kim *et al.*, 1985).

All the free radicals have been reported to cause damage to cells and are not likely activators *in vivo*. *In vivo*, the activation of L-SD, either through oxidation or through cleavage, or even by cross-links is quite likely to be achieved enzymatically. The *in vitro* activation is probably just an imitation of the enzymatic process by a chemical reaction.

4.1.c. Examples of thiol and metal requiring enzyme

As introduced earlier, the activation of L-SD might involve just DTT or DTT together with ME to reduce disulfide bonds in this protein. Thiols in the active site of the enzyme often play a role in the mechanism of action of the enyme. For instance, the *E. coli* FNR protein, which is the regulatory protein for activating gene expression in response to oxygen limitation, contains a cluster of cysteine and is only active in anaerobic condition when the cysteines are reduced (Spiro and Guest, 1988).

For another example, *E. coli* L-threonine dehydrogenase is a tetrameric protein with 6 half-cystine residues per subunit. When Mn^{2+} or Cd^{2+} are added, the activity of this enzyme is increased 5 to 10-fold, which is caused by the binding of Mn^{2+} and Cd^{2+} with one particular cysteine residue (Epperly and Dekker, 1991).

L-serine dehydratase from *Peptostreptococcus asaccharolyticus* also contains an Fe-S center. This enzyme functions only in anaerobic conditions, and loses activity when exposed to oxygen.. However, the activity of this enzyme can be regained in anaerobic

conditions with the addition of Fe^{2+} (Grabowski and Buckel, 1991). *E. coli* L-SD may need iron to bind at an iron-sulfur center just like L-serine dehydratase in *Lactobacillus fermentum* which was reported to have to bind Fe^{2+} to activate the enzyme through conformational change and through a possible interaction with enzyme bound pyridoxal-5'-phosphate (PLP) (Farias *et al.*, 1991). Iron requiring enzymes in *E. coli*, which need Fe^{2+} to bind and activate the enzyme activity, include D-altronate hydratase and D-mannonate hydratase (Dreyer, 1987).

4.2. Study of activation of L-SD through protein fusion

4.2.a. Purification and characterization of L-SD through protein fusion

Originally designed to overcome the difficulty of purifying L-serine deaminase, protein fusions of L-SD to β-galactosidase and the two-part fusion via a collagen sequence were used to study the activation mechanism of L-serine deaminase (Su *et al.*, 1993; Moniakis, 1992).

The L-SD gene *sdaA* was first cloned and fused in-frame with the β-galactosidase gene *lacZ* in two different forms to generate two protein fusions: the two-part fusion (L-SD - β-galactosidase) and the three-part fusion (L-SD - collagen - β-galactosidase). The product from a plasmid carrying *sdaA-lacZ* or *sdaA*-collagen-*lacZ* was then purified with the use of a β-galactosidase affinity column. Thus L-SD covalently bound to β-galactosidase could be isolated (Su, 1991).

Su found that L-SD purified through the method described above had no activity *in vivo*, even in cultures grown with glycine and L-leucine, which usually induce L-SD activity *in vivo* (Su, 1991). However, both the two-part and three-part fusion proteins along with the collagenase treated three-part fusion proteins could be activated *in vitro* by iron and DTT. One explanation of this result is that, with the protein fusions, *in vivo* the activating protein(s) simply can not reach L-SD when there is a fused bulk β-galactosidase, while *in vitro*, the much smaller molecules iron and DTT can comparatively easily reach L-SD in the fusion protein to activate it (Moniakis, 1992).

4.2.b. Two different levels of inactive L-SD

Moniakis' study on L-SD also revealed that there seem to be two different states of inactive L-serine deaminase (Moniakis, 1992). The first one is present in fresh crude cell extracts and freshly purifed proteins. The second one is formed after a few hours incubation at room temperature. L-SD after this treatment can not be activated by the usual DTT concentration of 4.45 mM, yet its activity can be restored by incubation with a higher concentration of DTT (45 mM) (Moniakis, 1992).

If reduction and conformational rearrangement are involved in the activation of L-SD, probably the L-SD is oxidized when left in room temperature. This oxidation may change the conformation of L-SD to a much less active form via the formation of new disulfide bonds or shuffling of already existing disulfide bonds (Moniakis, 1992).

4.2.c. Possible mechanism of conformation change for L-SD activation

Moniakis' study revealed that it is actually highly unlikely that a cleavage is involved in the activation of L-serine deaminase, since there is no detectable difference in the molecular weight of the inactive and active forms of L-SD (Moniakis, 1992). Also, from the tryptic digestion experiment, it was concluded that active and inactive L-SD have different susceptibilities to trypsin. Therefore a possible activation through conformational change was suggested (Moniakis, 1992).

Part 5. Example of Enzyme Subjected to Post-Translational Modification and Enzyme of Multiple Functions

One *E .coli* enzyme subjected to post-translational modification that has been studied in detail is *E. coli* pyruvate formate-lyase, which catalyses the non-oxidative cleavage of pyruvate to acetyl-CoA and formate (Knappe and Sawers, 1990). This enzyme is also important in controlling the anaerobic metabolism of glucose as a carbon source in *E. coli* (Knappe, 1987). During growth in aerobic conditions, the synthesis of this enzyme is inhibited, while anaerobic growth activates this enzyme. Furthermore, this enzyme needs post-translational modification to convert the initially inactive form into an active form (Knappe *et al.*, 1984; Conradt *et al.*, 1984). The active form contains an oxygen-sensitive organic free radical - a glycyl radical, located on the Gly-734 of the polypeptide chain, which is produced post-translationally by pyruvate formate-lyase-

activating enzyme (PFL activase), and is essential for catalysis (Wagner *et al.*, 1992). PFL activase is a monomer of 28 kDa requiring Fe^{2+}. The generation of the catalytically competent radical form of pyruvate formate-lyase occurs by a uniquely small covalent modification of the protein. Frey *et al.*'s results showed that a hydrogen atom is abstracted directly and stereospecifically from C-2 of Gly-734 by a 5'-deoxyadenosyl radical which is produced at the active site of PFL activase from the adenosylmethionine co-substrate (Frey *et al.*, 1994). Strict anaerobiosis is obligatory for this process since oxygen destroys the radical, with concomitant fragmentation of the polypeptide chain (Frey *et al.*, 1994).

The active form of PFL (PFL_a) can be turned into an inactive form (PFL_i) by a PFL deactivase under anaerobic conditions, through which PFL protein is saved from destruction by oxygen:

$$PFL_a \xrightarrow{NAD,\ CoA,\ Fe^{2+}} PFL_i$$

Later on this deactivase was identified as AdhE protein, which is a homopolymer of 96 kDa subunits harboring three Fe^{2+}-dependent catalytic functions: acetaldehyde-CoA dehydrogenase, alcohol dehydrogenase, and pyruvate formate-lyase (PFL) deactivase. The simultaneous occurrence on a single polypeptide of these various redox functions, all of which are specifically Fe^{2+}-dependent makes the AdhE protein an interesting multienzyme model (Kessier *et al.*, 1992).

Part 6. Serine Toxicity to *E. coli*

It has been known long ago that L-serine is toxic to *E. coli* (Amos and Cohen, 1954), though it is an essential cell component. When *E. coli* cells are grown on lactate or other carbon sources, an addition of serine to the medium causes growth inhibition. This inhibition is caused by inhibition by serine of homoserine dehydrogenase I, which is involved in threonine - isoleucine biosynthesis (Hama *et al.*, 1990). This inhibition can be released by homoserine, L-threonine and α-ketobutyric acid, which are intermediates in the biosynthesis of L-isoleucine after homoserine dehydrogenase reaction. The end-product L-isoleucine can also relieve the L-serine inhibition to growth. The pathway related to serine toxicity is illustrated in Fig. 2.

In this lab, all the strains in use are derivatives of MEW1, which carries an *ilvA* deletion (Newman *et al.*, 1985a). As we know this is the gene coding for threonine deaminase (TD), the first enzyme related to L-isoleucine biosynthesis (Umbarger, 1987). Because of this, isoleucine must be added to minimal medium for cell growth. Since according to Hama *et al.*, L-serine should not be toxic in the presence of L-isoleucine (Hama *et al.*, 1990), addition of L-isoleucine actually also serves to counteract L-serine toxicity.

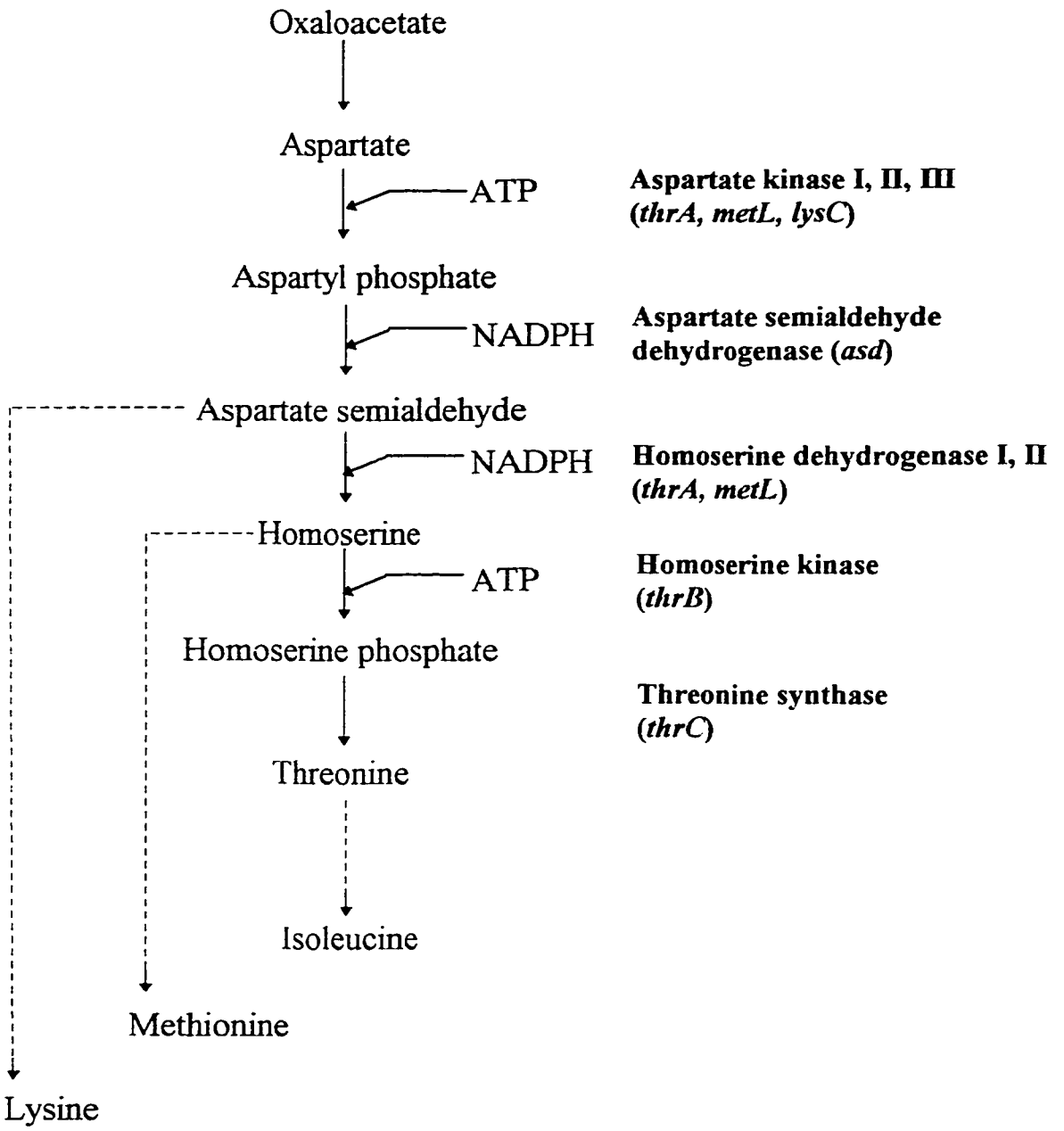


Fig. 2. Biosynthetic pathway to lysine, methionine, threonine and isoleucine (Cohen and Saint-Girons, 1987)

Broken lines represent multiple steps.

Part 7. Summary of Known Functions of Genes Identified in This Thesis

In this thesis three genes related to SGL- phenotype were identified. Here is a summary of the known functions of these three genes.

7.1. Known function of *torA*

Trimethylamine N-oxide (TMAO) is one of a variety of terminal electron acceptors that can be used by enterobacteria. Reduction of TMAO, which allows anaerobic growth of *E. coli* on nonfermentable sources like glycerol, is due mainly to the TMAO reductase pathway (Barrett and Kwan, 1985; Silvestro *et al.*, 1989). The TMAO reductase coding region was found to be an operon, the *torCAD* operon. Located at 22 min on the chromosome, the three genes of the operon are arranged in the order *torC*, *torA* and *torD*. The presence of five putative c-type haem-binding sites within the TorC sequence, as well as the biochemical characterization, indicate that *torC* encodes a 43 kD c-type cytochrome. The second gene *torA*, was identified as the structural gene for TMAO reductase (Mejean *et al.*, 1994), which can reduce TMAO to volatile trimethylamine (TMA) (Barret and Kwan, 1985). Immunological studies proved TMAO reductase can exist as a dimer or a monomer (Silvestro *et al.*, 1988).

The expression of *torCAD* operon was found only when TMAO is present in the medium, and it is induced to its maximum level under anaerobic condition (Pascal *et al.* 1984; Simon *et al.*, 1994).

7.2. Known function of *nuoM*

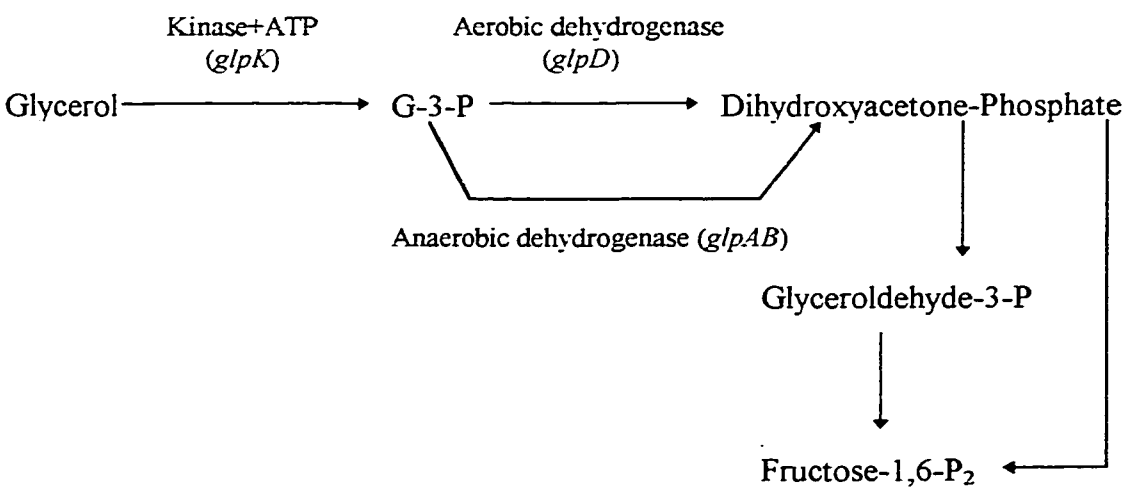
The aerobic respiratory chain of *E. coli* contains several dehydrogenases which catalyze the oxidation of various substrates. One of them, NADH dehydrogenase, donates electrons to the ubiquinone pool and two ubiquinol oxidases (the bo-type and bd-type oxidase) directly accept electrons from the pool and reduce molecular oxygen to water (Anraku and Gennis, 1987). This electron transport chain functions to generate a proton motive force across the membrane. This force, in turn, is responsible for a number of energy-dependent processes like ATP synthesis and active transport.

The locus of the 550 KD NADH dehydrogenase coding region was mapped at 49 min in *E. coli* genome and contains a cluster of 14 genes (*nuoA* to *nuoN*), each of which codes for a subunit of the NADH dehydrogenase (Weidner *et al.*, 1993).

7.3. Known function of *glpC*

In *E. coli*, the dissimilation of glycerol and glycerol-3-phosphate (G-3-P) with fumarate as the sole exogenous hydrogen acceptor requires the anaerobic G-3-P

dehydrogenase-fumarate reductase system (Kistler and Lin, 1971). *E. coli* codes for two distinct glycerol-3-phosphate (G-3-P) dehydrogenases coupled to electron transport. Under aerobic growth conditions an aerobic G-3-P dehydrogenase, coded by the *glpD* gene, is expressed, and under anaerobic growth conditions, an anaerobic G-3-P dehydrogenase, coded by *glpA* region, is synthesized (Lin, 1976). The purifed G-3-P dehydrogenase contains two subunits, which are of 62 KD and 43 KD in size (Schryvers and Weiner, 1982). Later on, the *glpA* region was cloned and sequenced by Cole *et al.* (Cole *et al.*, 1988). Their analysis revealed that the *glpA* locus actually comprised three genes arranged as an operon, which they named the *glpABC* operon. Also, they found that the two promoter-proximal genes encode the two subunits of anaerobic G-3-P dehydrogenase, while the third gene, *glpC*, seems to code for an iron-sulfur protein which serves as the acceptor of reducing equivalents from the GlpAB catalytic dimer in the electron transport chain (Cole *et al.*, 1988). The function of GlpAB protein is as follows (Lin, 1996):



Less is known about *glpC*. Kuritzkes *et al* first reported that loss of the specific membrane anchor protein coded by *glpC* causes the accumulation of G-3-P dehydrogenase in the cytoplasmic fraction of the cells (Kuritzkes *et al.*, 1984). Data from Varga et al. indicated that GlpC mediates electron transfer from the soluble GlpAB dimer to the terminal electron acceptor fumarate via the membrane bound menaquinone pool (Varga and Weiner, 1995).

Materials and Methods

A. Bacteria

All bacterial strains used are listed in Table 1.

B. Media and Solutions

B.1. Minimal Medium

Liquid minimal medium (+N)

5.4 g K_2HPO_4, 12.6 g KH_2PO_4, 2 g $(NH_4)_2SO_4$, 2 g $MgSO_4 \cdot 7H_2O$, and 0.1 g $CaCl_2$ in 1 liter distilled water (pH 7.0).

Solid minimal medium (+N)

Minimal medium with 20 g/liter Bactoagar.

Since MEW1 (*ΔilvA*) and all its derivatives require isoleucine and valine for growth, both isoleucine and valine were added to a final concentration of 50 μg/ml. The term NIV solution was used to refer to liquid minimal medium without carbon sources added.

Table1. Strains, Bacteriophages and Plasmids

Strain, phage and plasmid	Genotype	Source or reference
E. coli		
Cu1008	*E. coli K-12 ΔilvA*	Williams,L.S
MEW1	Cu1008 *Δlac*	Newman
Sp2	MEW1 *lrp*::λplacMu	Lin, R.
MEW84	MEW1 *glpC*::λplacMu9	Su
MEW128	MEW1 sda128::Mudx	Newman
MEW191	MEW1 sda191::Mudx	Newman
MEW20b	MEW1 *muoM*::λplacMu9	This work
MEWb1	MEW1 *torA*::λplacMu9	This work
CH22	MEW1 *sdaA*::Cm^r	Su
MEW84/lrp	MEW84, *lrp*::Tn10	This work
MEW20b/lrp	MEW20b, *lrp::lacZ*	This work
MEWb1/lrp	MEWb1, *lrp*::Tn10	This work
MEW1/torA	MEW1, *torA::lacZ*	This work
MC4100	*araD139 Δ(lacIPOZYA-argF)U169 rpsL thi*	Casadaban,M.J
LCB620	MC4100 *torA8*::MudII1734	Pascal, M.C.
TL661	MC4100 *glpB661*::λp1(209)	Larson, T.J.
TL684	MC4100 *sdh-9 ΔglpD102 glpA104*::λp1(209)	Larson, T.J.
ZK1556	W3110 *tna2 ΔlacU169* λMAV103(*bolA::lacZ*, kan) *muoF*::mini-Tn10 Cm	Kolter, R
ZK1557	W3110 *tna2 ΔlacU169* λMAV103(*bolA::lacZ*, kan) *muoM*::mini-Tn10 Cm	Kolter, R
Phages		
λplacMu9	λplacMu1 Km^r	Bremer, E.
λplacMu507	λcI ts857 Sam7 MuA+B+(Helper phage)	Bremer, E.
Plasmids		
pUC18-*torCA*	pUC18 with *torCA* region inserted at XbaI site	This work
pUC18-*glpABC*	pUC18 with *glpABC* operon inserted at BamHI site	This work
pBAD22-*muoMN*	pBAD22 with *muoMN* region inserted at HindIII site	This work

Carbon sources were added to the minimal media at the concentration of 0.2%. Minimal medium containing L-serine as the only carbon source was termed NSIV medium. Minimal medium containing 0.2% L-serine, 300 μg/ml glycine and 300 μg/ml leucine was referred to as SGL medium.

B.2. Luria Broth (LB)

10 g Bactotryptone, 5 g yeast extract, and 5 g NaCl in 1 liter of distilled water.

For making plates, 20 g/liter Bactoagar was added to the medium before autoclaving.

B.3. Phage Agar Plate for P1 Plate Lysate

10 g Bactotryptone, 1 g yeast extract, 8 g NaCl, and 17 g Bactoagar in 1 liter of distilled water.

B.4. R-Top Agar for P1 Plate Lysate

10 g Bactotryptone, 1 g yeast extract, 8 g NaCl, and 6 g Bactoagar in 1 liter distilled water. $CaCl_2$ and glucose were added to the medium to the final concentration of 2 mM and 0.1% respectively after being sterilized separately.

B.5. TB Medium for λplacMu Phage Lysate

10 g Bactotryptone, 5 g NaCl dissolved in 1 liter distilled water.

For making TB agar plate, 11 g/liter Bactoagar was added to the medium before autoclaving.

B.6. TB Top Agar for λplacMu Plate Lysate

10 g Bactotryptone, 5 g NaCl, 4 g Bactoagar was dissolved in 1 liter distilled water.

B.7. LB Citrate

LB with 0.5 M sodium citrate.

B.8. Antibiotics

Antibiotics were added to media to the final concentration as follows:

ampicillin (Ap) 100 μg/ml

chloramphenicol (Cm) 25 μg/ml

kanamycin (Km) 50 μg/ml

tetracycline (Tc) 20 μg/ml

Specific antibiotics were always added to the culture of strains with corresponding antibiotic resistance.

B.9. Buffers and Solutions

MC buffer for P1 transduction

0.100 M $MgSO_4$, 0.005 M $CaCl_2$.

TMG (λ dilute) for λplacMu phage lysate

1.21 g Tris base, 1.20 g $MgSO_4 \cdot H_2O$, 0.10 g Gelatin dissolved in 1 liter distilled water. Adjust pH to 7.5.

X-gal solution for selecting *lac*+ colonies

5-bromo-4-chloro-3indolyl-β-D-galactoside was dissolved in N-N-dimethyl-formamide at a concentration of 20 mg/ml.

IPTG solution for inducing *lacZ* in pUC18 vector

2 g IPTG dissolved in 8 g distilled water.

Z-Buffer for β-gal activity assay

16.1 g $Na_2HPO_4 \cdot H_2O$ or 8.52 g Na_2HPO_4, 5.3 g $NaH_2PO_4 \cdot H_2O$, 0.75 g KCl, 0.264 g $MgSO_4 \cdot 7H_2O$, 2.7 ml β-mercaptoethanol dissolved in 1 liter distilled water. Adjust pH to 7.0.

Phosphate buffer for whole cell L-SD assay

50 mM, pH 7.5

TBE (Tris-borate and EDTA) buffer for DNA agarose gel electrophoration

Concentration of stock solution (5 x)

Per 1 liter:

54 g Tris base

27.5 g boric acid

20 ml 0.5 M EDTA (pH 8.0)

TE (Tris and EDTA) buffer for dissolving DNA

10 mM Tris.HCl

1 mM EDTA (pH 8.0)

SOC buffer for electro-transformation

2% Bactotryptone, 0.5% Bacto yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM $MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose.

C. Enzyme Assays

C.1. β-Galactosidase Assay

β-galactosidase activity assay in whole cell was conducted according to the method of Miller (Miller, 1972) . One unit of β-galactosidase was the amount of enzyme that produces 1 mμ-mol/ml o-nitrophenol/min. in standard assay conducted at 28°C , pH 7.0.

C.2. Whole Cell L-SD Assay

The *in vivo* assay of L-SD was conducted as follows, which was the method of Isenberg and Newman (Isenberg *et al.*, 1974): overnight cell culture in minimal media was subcultured and chilled on ice after it reached log-phase. Cells were then resuspended in phosphate buffer (pH 7.5) to a density of OD = 100 at 540 nm. A 0.3-ml sample of resuspended cells, 0.1 ml L-serine (20 mg/ml), 0.02ml toluene were added to a clean glass test-tube at the next step. The reaction was stopped after 35 min incubation at 37°C water bath by adding 0.9 ml of 0.0025% 2,4-dinitrophenylhydrazine (dissolved in 4.1% HCl) to the reaction mixture. After being allowed to stand at room temperature for 20 minutes, 1.7 ml 10% NaOH was injected in the reaction mixture. L-SD was determined by measuring keto acid formation by a spectrophotometer at 540 nm.

One unit of L-SD as measured in the whole cell assay is defined as the amount of enzyme which catalyzed the formation of 1 μmol of pyruvate in 35 min. in whole cell assay conditions.

D. Selecting SGL- Mutants by λplacMu Insertion

λplacMu9 (kan^r) (Bremer *et al.*, 1985) was inserted into the *E. coli* chromosome and kanamycin-resistant cells, unable to grow on SGL, were selected as follows.

The host strain was infected with λplacMu9 and the 'helper phage' λplacMu507 as described by Weinstock and his colleagues (Bremer *et al.*, 1984, Bremer *et al.*, 1985). Cells were then subcultured in liquid glucose-minimal medium at 37°C for 3 hours, washed and immediately resuspended in liquid SGL medium for additional 3 hours. In the following 3-hour incubation at 37°C, ampicillin was added to concentration of 300 μg/ml to kill cells able to grow in this specific medium. Cultures were later plated on LB agar plate with kanamycin to select insertions. The resulting colonies were further screened for their inability to grow on SGL. SGL- kan^r colonies thus selected were then purified.

E. P1-Mediated Transduction

P1-mediated transduction was carried out by the method of Miller (Miller, 1972) with slight modification. An overnight cell culture in LB medium at 37°C was harvested and resuspended in MC buffer in half volume. A 0.1 ml aliquot of the resuspended cells

were mixed with different dillutions of P1 lysates in room temperature for 30 min. 1 ml LB citrate was then added to reaction mixtures to stop the P1 absorption and the mixtures were incubated at 37°C for one hour to allow the the transduced phenotype to be expressed. Appropriate selection plates were then used to select the corresponding transductants.

F. Chromosomal DNA Extraction

The extraction of chromosomal DNA from *E. coli* was modified from the method described by Silhavy *et al.* (Silhavy *et al.*, 1984). An overnight LB liquid medium culture of a given strain was subcultured into the same medium until cell density reached OD_{600nm} ≈ 0.8 - 1.2. The cell culture was then chilled on ice to stop growth and resuspended in 10 ml ice-cold solution of 10% sucrose with 50 mM Tris·HCl (pH 8.0) and 25 mM EDTA. 1.5 ml 10 mg/ml freshly prepared lysozyme dissolved in 10 mM Tris·HCl (pH 8.0), 0.6 ml 20% SDS and 60 μl 10 mg/ml RNAse were added to the solution, following which the reaction mixture was incubated in a 37°C water bath for 40 min. After adding 200 μl 1% $CaCl_2$ and 50 μl 20 mg/ml Proteinase K and incubating at 45°C for one hour, 10 ml phenol was added and mixed with the reaction mixture by gently inverting the reaction tube several times. Then the upper phase was carefully transferred to a new centrifuge tube after centrifugation at 10,000 r.p.m. for 5 min. The DNA was further extracted again by adding and mixing with 5 ml phenol and 5 ml chloroform. Centrifugation was repeated once, and the upper phase liquid was mixed with 25 ml 95 - 99% ethanol followed by

centrifugation at 10,000 r.p.m. for 15 min. In the last step, the DNA pellet was washed with 70% ethanol twice and dissolved in TE buffer.

The concentration of chromosomal DNA in TE was determined by the following equation: Concentration of chromosome DNA (μg/ml) = 50 x Dilution factor x Absorbance ($OD_{260\ nm}$).

G. Identification of SGL- Mutated Genes by Inverse PCR

The basic principle and method for identification of λplacMu insertion by inverse PCR were based on the method developed by Tchetina and Newman (Tchetina *et al.*, 1995) and are illustrated in Fig. 3.

Choice of primers for inverse PCR

One set of primers for carrying out the inverse PCR was as follow:

primer 3: 5'-GCAAGGCGATTAAGTTGGGTAACG-3'

primer 4: 5'-CCTGCTGATGAAGCAGAACAAC-3'

Primer 3 was homologous to the beginning sequence of the *lacZ* gene in λplacMu, while primer 4 was about 1,040bp downstream, near single restriction cutting sites of AvaII, BssHI, SacI, SstI and EcoRV and two multiple-cutting sites AvaI and MluI.

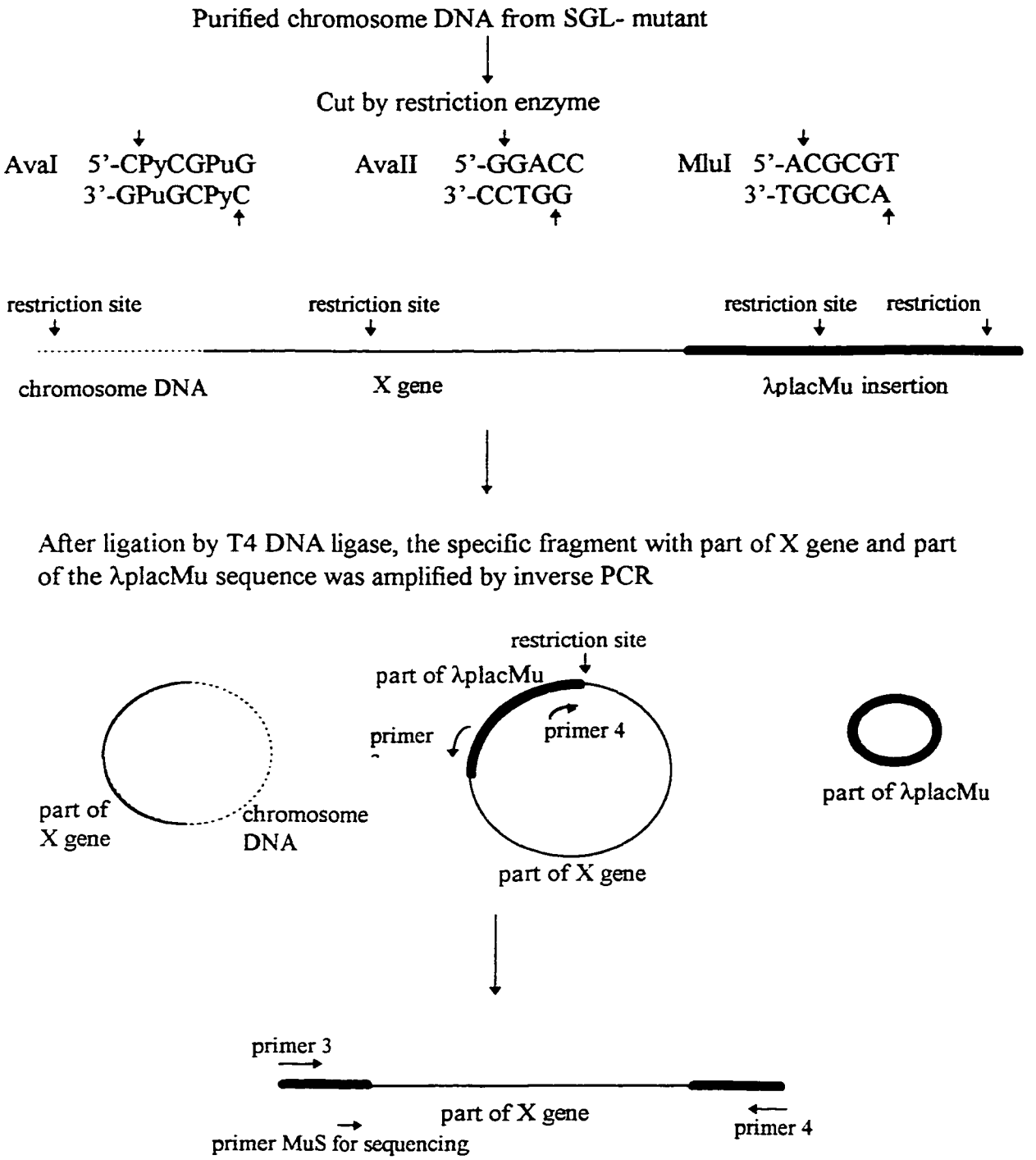


Fig.3. Identification of SGL- mutant (λplacMu insertion) by inverse PCR

Chromosome DNA extraction, digestion and purification

The extraction of chromosomal DNA from *E. coli* strains was conducted as the procedures described above. The TE dissolved DNA was digested with MluI in a 200 μl reaction mixture by using Promega enzyme and buffer product containing 6 mM Tris.HCl (pH 7.9), 150 mM NaCl, 6 mM $MgCl_2$, 1 mM DTT, 0.1 mg bovine serum albumin (BSA) per ml, 10 μg DNA and 50 U of enzyme, or AvaII in a 200 μl reaction mixture by using Promega enzyme and buffer package containing 10 mM Tris.HCl (pH 7.9), 50 mM NaCl, 10 mM $MgCl_2$, 1 mM DTT, 0.1 mg BSA per ml, 10 μg DNA and 50 U of enzyme, or AvaI in a 200 μl reaction mixture by using Promega enzyme and buffer product containing the same ingredient as to AvaII. After incubation at 37°C for 4 hours, the digestion was stopped by adding 2 μl 0.5 M EDTA.

The efficiency of digestion was checked out by using 15 μl of the digestion mixture to run agarose gel electrophoresis as describe below.

The completely digested chromosomal DNA was purified by QIAGEN product QIAquick Gel Extraction Kit as described below.

DNA ligation

The purified DNA fragments were ligated by using T4 DNA ligase (MBI) in the 20 μl reaction mixture containing 2 μl 10 x T4 buffer [with 400 mM Tris·HCl, 100 M $MgCl_2$, 100 mM DTT, 5mM ATP (pH 7.8)], a convenient amount of DNA and 1 U T4 ligase. The ligation mixture was incubated for 8 hours at 16°C.

Inverse PCR

Aliquots of the ligation mixture were added to the inverse PCR reaction mixture up to a total volume of 40 μl containing 4 μl 10 x Taq Buffer [MBI, containing100 mM Tris·HCl (pH 8.8), 500 mM KCl, 0.8% Nonidet P40], 2.2 μl 25 mM $MgCl_2$, 0.8 mM each of the four deoxynucleoside triphosphates (dNTPs), 0.6 μM each of the primer 3 and 4. Once overlaid with mineral oil, the reaction mixture was subjected to the following PCR cycles conducted in the PCR machine (Interscience):

96°C 3min to denature DNA

69°C to allow the annealing of primers. At this moment 2.5 U of *Thermus aquaticus* DNA polymerse (MBI) was added and then cycles started.

First 10 cycles:	94°C	1 min
	57°C	1 min
	71°C	4 min
Second 10 cycles:	94°C	1 min
	59°C	1 min
	71°C	4 min
Third 10 cycles:	94°C	1 min
	60°C	1 min
	71°C	4 min
Last 2 cycles:	95°C	1 min
	70°C	8 min

The PCR products were visualized by ethidium bromide staining after loading 10 μl samples in 0.9% agarose gel and electrophoresing.

Sequencing the PCR products

Sequencing double-stranded DNA was performed by Gibco BRL ds DNA Cycle Sequencing System (Life Technologies), which was based on the method of Sanger *et al.* (Sanger *et al.* 1978). The primer used for sequencing was termed as MuS primer (5'-CTTTCGCGTTTTTCGTGC-3') since its sequence was homologous to the beginning of the MuS sequence which was preceding the *lacZ* gene. To end-label the MuS primer, a certain amount of MuS was incubated at 37°C for 10 min and then at 55°C for 5 min in a reaction mixture containing, in 5 μl, 30 mM Tris·HCl (pH 7.8), 5 mM $MgCl_2$, 0.1 M KCl, 0.5 μM primer, 2 μM [γ-^{32}P]ATP, and 1 U of T4 polynucleotide kinase. Later on the set of sequencing reaction mixture contained, in 10 μl, 30 mM Tris·HCl (pH 9.0), 5 mM $MgCl_2$, 30 mM KCl, 0.05% W-1, 0.5 to 1 μM template DNA, 0.5 μM labeled primer MuS, and 0.6 U of *Taq* polymerase. After overlaying with mineral oil, the following PCR program reaction was carried out to perform the primer extension-termination.

95°C	10 sec to denature DNA	
First 10 cycles:	95°C	55 sec
	54°C	50 sec
	71°C	60 sec
Second 10 cycles:	95°C	55 sec

	60°C	60 sec
	72°C	60 sec
Third 10 cycles:	95°C	60 sec
	69°C	80 sec

5 μl of stop solution was added to each reaction tube right after the PCR reaction.

The end-labeled fragments from the previous step were loaded in and analyzed by electrophoresis on thin (0.4 mm) acrylamide sequencing gel (8%).

Sequences deduced from the gel were sent to NCBI BLAST network (Altschul et al., 1990) for homologous sequence searching.

H. Cloning of SGL- Genes.

H.1. Cloning the *glpABC* Operon.

The whole procedure of cloning the *glpABC* operon is illustrated in Fig.4.

First, genomic DNA from wild type *E. coli* MEW1 was extracted and purified as described above. Primer glpU (5'-CTGGATCCTCAACATTGATAGCCGTG) and primer glpD (5'-CGGGATCCTTTGACATCTGCCAGTTTCTGC), which covered 259-278 bp upstream of starting codon ATG and 96-117 bp downstream of TAA stop codon of *glpABC* operon (Cole *et al.*, 1988) respectively were selected to amplify this DNA

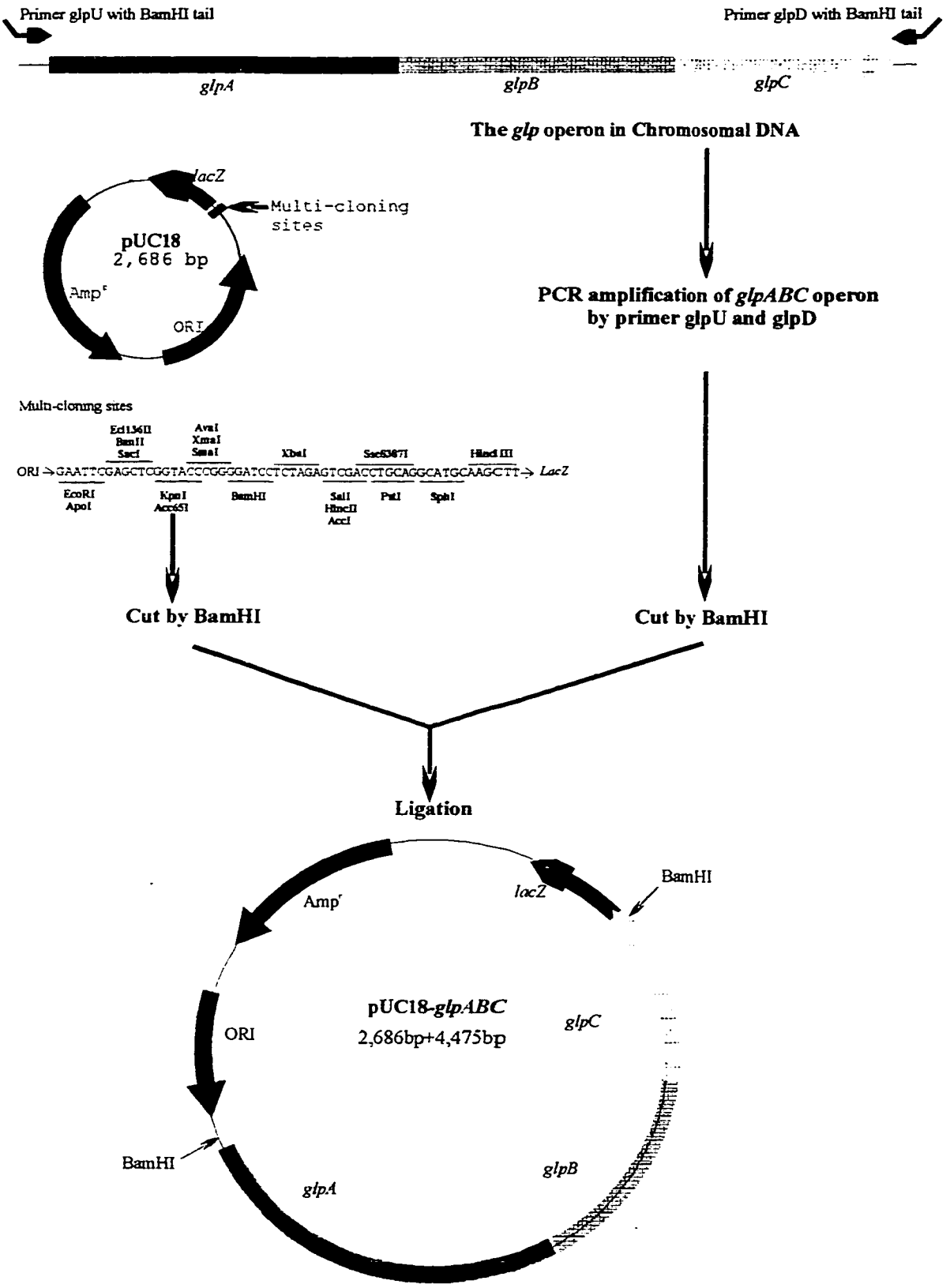


Fig. 4. Cloning the *glpABC* operon

fragment. Restriction enzyme BamHI site was added to the end of each primer (underlined) together with a few additional base pairs to facilitate the enzyme cutting.

Following BamHI treatment, 1 μl genomic DNA template (contained approximately 1 μg DNA) was mixed with 0.8 μl dNTPs (10mM each), 2.5 μl 10 pmol/μl of primer glpU and glpD, 4 μl 10 x *Taq* buffer (MBI) [with 100 mM Tris·HCl (pH8.8), 500 mM KCl, 0.8% Nonidet P40] and 30 μl distilled water. After covering the reaction mixture with a layer of mineral oil, the following PCR program was used to carry out the amplification:

96°C 5min. to denature DNA template

69°C to allow the annealing of primers, and at this point of time, 0.5 U of *Taq* DNA polymerase was added to the reaction mixture.

First 3 cycles:	94°C	1 min
	56°C	1 min
	71°C	5 min
Second 26 cycles:	94°C	1 min
	58°C	1 min
	71°C	5 min
Third 2 cycles:	94°C	1 min
	7 0°C	8 min

The result of PCR amplification was further visualized by loading 10 μl of the reaction mixture and running DNA electrophoresis in 0.9% agarose gel.

The 4475 bp DNA fragments amplified by PCR were later purified by QIAquick PCR purification kit (QIAGEN), cut by BamHI and purified again. Meanwhile, plasmid pUC18 was cut by BamHI and purified by QIAquickGel Extraction Kit (QIAGEN). To prevent self-ligation of BamHI cut pUC18, the linearized plasmid was subjected to dephosphoralation by incubation with calf intestinal alkaline phosphatase (CIP) as described by Maniatis (Maniatis *et al.*, 1982) and purified again by the same purification kit and procedure.

Ligation was set up in a 20 μl reaction mixture containing equal amount of amplified *glpABC* fragments and pUC18 vectors together with 2 μl 10 x T4 DNA ligase [containing 400 mM Tris·HCl, 100 mM $MgCl_2$, 100 mM DTT, 5 mM ATP (pH 7.8)] and 1 U T4 ligase. The reaction mixture was incubated at 16°C for 8 hours.

H.2. Cloning the *torCA* Fragment

The cloning of the *torCA* fragment of the *torCAD* operon is illustrated in Fig.5.

Primer torU (5'-CGTCTAGAGGGTTTTACTCATTCTGTTC) and primer torD (5'-GCTCTAGATTTGCGTCAGTTGTTCATCG), which covered the 116-125 bp upstream of the ATG starting codon and 61-90 bp downstream of the TGA stop codon of *torA* gene (Mejean *et al.*, 1994) respectively, were selected to amplify the *torCA* fragment of the *torCAD* operon. Restriction site XbaI (underlined) together with a few extra base pairs were added to the end of each primer to facilitate the enzyme cutting.

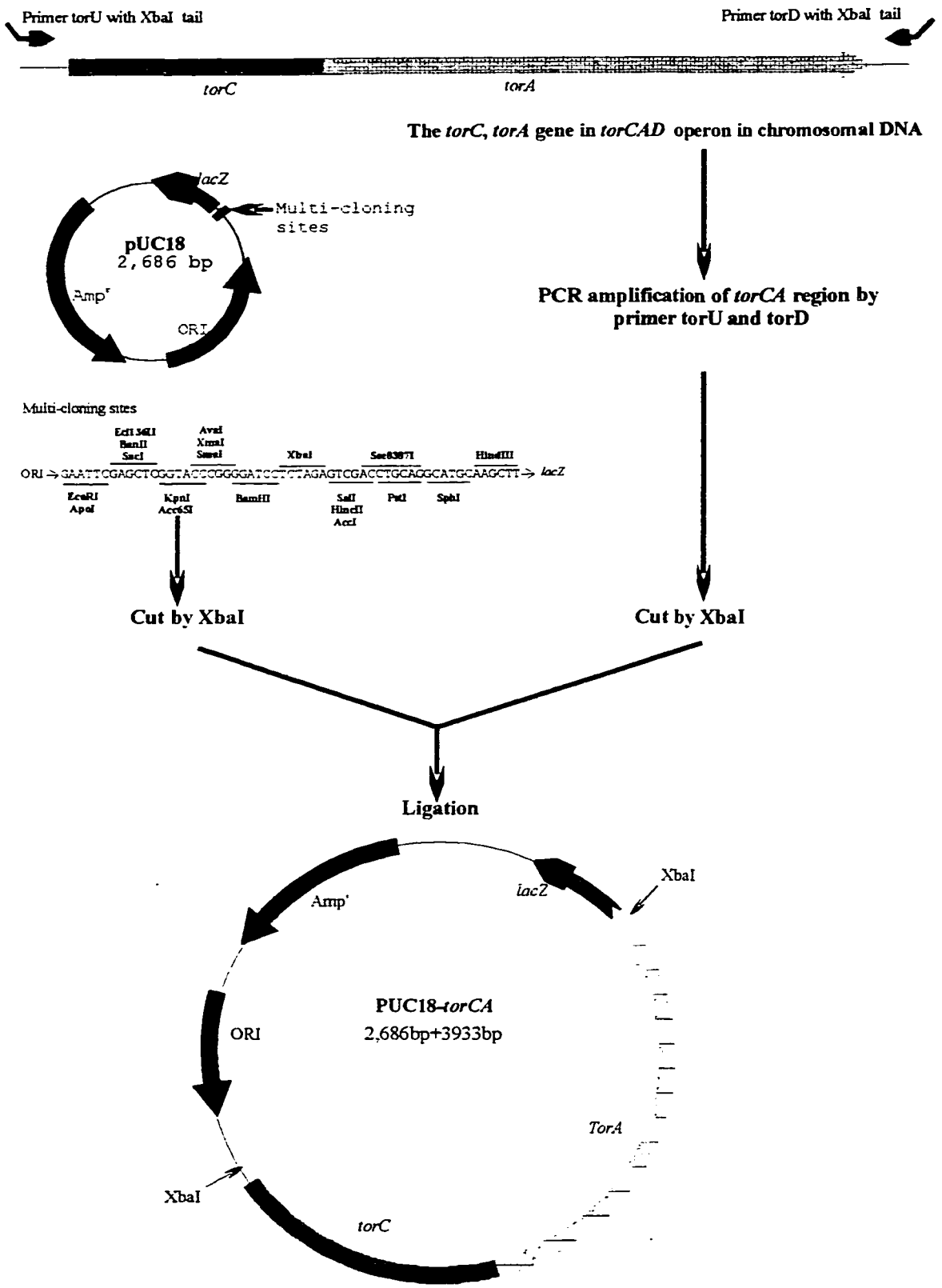


Fig. 5. Cloning the *torCA* fragment

PCR reaction was set up similar to the amplification of *glpABC* fragment: *torCA* fragments were also amplified by using wild-type genomic DNA as template, but by torU and torD primers. PCR reaction program was modified by using 54°C, 56°C and 58°C as annealing temperature in three rounds of cycles.

Amplification result was subjected to the same procedure of purification, enzyme cutting, purification and ligation with the properly treated pUC18 (XblI cut) vectors.

H.3.Cloning the *nuoMN* Fragment

Fig.6 shows the process of the cloning of the *nuoMN* fragment.

Primers for amplification of this specific sequenc were termed nuoU(5'-CGAAGCTTGCAGGTATAAGAACGTAGGTC) and nuoD (5'-TGAAGCTTAACGCTCTGCGTCTATCTA), which confined a 3218 bp DNA sequence containing the intact *nuoM* and *nuoN* gene from 12255 bp to 15473 bp (Weidner *et al.*, 1993). HindIII cutting site tails were added to the end of each primer.

By using primer nuoU and nuoD, the PCR reaction was about the same as the amplification of *torCA* fragments.

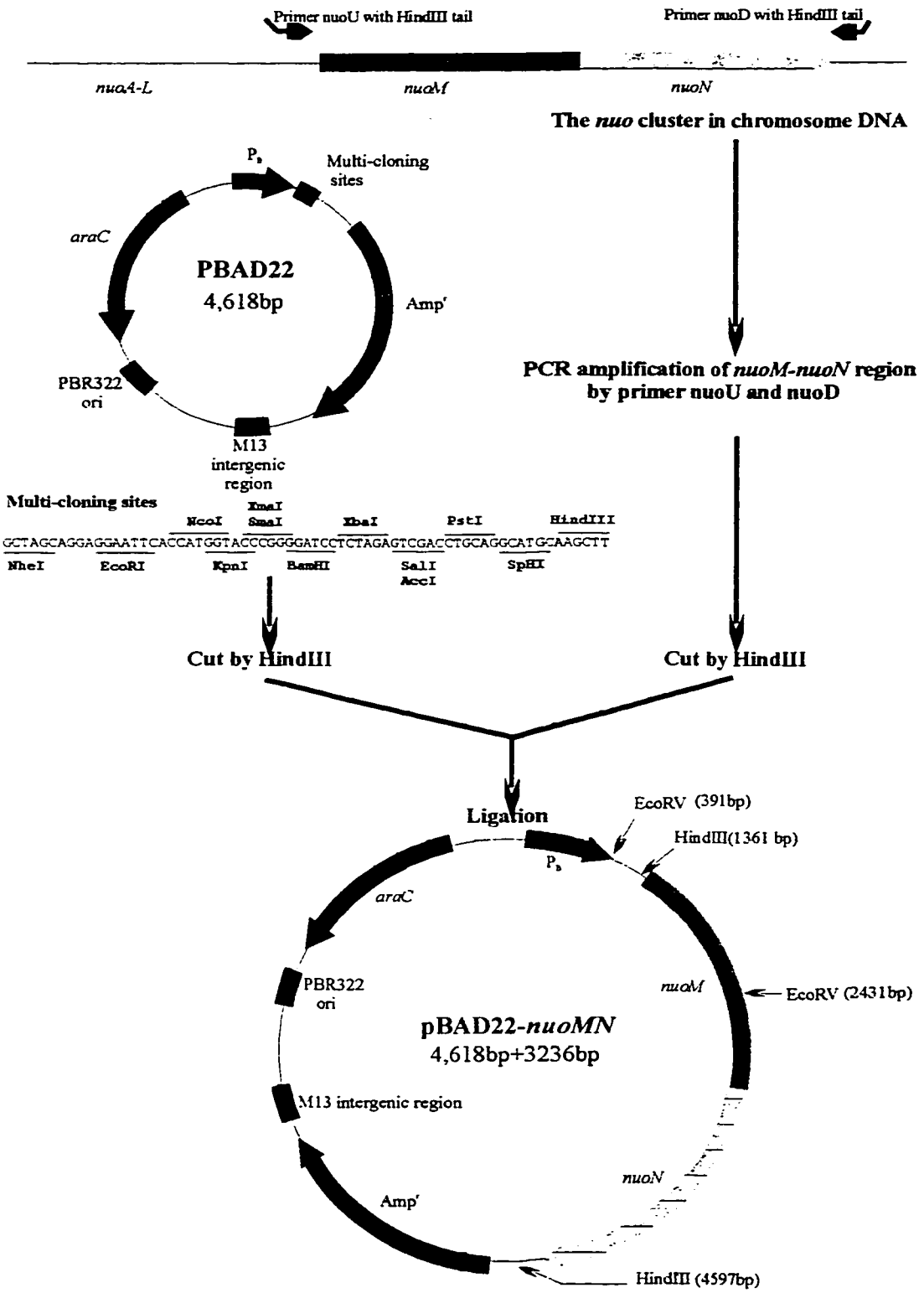


Fig.6.Cloning the *nuoMN* fragment

The vector selected for this cloning task is pBAD22 (Amp^r) (Guzman *et al.*, 1995). The sequence of enzyme cutting, purification and ligation steps were the same as used for the cloning of *glpABC* and *torCA* fragments.

H.4. Selection of the Clones

The ligation of the target DNA fragments with vectors were transformed into host strain XL1-Blue. For pUC18-*glpABC* and pUC18-*torCA*, transformants with the correct clone were selected on LB agar plate with the addition of X-gal, IPTG and ampicillin. White transformants presumably containing the insertion of amplified fragment were selected and further processed to extract plasmids. Plasmids which proved to be correctly inserted with the target fragment were then transformed into mutant strain MEW84 and MEWb1.

Transformants of pBAD22-*muoMN* ligation were selected on LB ampicillin agar plate. Since there is no easy color selection for the right clone, plasmids were extracted from randomly picked transformants and tested with EcoRV restriction enzyme. There is one EcoRV site in the original pBAD22 plasmid and one in the *muoM* gene. If the *muoMN* fragment is ligated in the correct orientation as illustrated in Fig.6., the EcoRV cut should produce two bands with the size of 2040 bp and 5814 bp. Otherwise, the pBAD22 with the inversely cloned *muoMN* sequence would give two bands (3140 bp and 4714 bp) after being cut by EcoRV. The correct clone was subsequently transformed into MEW20b.

I. Gel Electrophoresis

DNA agarose gel ectrophoresis was carried out by the method presented by Maniatis *et al.* (Maniatis *et al.*, 1982). Agarose gels usually used were from 0.7% to 1.0%.

J. Plasmid Isolation

Plasmids were isolated and purified by QIA Standard Plasmid Minipreparation Kit from QIAGEN and the procedure was carried out according to their protocol.

K. Recovery of DNA from Agarose Gel Electrophoresis.

DNA fragments were recovered and purified from agarose gels using QIAquick Gel Extraction Kit (QIAGEN) and the protocol provided with the kit.

L. Purification of PCR Amplified Fragment.

PCR amplification products were subjected to QIAquick PCR Purification Kit (QIAGEN) following the protocol provided together with the kit.

M. Transformation

Transformation was either done according to the Maniatis *et al* (Maniatis *et al.*, 1982), or performed by electro-transformation using Gene Pulser (Bio-Rad) and the instruction manual together with the apparatus.

N. Testing for Growth

N.1.Effect of Amino Acids on Cell Growth

Unless otherwise stated the following concentration of amino acids were used for testing their effect on cell growth: L-aspartic acid, 100 μg/ml; glycine, 100 μg/ml; L-histidine, 50 μg/ml; L-leucine, 50 μg/ml; L-lysine, 50 μg/ml; L-methionine, 50 μg/ml; L-proline, 50 μg/ml; L-serine, 100 μg/ml; L-threonine, 80 μg/ml and L-tryptophan, 50 μg/ml.

N.2.Comparing the Cell Growth by Absorbance Measurement

Strains were cultured in aliquots in minimal media supplied with conpounds noted at 37°C with shaking. Differences of growth were compared by cell density differences judged by the spectrophotometer reading at 600 nm.

N.3. Testing of Growth Rate.

Overnight cell cultures in minimal medium were subcultured in 20 ml of the same medium to a cell density of $OD_{600nm} \approx 0$. Cell cultures were then shaken vigorously at 37°C to ensure good aeration. Optical density was determined every 60 min. at 600 nm by spectrophotometer.

Results

Part 1. Isolation and Characterization of SGL- Mutants

Wild type *E. coli* can not use L-serine as a sole carbon source unless glycine and leucine are also supplied in the growth media (Newman *et al.*, 1982). Since the ability of *E. coli* to grow with these three amino acids is dependent on L-serine deaminase (L-SD), in this thesis I tried to reveal the mechanism of the activation of L-SD by studying strains unable to grow with L-serine, glycine and leucine (SGL).

1.1. Isolation of SGL- mutants

As described in the Materials and Methods section, the isolation of SGL- mutants by λplacMu insertion was achieved through a double selection: first kanamycin resistance, which was conferred by the insertion of λplacMu, was selected; then cells able to grow on SGL were killed by ampicillin. After these two steps of selection, most of the colonies formed on LB-kanamycin agar plates were unable to grow with SGL, i.e. SGL-.

After purification and further tests on SGL media, two of the SGL- mutants: MEW20b and MEWb1 were kept for further study, together with another mutant strain of *E. coli K-12*, MEW84, which was also a SGL- mutant carrying a λplacMu insertion

isolated by Hongsheng Su, a former Ph.D student in this lab. It seemed likely that these mutants might affect the L-serine deaminase activation system.

1.2. Further characterization of strain MEW84, MEW20b and MEWb1

Strain MEW84 was considered to define a locus affecting the L-SD activation system (Feng, 1990; Su, 1991). Its physiological character has been studied by a former graduate student Xiaopeng Feng, who determined its genetic location by conjugation between 45- 60 min in the genome of *E. coli* (Feng, 1990). However, she did not identify or clone the gene interrupted by the insertion. At the beginning of my project I repeated some of her experiments to verify her observation and make sure that strain MEW84 had not changed on storage.

1.2-A. L-serine deaminase activity of strain MEW84, MEW20b and MEWb1

Most of the previously described SGL- mutants have little or no L-serine deaminase activity when grown in glucose-minimal medium, with or without the inducers glycine and leucine (Newman *et al.*, 1985a; Newman *et al.*, 1985b and Su *et al.*, 1989). Feng showed clearly that strain MEW84 was deficient in L-serine deaminase under both conditions (Feng, 1990). To verify this and also test the L-serine deaminase in MEW20b and MEWb1, the whole cell L-SD activity assay of cells grown in glucose minimal media with and without glycine and leucine was carried out. The result showed that MEW84 had no significant L-SD activity when cells were grown in glucose minimal-medium, but

could be induced when inducers glycine and leucine were added to the growth medium. The other two mutants MEW20b and MEWb1 had L-SD levels a little bit lower than their parent wild-type strain MEW1 when grown in glucose minimal medium. This could also be increased by inducers (Table 2).

To judge the significance of the preceding L-SD levels, I also tested strain MEW1, Sp2 and CH22. In strain Sp2, with the destruction of *lrp* gene by λplacMu insertion, the level of L-SD (product of the *sdaA* gene) should be about 5 - 6 times higher than that of wild type MEW1 in glucose minimal medium (Newman, personal communication). Meanwhile, in strain CH22, there should be no L-SD activity since the L-SD coding gene *sdaA* was interrupted by insertion (Su, 1991).

1.2-B. β-galactosidase activity of mutant strains MEW84, MEW20b and MEWb1

When λplacMu insertion was carried out, the *lacZ* gene carried by λplacMu was also inserted into the target gene. If the *lacZ* of the phage was inserted in a gene in the correct orientation to be transcribed by the promoter of the gene, β-galactosidase might be synthesized. As a matter of fact, this was indeed the case in strain MEW84 (Feng, 1990; Su, 1991). I also determined the β-galactosidase activity of MEW 84 and the result was consistent with Feng's result, which showed that MEW84 had higher β-galactosidase activity in LB than in glucose minimal medium (Table3).

Table 2. Whole-Cell L-SD Activity of Mutants MEW84, MEW20b and MEWb1

Strains	Relevant genotype	L-SD Activity in Glucose Minmal Medium	
		without Gly and Leu	with Gly and Leu
MEW1	wild type	0.104	0.510
Sp2	*lrp*::λplacMu	0.557	0.935
CH22	*sdaA*::λplacMu	0.029	0.034
MEW84	*glpC*::λplacMu	0.023	0.162
MEW20b	*nuoM*::λplacMu	0.083	0.450
MEWb1	*torA*::λplacMu	0.100	0.500

Cells were inoculated into glucose minimal medium with or without the addition of 300 μg/ml glycine (Gly) and 300 μg/ml leucine (Leu) from LB plates and incubated overnight in a 37°C shaker, and subcultured in the same medium. Log phase cells were collected and subjected to whole-cell L-SD assay as described in Materials and Methods section. L-SD activities were expressed in spectrophotometer units at $OD_{540\ nm}$.

The results presented are the averages of three experiments.

Table 3. β-galactosidase Activities of MEW84, MEW20b and MEWb1

Strains	Relevent Genotype	β-gal activity	
		in LB medium	in glucose minimal medium
Sp2	*lrp*::λplacMu	150	1300
MEW1	wild type	30	35
MEW84	*glpC*::λplacMu	1231	820
MEW20b	*muoM*::λplacMu	26	32
MEWb1	*torA*::λplacMu	28	30

Cells were inoculated from patches on LB agar plates into LB or glucose minimal medium and grown overnight at 37°C with shaking. Overnight cultures were then subcultured in the same growth media and incubated in the same conditions.

β-galactosidase activity was assayed as described in the Materials and Methods section after cells were grown to mid-log phase.

The results presented are the averages of three experiments.

The other two mutant strains MEW20b and MEWb1 did not show any blue color on LB agar plates supplied with X-gal, nor did the β-galactosidase activity assay show obvious activity of this enzyme (Table 3). This might be because the *lacZ* insertion was not in the proper orientation, or because the two target genes did not express at a very high level under the test conditions.

1.2-C. Demonstration that the SGL- phenotype of the mutants was due to λplacMu insertion

To confirm that the SGL- phenotype of the mutants was due directly to the insertion of λplacMu instead of other point mutations in the genome, P1 tranductions were carried out. P1 phage grown in mutant strains MEW84, MEW20b and MEWb1 were collected and transduced into the parent strain MEW1. Transductants were first selected on LB-kanamycin agar plate for the transduction of the kanamycin resistance brought in by λplacMu and then screened on SGL plates to test their ability to grow with SGL.

For MEW84, almost 90% of the 60 blue transductant colonies formed on the LB-kanamycin agar selection plate with X-gal proved to be SGL-. A repeat of this same transduction showed about the same result.

The SGL- phenotype of strain MEW20b and MEWb1 was also found cotransducible with kanamycin resistance. An average of about 70-80% of the kanamycin

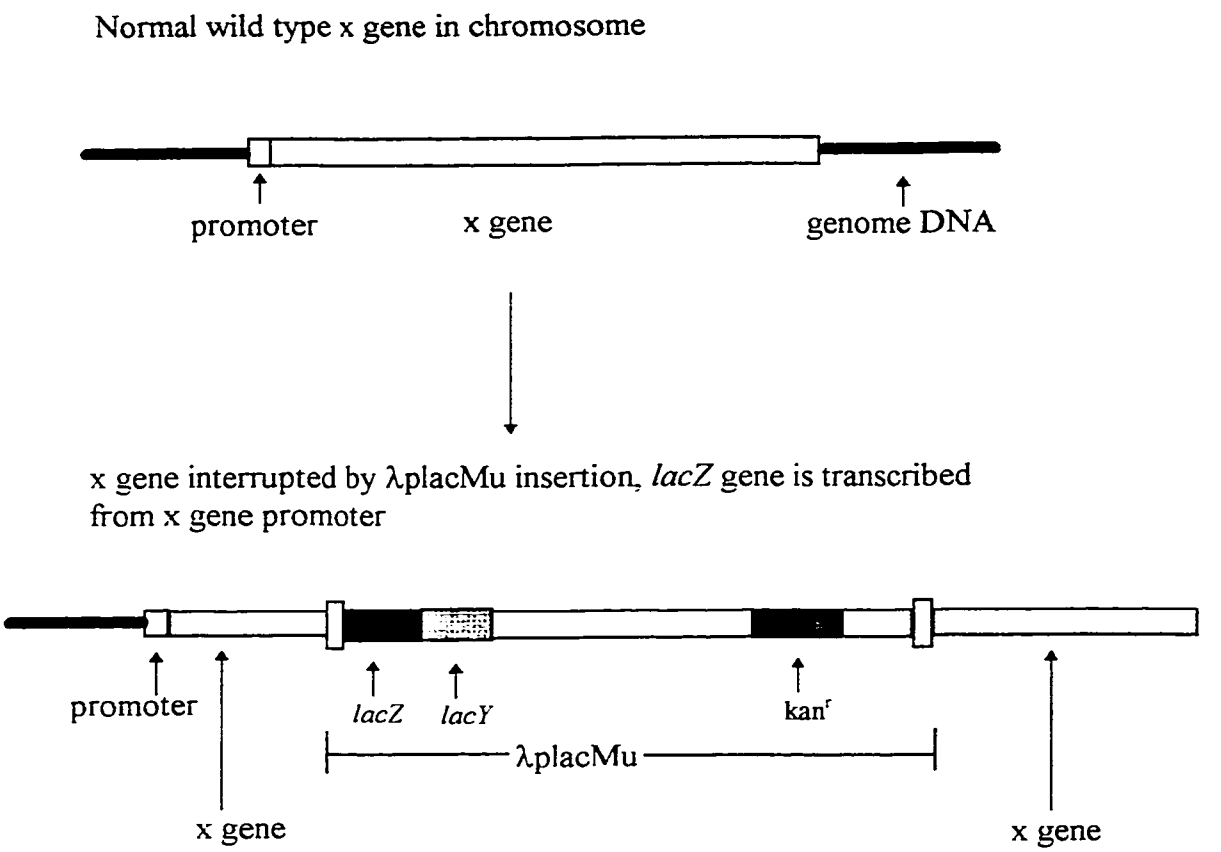


Fig.7. In-frame fusion of *lacZ* by λplacMu insertion

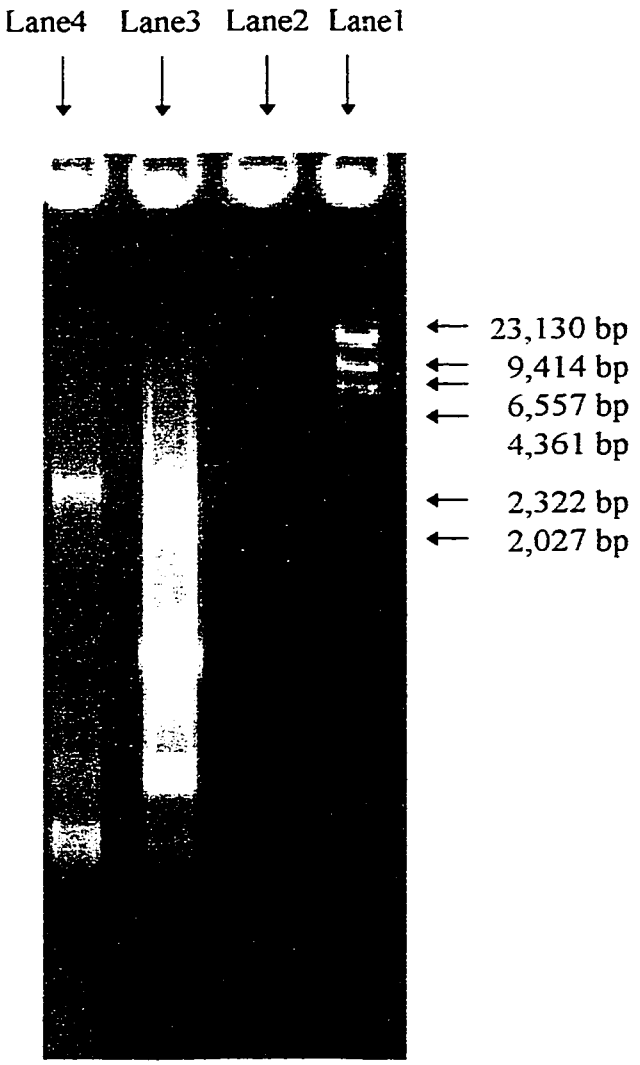
resistant MEW1/MEW20 or MEW1/MEWb1 transductants were also unable to grow on SGL plates.

These transductions clearly showed that the insertion of λplacMu was the immediate cause for the SGL- phenotype of the mutant strains MEW84, MEW20b and MEWb1.

1.3. Identification of the SGL- genes by inverse PCR

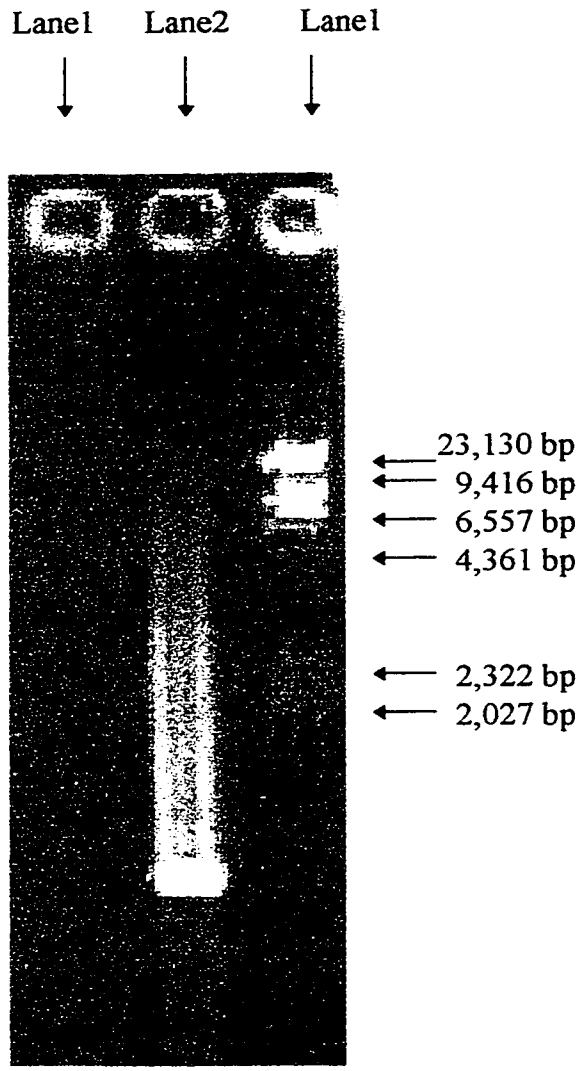
The genomic DNA from mutant strains MEW84, MEW20b and MEWb1 was extracted, purified and digested by AvaI, AvaII, MluI restriction enzymes, and amplified with inverse PCR as described in the Materials and Methods section. The AvaI-digested MEW84 chromosomal DNA, MluI-digested MEWb1 and the MEW20b chromosomal DNA gave the best inverse PCR amplification result (Fig.7, Fig.8). Samples from MEW84 and MEWb1 never gave a single sharp band like that of MEW20b, when the inverse PCR results were visualized on gel electrophoresis, although the reaction condition has been optimized. But considering that there must be a specific band composed of the ends of both λplacMu and the target gene, I decided to purify the whole inverse PCR product and went on to the sequencing procedure.

The sequencing films of mutant strains were exposed overnight and are shown in Fig.9, Fig.10 and Fig.11.



Lane1. 10 μl Lambda/HindIII Marker (about 1μg DNA).
Lane2. Inverse PCR amplification with primer3 and primer4,but no template DNA was added
Lane3. Inverse PCR amplification of MEW84 with primer3 and primer4.
Lane4. Inverse PCR amplification of MEWb1 with primer3 and primer4.
DNA electrophoresis was carried out on 0.9% agarose gel. From the 40 μl PCR reaction mixtures, 12 μl of each sample was loaded in the gel.

Fig.8. Amplification of completely digested MEW84 and MEWb1 by inverse PCR



Lane1. 10 μl Lambda/HindIII Marker (containing about 1 μg DNA).
Lane2. Inverse PCR amplification of MEW20b with primer3 and primer4.
Lane3. Inverse PCR amplification with primer3 and primer4 with no DNA template as a control.
DNA electrophoresis was carried out on 0.9% agarose gel. 12 μl of each PCR amplification sample from 40 μl reaction mixtures were loaded on the gel.

Fig.9. Inverse PCR amplification of completely digested MEW20b by primer3 and primer4

Fig.10. Sequencing of inverse PCR amplification product from MEW84.

Fig.11. Sequencing inverse PCR amplification product from MEW20b

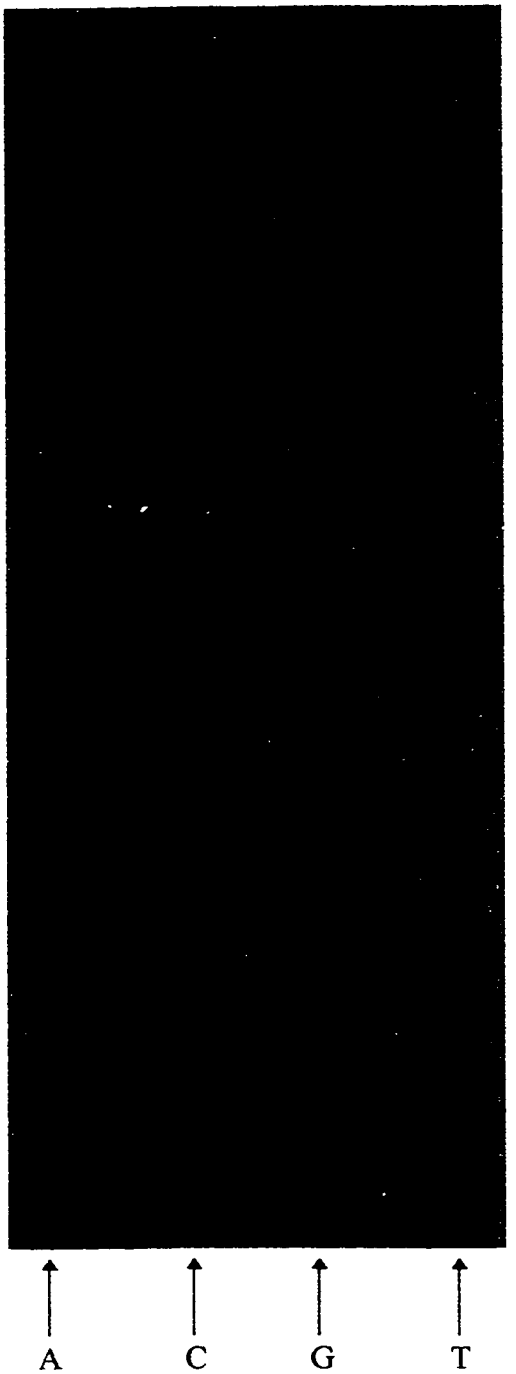


Fig. 12. Sequencing of inverse PCR amplification product from MEWb1

After sending the sequences to BLAST network service, I found sequences of MEW84, MEW20b and MEWb1 homologous to gene sequences of *glpC*, *muoM* and *torA* respectively, i.e. the λplacMu was inserted in those genes (Fig.12.). The denomination of the *glp* operon was inconsistent by different studying groups. In this thesis I followed GeneBank and the Cole *et al.*'s way of naming the distal gene in *glpABC* operon as *glpC* gene (Cole *et al.*, 1988)

.

By close examination of the sequences from the sequencing gels, the exact λplacMu insertion position in MEW84, MEW20b and MEWb1 was located at: 460 bp downstream of the ATG starting codon of *glpC*; 61 bp upstream of the stop codon of *muoM* and 37 bp downstream of the starting point of *torA* (Fig.13.).

Part 2.Cloning the SGL- Genes

2.1. Cloning and expressing the *glpABC* operon in pUC18 vector

The *in vitro* amplification of wild type *glpABC* operon by PCR was carried out using primers and programs described in the Materials and Methods section. The *glpC* gene was shown to be the distal gene in the *glpABC* operon and to be transcribed under the same promoter upstream of *glpA* (Kuritzkes *et al.*, 1984; Cole *et al.*, 1988). The set of primers, primer glpU and glpD, was chosen to flank the whole *glpA*, *glpB* and *glpC* gene with an extra 278 bp upstream of *glpA* and 117 bp downstream of *glpC*, hoping to include the promoter region.

Part of the sequence from sequencing gel of MEW84

```
TGCTGCGGCGTACCGCTAATCGCCAACGGCTTTNCCGATAAANCACGCA
                  | |                       |
TGCTGCGGCGTACCCGTAATCGCCAACGGCTTTACCGATAAAGCACGCA
```

Sequence found homologous through BLAST search
(gb|M20938| ECOGLPA *E. coli glpABC* operon)

Part of the sequence from sequencing gel of MEW20b

```
CTCCGCGATTGGCAATATCCAGCCAGCAGTCGTTTGTTAATTCCGTTAC
                                   |
CTCCGCGATTGGCAATATCCAGCCAGCAGTGGTTTGTTAATTCCGTTAC
```

Sequence found homologous through BLAST search
(emb|X68301| ECNUO *E. coli* DNA sequence of *nuo* operon)

Part of the sequence from sequencing gel of MEWb1

```
TTGCNTTACNAGTCTCGATAAACGTGAAGAACGGAAC
 |   |     |                     | |
TGGCTTTACCAGTCTCGATAAACGTGAAGAACGCACC
```

Sequence found homologous through BLAST search
(emb|X73888| ECTOR *E. coli torC* and *torA* gene)

Fig. 13. Identify the gene sequence through BLAST search

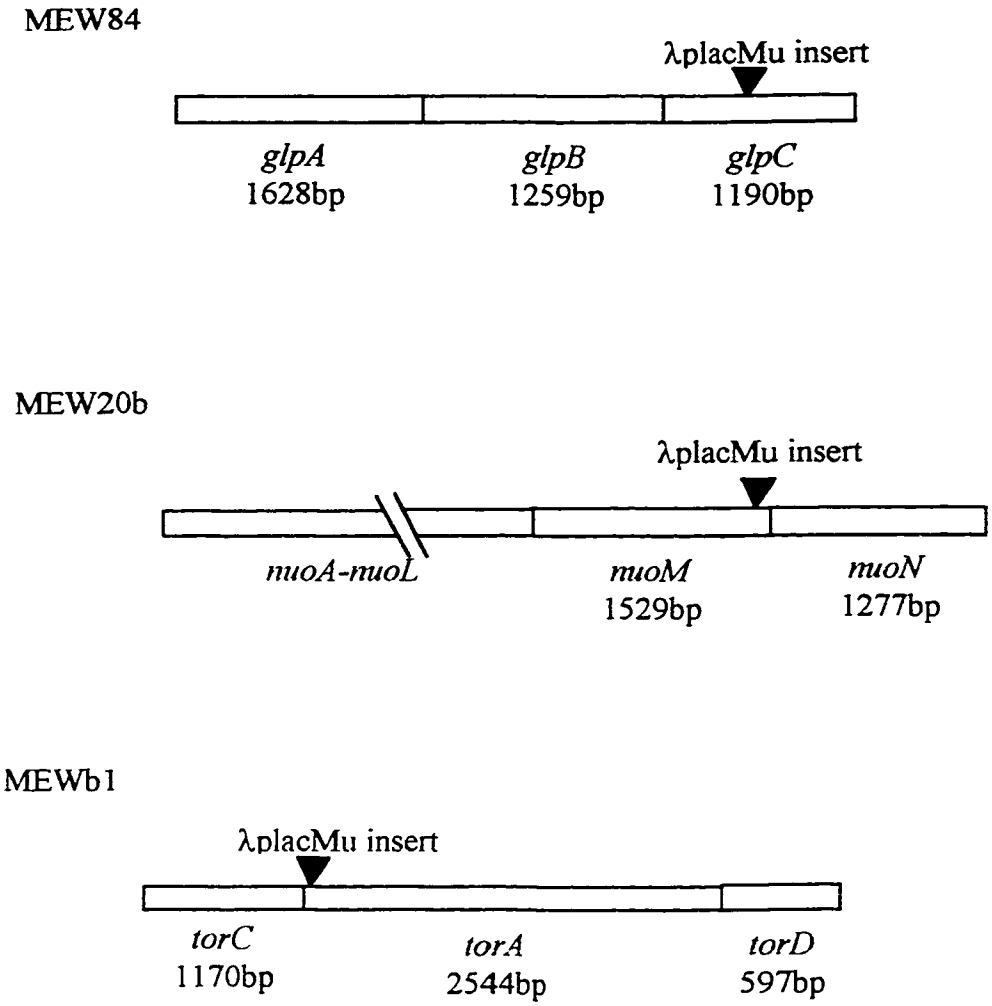


Fig.14. Location of λplacMu inserts

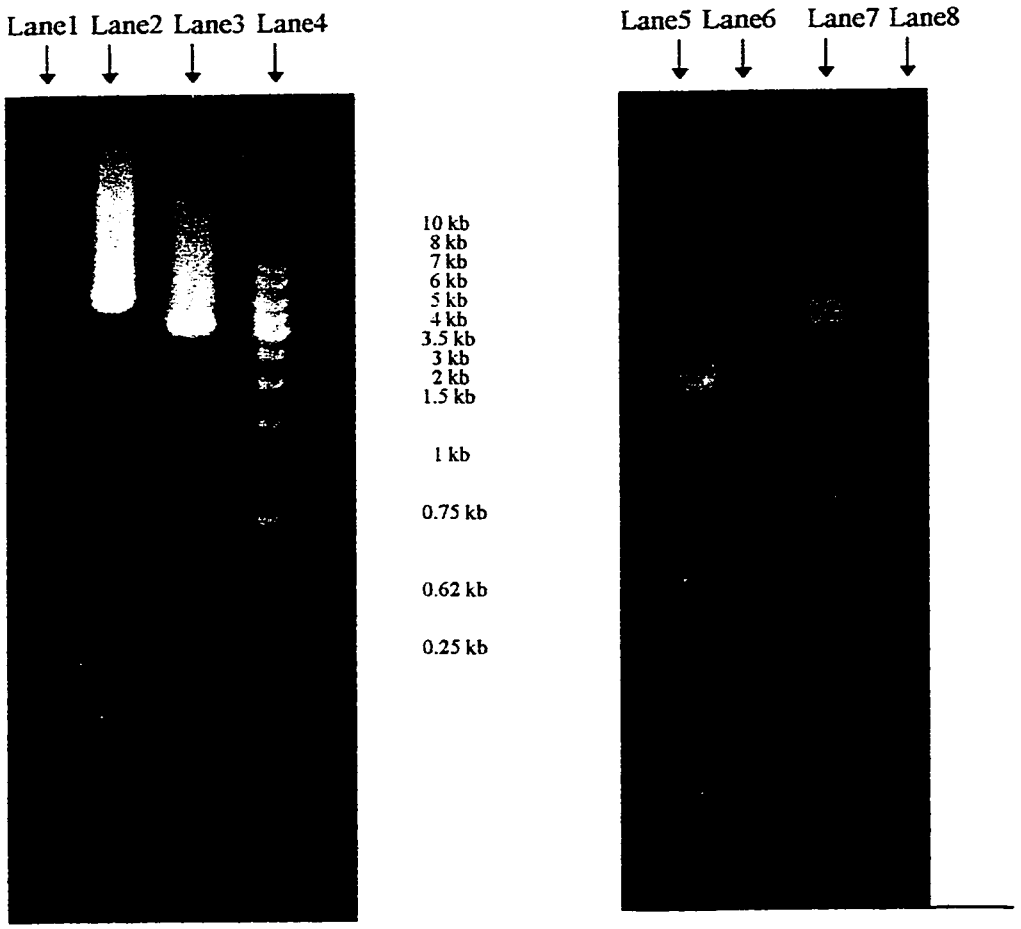
pUC18 was chosen to be the host vector because it was simple, easy and efficient to select a strain with a cloned fragment. Since there was only one BamHI site in the multi-cloning site in pUC18, but none within the *glpABC* amplification fragment, the BamHI site tails were added to the ends of each primer. To facilitate the restriction digestion, 2 additional base pairs were also added to the primers' ends.

The 4475 bp *glpABC* fragment was succesfully amplified by PCR reaction. As shown in Fig.15., with the control of amplification without DNA template, the amplification was specific. Since the λplacMu9 was inserted, the control amplification with genome DNA from MEW84 should give either a much bigger fragment or fail to amplify this huge piece of DNA. Obviously the result was the latter one.

After necessary purification and ligation as described in the Materials and Methods section, plasmids with the correctly cloned *glpABC* operon were collected and transformed into mutant strain MEW84. These plasmids failed to compensate the SGL- phenotype. This was not pursued further.

2.2. Cloning and expressing the *torCA* fragment in pUC18 vector.

The λplacMu insertion in mutant strain MEWb1 was identified as being in the *torA* gene, which is the second gene in the *torCAD* operon and is transcribed under the control of the same promoter immediately upstream of the *torC* gene. The wild-type *torCA* fragment was amplified by PCR. The chosen primers, primer torU and primer torD,



Lane1. 10 μl PCR amplification of *torCA::lacZ* from MEWb1 by primer torU and torD

Lane2. 10 μl PCR amplification of wild type *torCA* region from MEW1 by primer torU and torD

Lane3. 10 μl PCR amplification of wild type *nuoMN* region from MEW1 by primer nuoU and nuoD

Lane4 & Lane5. 10 μl 1kb Marker (containing about 1μg DNA)

Lane6. 10 μl PCR amplification of *glpABC::lacZ* from MEW84 by glpU and glpD

Lane7. 10 μl PCR amplification of wild type *glpABC* from MEW1 by primer glpU and glpD

Lane8. 10 μl PCR amplification by using primer glpU and glpD but without adding any DNA template

Fig.15. Amplification of *torCA*, *nuoMN* and *glpABC* fragment by PCR

defined a 3933 bp DNA fragment containing *torC*, *torA*, a 125 bp upstream of *torC* sequence and 90 bp downstream of *torA*.

Again pUC18 was chosen for cloning the target DNA fragment. The sequence of a unique single cutting site XbaI in pUC18 was added to the end of each primer together with 2 extra base pairs to facilitate the restriction digestion.

The PCR amplification result is shown in Fig.15. The single band, which appeared on agarose gel electrophoresis, was the correct size. The fact that the amplification reaction without DNA template gave no visible product clearly showed that the PCR reaction product of *torCA* fragment was specific.

Plasmids with the correct clone of *torCA* sequence were collected after the procedure of XbaI digestion, purification and ligation detailed in the Materials and Methods section, and transformed into strain MEWb1. They were able to compensate for the SGL- phenotype of the host.

2.3. Cloning and expressing the *nuoMN* in pBAD22 vector.

nuoM is the thirteenth gene in a cluster of 14 genes coding for NADH dehydrogenase I. All these genes are arranged as a single, large operon that is expressed from a complex promoter region upstream of *nuoA* (Archer *et al.*, 1995; Weidner *et al.*, 1993). There is no way to amplify such a huge operon containing more than 15 Kb by

PCR due to the limitation of the size of fragment that PCR can successfully amplify. Thus only the last two genes in this operon, wild-type *nuoM* and *nuoN* were to be amplified. Primer nuoU and nuoD were chosen to amplify this 3,236 bp fragment.

Considering that there is no promoter of nuoM within the sequence being amplified, a vector with a convenient promoter (pBAD22) was chosen to house the target fragment. The pBAD vector series contained the pBAD promoter of the *araBAD* (arabinose) operon and the gene encoding the positive and negative regulator of this promoter, *araC*. Thus under the control of pBAD promoter, the cloned gene can be efficiently and rapidly turned on and off (Guzman *et al.*, 1995).

Restriction site HindIII is a unique site in the multicloning site of pBAD22, and does not exist in the target *nuoM nuoN* sequence. Thus an 8 bp tail containing a HindIII cutting site and two extra base pairs to facilitate the restriction digestion were added to each end of the two primers. By using the method described in Materials and Methods, a sequence containing *nuoM* and *nuoN* was specifically amplified as shown in Fig.15.

After necessary checking of several restriction digestions, including HindIII and EcoRV, plasmids with the appropriate clone were transformed into MEW20b. It was shown that MEW20b with the pBAD22-*nuoMN* clone could grow on SGL plate supplied with 5 μg/ml arabinose, but could not grow on SGL plate without arabinose, i.e. when *nuoMN* was transcribed by the induction of arabinose, it could compensate for the SGL-

phenotype of MEW20b. Meanwhile, as a control, MEW20b transformed with original pBAD22 could not grow on SGL plate either with or without the addition of arabinose.

Strain MEW20b is an *ara*+ strain. However, the addition of only 5 μg/ml arabinose should not be able to be used as carbon source to support its growth. To prevent the slight possibility of an error due to this, an *ara*- MEW20b was constructed. This was achieved through the P1 transduction of the λplacMu interrupted *nuoM* into the *ara*- strain CuA. Thus the correct CuA/MEW20b transductants (i.e., those which could not grow on either SGL or arabinose minimal agar plates) were transformed with pBAD22-*nuoMN* plasmid. Here I got the same result as was obtained earlier, that is with the addition of arabinose (5-20 μg/ml): the induction of the *araBAD* promoter resulted in transcription of wild-type *nuoM* and *nuoN* and restoration of the ability to grow on SGL.

Part 3.Further Evidence that the SGL- Phenotype Was Caused by Insertion in *nuoM* or *torA* Genes.

3.1. SGL- and *torA* gene

The *torA* gene codes for trimethylamine-N-Oxide (TMAO) reductase, and work on *torA* has been focused on TMAO reduction for years since this function was first described. A *torA::lacZ* fusion has also been made to study the expression of this gene

(Mejean *et al.*, 1994). This provided an opportunity for me to confirm the relation of *torA* and SGL- phenotype by comparing my isolate with a known *torA* mutant.

Strain LCB620, an *E. coli MC4100* derivative obtained from Dr. Chippaux, also carries a *torA::lacZ* inframe fusion (Kan^r) (Pascal et al., 1984). It did not grow on SGL medium.

In strain LCB620, *torA::lacZ* was in the MC4100 background whereas my mutant was derived from strain Cu1008. To avoid background effect, the *torA::lacZ* from LCB620 was then transduced by P1 phage into my wild-type strain MEW1, which was also the parent strain of MEWb1. Of all the 200 transductants being tested, about 20% were SGL-. This clearly showed that a *torA* mutation caused the inability to grow on SGL medium.

3.2. SGL- and *nuoM* gene

Fortunately the *nuo* system also has been studied in detail. Various insertions have been made in different *nuo* genes. One strain, ZK1557, carries a mini-Tn10 Cm^r insertion in *nuoM* (Kolter, unpublished data). When tested in both SGL liquid and agar media, it was not able to grow.

Again P1 transduction was carried out to select a MEW1/*nuoM*::mini-Tn10 Cm^r transductant. Of about 60 chloramphenicol resistant colonies selected, nearly 10%

showed no sign of growth on SGL agar plate. Therefore we can also conclude that a *nuoM* mutation causes an inability to grow on SGL medium.

3.3. SGL- and *glpC* gene

Coding for anaerobic sn-glycerol-3-phosphate dehydrogenase, the *glpABC* operon has also been studied for a long time. I was able to obtain an insertion mutant in *glpABC* operon - mutant TL684 from Dr. Larson's group (Ehrmann *et al.*, 1986) Although the insertion of λ p1(209) is in *glpA* of this MC4100 derivitive, it is quite possible that the transcription of *glpC* is also interrupted since the whole operon is transcribed from the same promoter upstream of *glpA*.

When tested on SGL agar plate, TL684 showed poor growth after very long incubation in 37°C. I worried that the background of TL684 might have an effect on the experimental result. However, since there is no selectable marker for the insertion, there is no simple way to transduce this *glpA*::λ p1(209) into my parent strain MEW1. Thus further investigation of the relation between SGL- phenotype and the *glpC* gene was set aside.

Part 4. Physiological Study of SGL- Mutants

4.1. The use of different carbon sources by SGL- mutants

To find out whether the SGL- phenotype has an effect on *E. coli*'s ability to use different carbon sources other than L-serine, several carbon sources have been tested. The results are shown in Table 4.

It is obvious that the SGL- mutants have no significant difference from their parent strain in using glucose, succinate, glycerol, gluconate, fructose, pyruvate, maltose and L-alanine.

4.2. Azaleucine toxicity to strain MEW84

It was first observed by Feng that azaleucine is toxic to strain MEW84 (Feng, 1990). As one of the unique characteristics of this mutant strain, this observation was repeatedly tested. To measure azaleucine toxicity, overnight glucose-minimal cultures were first heavily plated (0.1 ml) on glucose-minimal agar plate. Then, a paper disc about 0.6 cm in diameter containing azaleucine was placed on top of the lawn of cells and incubated overnight. As shown in Table.5, as in Feng's experiments, azaleucine was toxic to all tested strains including SGL- mutants MEW20b, MEWb1, MEW84, their parent strain MEW1, a *sdaA*- strain CH22 and a *lrp*- strain Sp2, when added in very high amount. However, MEW84 showed a much higher sensitivity to azaleucine than did the other strains.

Table 4. Ability of Using Different Carbon Sources by SGL- Mutants

	MEW1	Sp2	MEW84	MEW20b	MEWb1
Glucose & Succinate	+	+	+	+	+
Succinate	+	+	+	+	+
Gluconate	+	+	+	+	+
Glycerol	+	+	+	+	+
Fructose	+	+	+	+	+
Pyruvate	+	+	+	+	+
Maltose	+	+	+	+	+
L-Alanine	+	+	+	+	+
Glucose	+	+	+	+	+

Cells were streaked from patches on LB agar plat onto minimal medium agar plate supplied with different carbon sources (all to a final concentration of 0.2%). The growth result was checked after overnight incubation at 37°C.

Table 5. Azaleucine sensitivity of SGL- strains

Strains	Inhibition circle (cm)	
	+10 µl 100 mM Azaleucine	+50 µl 100 mM Azaleucine
MEW1	0	1.3
Sp2	0	1.5
CH22	0	1.4
MEW84	3.0	4.7
MEW20b	0	2.2
MEWb1	0.2	1.7

Strains were grown overnight in glucose-minimal medium in a 37°C shaker. Aliquots of 100 µl cells were then evenly spread on glucose minimal agar plates. Whatman filter paper discs, 0.8 cm in diameter, were placed on top of the plates and different amounts of azaleucine added to them.

The plates were then incubated in 37°C overnight. The measure of inhibition circle was taken as the diameter of the nongrowth area minus 0.8 cm.

Data were from a single experiment, typical of three such experiments.

Since strain CH22, which has no L-SD activity, has the same sensitivity level to azaleucine as other strains (Table 5), azaleucine sensitivity can not be due to low L-serine deaminase activity. Therefore, it seems that the higher azaleucine sensitivity is caused by the mutated gene in MEW84. We can not explain why an insertion in *glpC* would cause azaleucine sensitivity.

4.3. L-serine toxicity to MEW84

As described in the Introduction, L-serine was known to be toxic to *E. coli* due to its interference with the sythesis of homoserine dehydrogenase I (Hama *et al.*, 1990). All the strains being studied in our lab are derivatives of MEW1, which is an *ilvA*- strain and thus needs an exogenous supply of isoleucine. Therefore L-serine toxicity should be able to be avoided, since L-serine shouldn't be toxic in the presence of isoleucine (Hama *et al.*, 1990). Indeed neither MEW1 nor Sp2 has been found sensitive to L-serine.

However, MEW84 was found to be very sensitive to L-serine even in the presence of glucose (Feng, 1990). To be sure that toxicity of SGL medium was also due to L-serine, I tested the growth of the three SGL- strains and their parent strain MEW1 together with *sdaA*- mutant CH22 on different combinations of glucose, glycine, L-leucine and L-serine. Finally the toxic effect was found to be due to L-serine as shown in Table 6. Furthermore, it seemed that the serine sensitivity is caused by low L-SD since L-serine was found toxic to both MEW84 and CH22, suggesting that one of the roles of L-SD is to keep the serine pool sufficiently low.

Table 6. Testing of L-serine Toxicity to MEW84

	MEW1	CH22	MEW84	MEW20b	MEWb1
Glucose	+	+	+	+	+
Glucose + Glycine	+	+	+	+	+
Glucose + L-Leucine	+	+	+	+	+
Glucose + Glycine + L-Leucine	+	+	+	+	+
Glucose + L-Serine + Glycine	+	+-	-	+	+
Glucose + L-Serine + L-Leucine	+	+-	-	+	+
Glucose + L-Serine	+	+-	-	+	+
SGL + Glucose	+	+-	-	+	+
SGL	+	-	-	-	-

Strains were streaked from patches on LB agar plate on minimal agar plate supplied with different carbon sources and inducing amino acids and let grow overnight in 37°C.

Glucose and L-serine were added to final concentration of 0.2%. Glycine and L-leucine were added to final concentration of 300 μg/ml. + indicate strains grew well, - indicate strains did not grow, +- indicate strains grew slowly.

To further demonstrate the serine toxicity to MEW84, growth in liquid minimal glucose medium with additon of L-serine was tested. Strain MEW1, MEW84, MEWb1, MEW20b and CH22 were first inoculated in glucose-minimla medium and grown overnight in a 37°C shaker. Strains were then diluted 1/1000 times and subcultured in glucose-minimal medium with different concentrations of L-serine. After 16 hours incubation, the data clearly showed that concentration as low as 50 μg/ml-L-serine can inhibit the growth of both strain MEW84 and CH22, while other strains can grow normally even with the addition of 1000 μg/ml serine (Data not shown).

To find out which amino acid might relieve the L-serine toxicity to MEW84, amino acids were tested alone and in combination. It seemed that threonine could partially release the L-serine toxicity to MEW84. This is also consistent with Feng's observation (Feng, 1990).

4.4. Effect of incubation in SGL medium on L-SD activity

All experiments quoted up to this point deal with L-serine deaminase as judged by L-SD assay on cultures in glucose-minimal medium. Since SGL- mutants can not grow in SGL medium, little is known about effects of serine, glycine and leucine combined together. I managed to test the whole cell L-SD activity after resuspending the cultures from glucose-minimal medium (with glycine and leucine) in SGL for a short period of time (one hour). Surprisingly, all SGL- mutants including MEW84 showed very high L-SD activities.

Subsequently more detailed experiments were carried out comparing strain MEW1 and CH22 with the SGL- mutants. All strains were inoculated in liquid glucose-minimal medium with glycine and leucine, and cultured overnight in a 37°C shaker and then subcultured into the same medium and grown in the same condition until the OD_{600nm} reached 0.5. Cells were then chilled on ice for 15 min, washed with H_2O and then resuspended in liquid glucose-minimal medium, SGL medium and minimal medium without any carbon source. After incubating in a 37°C shaker for various periods of time, L-SD activity was tested with the result shown in Table 7.

Quite unexpectedly, the L-SD activity of all SGL- mutant strains was increased noticeably in SGL medium in which the cells could not grow. Compared to the L-SD assay conducted with cells taken directly from glucose-minimal medium culture (Table 2), the L-SD of MEW84 was induced more than 20 - fold, while that of MEW1 was also increased about 10 times. The other two SGL- mutants MEW20b and MEWb1 also showed elevated L-SD (about 6 times higher). On the other hand, L-SD of all strains except CH22 decreases when strains were incubated in minimal medium. The result of zero time L-SD assay is close to the results obtained in glucose-minimal medium with glycine and leucine (Table 2).

This produced even more questions. Even L-SD of CH22 was significantly increased, while this *sdaA*- strain shouldn't be able to synthesis any L-SD #1. This may be due to the induction of *sdaB*, the coding gene for L-SD #2 that was originally found to be

Table 7. Effect of Incubation in SGL Medium on L-SD Activity

Strains		NIV-Glucose		NIV-SGL		NIV	
	0 hour	1 hour	2 hour	1 hour	2 hour	1 hour	2 hour
MEW1	0.360	0.427	0.342	1.123	0.990	0.490	0.275
CH22	0.014	0.036	0.051	0.144	0.170	0.002	0.035
MEW84	0.232	0.243	0.237	0.604	0.523	0.068	0.041
MEW20b	0.425	0.492	0.415	0.696	0.765	0.205	0.266
MEWb1	0.410	0.502	0.421	0.709	0.757	0.188	0.210

Strains were first inoculated into glucose-minimal medium with glycine (300 μg/ml) and leucine (300 μg/ml), and incubated in a 37°C shaker overnight. Cultures were then subcultured into the same medium and incubated in the same growth conditions until the cell density reached 0.5 in OD_{600nm} reading on spectrophotometer. Cells were then chilled on ice to stop growth and washed with ice-cold water. Following this step, cells were further washed with and resuspended in NIV-glucose, NIV-SGL and NIV without any carbon source.

L-SD was tested right after the ice-cold water wash and then tested after the incubation in different media for one hour and two hours. L-SD activities were measured as OD_{540nm} spectrophotometric readings on.

The result given is from a single experiment typical of three such experiments.

only expressed in LB medium. It is still puzzling that with such high L-SD was observed in SGL medium, while none of these mutants could utilize this carbon source for growth.

4.5. Study of effect of Lrp on SGL- mutants

The global regulator Lrp (leucine responsive regulatory protein) is known to control the expression of a large number of genes including the L-SD #1 coding gene *sdaA* (Su *et al.*, 1989). In fact the Lrp coding gene *lrp* was first identified through a mutant able to grow on L-serine as sole carbon source presumably due to the increased L-SD in this *lrp*- strain. Thus it is natural to ask whether any of these SGL- genes is under the control of Lrp, or whether Lrp may have other effects on these strains.

To answer this, an *lrp* mutation was first introduced into the three SGL- strains MEW84, MEW20b and MEWb1 through P1 transduction from donor strain CT4, which carries a Tn10 (Tet^r) insertion in *lrp*. Transductants on selection plates were chosen randomly and then purified and their L-SD activity tested. The result is shown in Table 8.

Table 8. L-SD Activity of Transductants from CT4 to SGL- Mutants

Strains	L-SD activity
MEW1	0.095
Sp2	0.621
CH22	0.023
Transductant MEW84/*lrp*- #1	0.212
Transductant MEW84/*lrp*- #2	0.195
Transductant MEW84/*lrp*- #3	0.213
Transductant MEW84/*lrp*- #4	0.293
Transductant MEW20b/*lrp*- #1	0.801
Transductant MEW20b/*lrp*- #2	0.721
Transductant MEW20b/*lrp*- #3	0.748
Transductant MEW20b/*lrp*- #4	0.818
Transductant MEWb1/*lrp*- #1	0.679
Transductant MEWb1/*lrp*- #2	0.696
Transductant MEWb1/*lrp*- #3	0.809
Transductant MEWb1/*lrp*- #4	0.704

Transductants were obtained by using P1 lysate gathered from CT4 (*lrp*::Tn10 Tet^r) to transduce into host MEW84, MEW20b and MEWb1, and selecting on LB agar plate added with tetracycline and kanamycin. Transductants were randomly picked and purified from the colonies resistant to both antibiotics. Strains MEW84, MEW20b and MEWb1 showed L-SD activities of 0.023, 0.083 and 0.100 (data taken from Table 2.)

L-SD were assayed as in Table 2.

Results are from a single experiment typical of three such experiments.

Discussion

Part 1. Relation between *glpC*, *torA*, *nuoM* and SGL- phenotype

1.1. The relation between *glpC*, *torA*, *nuoM* and the activation system of L-SD

In this thesis, I described the isolation and characterization of two new mutants MEW20b and MEWb1, which are unable to use the combination of three amino acids: L-serine, glycine and L-leucine, as carbon source. Together with a formerly isolated SGL- mutant MEW84, all three mutations were located by inverse PCR in genes involved in oxidation-reduction reactions - *nuoM*, *torA* and *glpC*. These three genes code for a subunit of NADH dehydrogenase, TMAO reductase and a membrane protein involved in glycerol-3-phosphate dehydrogenase activity respectively. That mutations in these genes are responsible for the phenotype was shown by the fact that in each case, transduction of the insert into wild-type *E. coli* produced the SGL- phenotype. For the first two genes, *nuoM* and *torA*, a wild-type clone was shown to complement the mutant and restore the ability to use SGL for growth.

The involvement of dehydrogenases specifically in serine metabolism is a considerable surprise. The three mutants all can grow with L-alanine and pyruvate as carbon source (Table 4). The metabolism of L-alanine only involves a single metabolic pathway, in which L-alanine is converted to D-alanine by a specific racemase and the D-

alanie is then converted to pyruvate and ammonia by a membrane-bound D-amino acid dehydrogenase of broad specificity (McFall and Newman,1996). One can assume therefore that the problems of all three SGL- mutants' inability to grow on SGL are not in the use of pyruvate and must therefore be either in the generation of pyruvate from serine, or some reaction involved in the assimilation of carbon from serine into the anabolic pools.

The synthesis of L-SD #1 is unusual for *E. coli* in that it is made in an inactive form, and can be activated by a post-translational mechanism. Mutations in two as yet unidentified genes, as defined in MEW128 and MEW191, were shown to prevent activation but permit the synthesis of an inactive enzyme which could be activated *in vitro* through the incubation with iron and DTT. This process may mimic the *in vivo* enzymatic activation of L-SD.

The L-SD activity of MEW20b and MEWb1 is close to wild-type levels in cells from both with and without inducers glycine and leucine. It seems they are not likely to have any problem in being activated. However, little is known about assimilation of serine carbon. Meanwhile, L-serine degradation involves an enzyme, L-serine deaminase #1 (L-SD #1), which converts it to pyruvate which could then be used for both energy production and biosynthesis. This enzyme is essential for the use of serine as carbon source. Mutants of in *sdaA*, the gene coding for L-SD, are unable to grow with serine as sole carbon source or with the mixture of L-serine, glycine and L-leucine.

All the L-SD tests are done in glucose-minimal medium, and the relevant question is how much activity can be induced in SGL medium. Since we have at least one example of a strain which makes huge amounts of L-SD in glucose-minimal medium but can not grow in SGL, it seems quite possible that strains can be mutated in the induction signals as well (Newman, personal communication).

On the other hand, the fact that SGL- strains carry mutations in dehydrogenases suggests strongly that these mutations are involved in activation. The next section of this discussion considers how this activation might take place. However, one should keep in mind that the redox reactions might be involved somehow in making the inducer of the enzyme, or in the assimilation pathway, or in still another as yet unimagined way.

1.2. The possible relation of *glpC*, *torA* and *nuoM* to L-SD activation

The conversion of serine to pyruvate does not involve a change in redox state. Unless a very complex mechanism is postulated, there is no place in this reaction for electron transport systems such as those in which the three genes are involved.

On the other hand, L-SD is synthesized in an inactive form and can not function without activation. The activation of L-SD may very well involve a redox reaction in *E. coli* since it is mimicked by iron and DTT. In fact the activation of L-SD is a redox reaction in anaerobic bacteria and may be the same in *E. coli*. In another Gram-positive bacterium *Clostridium propionicum*, L-SD contains a 4Fe-4S cluster and is active when

the cluster is fully reduced, and inactive when it is partially oxidized (Hofmeister *et al.*, 1993). The *E. coli* enzyme contains an unusually high number of cysteines concentrated near the C-terminal end (Su, 1991; Su *et al.*, 1989). These are not arranged with the spacing expected for 4Fe-4S clusters, but their existence suggests some sort of redox reaction must also be involved in activation in *E. coli* L-SD.

1.2.a. A description of L-SD activation in anaerobic bacteria

L-SD activation in anaerobic bacteria has been shown to involve a change in oxidation state of an iron atom. Nothing, however, is known about the enzyme(s) which carry this out.

The L-SD from *Peptostreptococcus asaccharolyticus* is functionally similar to L-SD from *E. coli* and possess similar chemical characteristics (Hofmeister *et al.*, 1997). L-SD from *P. asacchariolyticus*, like that from *E. coli*, can be air-inactivated and can be reactivated specifically by Fe^{2+} under anaerobic conditions (Grabowski and Buckel, 1991). According to Muller and Buchel, the iron-sulfur clusters present in the dehydrase may have two functions, as electron carrier and as a Lewis acid, which coordinates the hydroxyl group and facilitates the elimination (Muller and Buchel, 1995). Study of L-SD from *P. asaccharolyticus* already confirmed the presence of a $[3Fe\text{-}4S]^{+}$ cluster in the inactive enzyme (Grabowski and Buckel, 1991). Furthermore, it has been suggested the paramagnetic $[3Fe\text{-}4S]^{+}$ cluster of the inactive enzyme was originated from a diamagnetic $[4Fe\text{-}4S]^{2+}$ cluster by oxidation and loss of one iron ion (Hofmeister *et al.*, 1994).

Conversely, the change from the inactive form of L-SD to the active form is quite likely require the transfer of electrons into L-SD. The study of Hofmeister *et al.* also indicated that the $[4Fe\text{-}4S]^{2+}$ cluster of the active enzyme is protected by L-serine against oxidation and loss of an iron ion. Also, the $[4Fe\text{-}4S]^{2+}$ cluster exerts an essential function in catalysis by direct interaction with the substrate (Hofmeister *et al.*, 1994).

The derived amino acid sequence of L-SD from *P. asaccharolyticus* shares similarities with both monomeric L-SD #1 and L-SD #2 from *E. coli* suggests that these two enzymes are also iron-sulfur proteins (Hofmeister *et al.*, 1997). Considering the high degree of similarity between these two L-SD enzymes from different hosts, there is probability that L-SD in *E. coli* needs electron donation to be activated.

1.2.b. A possible role for oxidative enzymes in activation of *E. coli* L-SD

Assuming that the three gene products identified in this study are indeed involved in L-SD activation, it seems likely that they would be involved in establishing the oxidation state of iron required for activity. An involvement of iron in *E. coli* L-SD has not been directly shown though there is a great deal of suggestive evidence. On the other hand, in this work, three enzymes which may be involved in the reaction have been identified, even if their metabolic role in activation is not obvious.

Two of the enzymes, glycerol-3-phosphate dehydrogenase and TMAO reductase are anaerobic enzymes and are not thought to function in aerobic metabolism. If they are involved in L-SD activation in aerobically grown cells, they must be expressed at least to a minor extent, and that expression seen under aerobic conditions must be critical for serine utilization.

TMAO reductase is named for its activity in transferring electrons to trimethylamine. This is not necessarily its primary substrate, but may simply be the only relevant substrate tested. In any case no TMAO is given to the cells, and there is no evidence that any is generated during metabolism. It seems then that TMAO reductase in this context is working on an alternative substrate.

The first puzzle is that all three redox enzymes are required for L-SD activation, suggesting that they work sequentially and form a pathway. As classified by Gennis and Stewart, electron transport chains consists of three types of components: 1) substrate-specific dehydrogenases, which carry out the oxidation of organic substrates and feed electrons into the mobile quinone pool; 2) quinones, which deliver reducing equivalents to the terminal oxidoreductasese; and 3) terminal oxidoreductases, which reduce the terminal electron acceptors being utilized (Gennis and Stewart, 1996). The genes considered here code for two enzymes in the first group, and one terminal electron acceptor.

This suggests that serine deaminase activation involves at least two substrate-linked dehydrogenases and a terminal oxidase. Considering that the iron in L-SD has to be reduced, the pathway should be considered in reverse, i.e. provision of a reducing agent specific to L-SD-Fe^{3+}. This pathway must be biochemically specific since specific mutations disrupt its function, but it may handle a very low electron flow since it only has to function to keep the relatively few molecules of L-SD supplied with reduced cofactor.

In what step does this specificity lie? Electrons are apparently taken from some substrate by the *torA* gene product, at a midpoint electron potential of +130 mv (Barret and Kwan, 1985), considerably lower than that of the nitrate/nitrite couple (+433 mv) and handed to some quinol or haem-containing electron carrier. This may not be due simply to the potential being convenient. TMAO reductase may be one of the few or only enzymes which is made in sufficient amount aerobically to function in this pathway. In fact, glycerol-3-phosphate and NADH are all effective electron donors for TMAO reduction (Gennis and Stewert, 1996). Therefore electrons may be funneled in some way from glycerol-3-phosphate and NADH to TMAO reductase and these electrons may then further transferred to L-SD to activate it.

Another possibility is that the products of the three genes react separately yet are all necessary to activate L-SD. Moniakis' study already revealed that the activation of L-SD quite likely involves the folding of this enzyme (Moniakis 1992). Maybe the electrons generated by NADH dehydrogenase are needed to react with a number of cysteines in L-

SD and changed the conformation of this protein. Then, electrons produced from TMAO reductase and glycerol-3-phosphate dehydrogenase may react with other cysteine residues to further change the conformations.

Part 2. Unique Characteristics of MEW84

2.1. Azaleucine sensitivity

Azaleucine is toxic to all strains tested, including other SGL- mutants MEW20b and MEWb1. However, MEW84 proved to be more sensitive to it. Feng's study already excluded the possibility that this increased azaleucine sensitivity is caused by the decreased L-SD activity in MEW84, since some strains showed high level of L-SD activity also has the same sensitivity to azaleucine as MEW84 (Feng, 1990).

As an analogue of leucine, azaleucine is toxic to *E. coli* and some other bacteria since it can be incorporated into protein in place of leucine, resulting in inactive enzymes. Also, it can repress the biosynthetic enzymes for leucine, isoleucine and valine (Rabinovitz et al., 1969). Azaleucine can be transported into the cell by a branched-chain amino acid (LIV-I) transport system or the aromatic amino acid (*aroP*) transport system (Harrison *et al.* 1975). Yet, how a mutation in *glpC* can possibly interfere the azaleucine transportation still remains to be answered.

It has been reported that a strain with a much higher sensitivity to azaleucine than the wild-type strain had the same kinetic parameters for azaleucine uptake and so must be altered in some other functions. Therefore, in MEW84, it is possible that the leucine biosynthetic pathway is somehow slowed down, or the leucine degrading process is speeded up; this latter response is another process that can effect the azaleucine sensitivity.

2.2. L-serine sensitivity

As indicated in the Introduction section in detail, L-serine is toxic to *E. coli* because of an inhibition of branched-chain amino acid biosynthesis. However, since all the strains in use in this lab carry an *ilvA* deleation, and isoleucine and valine are added to all media, L-serine toxicity should not be due to this mechanism (Hama *et al.*, 1990). Indeed, L-serine does not have any interference on the growth of MEW1, which is the parent strain of all the SGL- mutants, nor is it toxic to the other SGL- mutants MEW20b and MEWb1.

One explanation of the increased L-serine sensitivity of MEW84 can be the significantly decreased L-SD activity, leading to excess internal serine. This is supported by the fact that CH22, a strain lacking L-SD activity is also sensitive to L-serine. This experimental result seems to agree with the suggested functions of L-SD presented by Su, who regarded L-SD in *E. coli* as functioning to maintain the L-serine pool at an appropriate level, serving as a catabolic enzyme to convert excess L-serine to carbon and

nitrogen sources (Su, 1991; Su and Newman, 1989). Thus it seems logical that without enough L-SD to eliminate the toxicity of extra L-serine, it severely interferes with the growth of MEW84.

Threonine, but not aspartic acid can partially release serine toxicity in strain MEW84 indicating that the site of serine inhibition is before threonine but after aspartic acid in the branched-chain amino acid biosynthetic pathway (Fig.2). This finding also agrees with Hama *et al.*'s report that the target of L-serine inhibition in *E. coli* is homoserine dehydrogenase I (Hama *et al.*, 1990). However, since threonine can not completely rescue strain MEW84 and CH22 from serine toxicity, it seems that there exist other serine inhibition sites in *E. coli*.

Part 3. Effect of SGL medium on L-SD

It is completely unexpected that the SGL medium actually dramatically induced L-SD activity of all strains tested. After being resuspended in SGL liquid medium, MEW1 cells showed L-SD activity elevated more than 10 fold to normal L-SD activity from the same strain cultured in glucose-minimal medium. Similarly, compared to L-SD tested from cultures prepared in glucose-minimal medium, L-SD of MEW84 goes up more than 20 times; L-SD of MEW20b and MEWb1 is increased about 6 times after incubation in SGL medium in which they can not grow.

Yet strangely, strain CH22, which is supposed to express no L-SD in minimal medium because of the null mutation of L-SD coding gene *sdaA*, also showed about 10 times higher L-SD activity than that seen in glucose-minimal medium.

The only explanation for this phenomenon is that somehow the second L-SD, L-SD #2, is expressed when CH22 was resuspended and incubated in SGL medium, since the L-SD #2 coding gene *sdaB* still remains intact in this strain, and this is the only other gene in *E. coli* which is known to code for product of L-SD activity (Su and Newman, 1991; Shao, 1993).

As stated in the Introduction, although the coding genes are located in very different positions, L-SD #1 and L-SD #2 are quite similar to each other both in amino acid sequence and in chemical characteristics. L-SD #2 is only expressed in LB medium and the L-SD activity tested in minimal media is actually the activity of L-SD #1 (Newman, personal communication). Shao's detailed study on *sdaB* and its product L-SD #2 indicated that the mRNA from *sdaB* actually should exist in *E. coli* even in minimal medium culture. Moreover, he clearly proved that *sdaB* is under catabolite repression (Shao, 1993).

Catabolite repression is mediated by the cyclic AMP/cAMP receptor protein (Crp) system in *E. coli*. It was proved that growing cells in minimal medium with other carbon sources does not induce the expression of *sdaB*, which indicate that cAMP and Crp alone

can not activate *sdaB* expression. Addition of exogenous L-serine did not activate the *sdaB* expression either. Therefore, if *sdaB* is expressed in MEW1, MEW84, MEW20b, MEWb1 and CH22, either glycine or L-leucine act as a signal to start the expression of *sdaB*, or the washing and resuspension treatment which represents a carbon starvation actually activated L-SD #2.

Since the L-SD of MEW84 was induced to such a high extent, it is also possible that the inactive form L-SD #1 from this strain was also activated. But then there still remains the question why the high level L-SD activity from all the SGL- mutants in SGL medium still could not allow them to grow under this condition. It is probably because the L-SD activity in all these three strains is still not high enough to sustain growth. After all, the L-SD activity of the three SGL- mutants are all lower than that of MEW1 in SGL medium.

Part 4. The Lrp effect on L-SD activity

Lrp protein, as the product of *lrp* gene is a global regulator that can control the expression of a number of genes in *E. coli*. It is already known that *sdaA* expression is under the negative control of Lrp. Therefore in a *lrp* null mutant Sp2, the L-SD activity is about 6 times higher than in the wild-type strain (Newman, personal communication). Lrp also decreases the transcription of *serA*, which is the first enzyme functioning in L-serine synthesis (Lin *et al.*, 1991). Consequently, we can postulate that the size of the serine

pool in an *lrp-* strain should be small. Under the *lrp-* background, all the SGL- mutants have their L-SD activity increased, with that of MEW20b/*lrp-* and MEWb1/*lrp-* changed the most, being about 7 times higher than in *lrp+* background. Since the λplacMu insertion in these two strains do not seem to produce an in-frame *lacZ* fusion, it is hard to tell whether Lrp has an effect directly on the *nuoM* or *torA* genes. It has already been proven that *sdaB* is not under the control of Lrp (Shao, 1993), so the possibility of the expression of L-SD #2 can be excluded.

Part 5. Summary

Wild type *E. coli* can not use L-serine as a carbon and energy source unless glycine and L-leucine are also provided. The study of strains that can not grow on SGL medium provided a opportunity to investigate some of the interesting features of L-serine deaminase.

Previously, few SGL- mutants have been isolated. All of them, MEW128, MEW191 and MEW84 have been shown to have very low L-SD activity *in vivo*, yet this low L-SD can be restored by incubation with iron and DTT *in vitro*. In particular, the mutated genes within these few mutants all have been mapped at completely different locations in *E. coli* genome. This indicates that there exists a rather complicated activation system of L-SD which may involve a number of gene products.

To further study the activation system of L-SD, I began with isolation of SGL- mutants through the insertion of λplacMu. One might expect that SGL- mutants would have malfunctioning L-SD. The newly isolated SGL- mutants fall into a new class which have about the same level of the L-SD activity as their parent strain MEW1 when cells were cultured in glucose-minimal medium. Yet they still are not able to grow on SGL medium. This suggests the L-SD activity of these two mutants has interfered. After demonstrating that the loss of the ability to grow on SGL medium of MEW20b, MEWb1 and MEW84 was caused directly by the insertion of λplacMu, I went on to try to identify the mutated gene in all these three mutants.

Applying the inverse PCR technique, the mutations in the newly isolated SGL- mutants MEW20b, MEWb1 and the formerly isolated MEW84 were successfully identified as being in *muoM, torA and glpC*, three known genes not previously thought to be involved in serine degradation but have been to reported to exist as parts of operons. To confirm the relationship between these genes and the SGL- phenotype, I tried to amplify and clone DNA carrying the corresponding wild-type *muoMN, torCA and glpABC* genes. In order to expressing *torCA* and *glpABC*, I designed the primers and selected appropriate vectors to amplify the operon including the promoter region, so that when I cloned them into a simple plasmid like pUC18, they should be expressed. As for *muoM*, since this operon is too large in size to amplify by PCR, I only amplified the *muoM muoN*

genes and put this DNA fragment under the ara pBAD promoter in the $pBAD_{22}$ plasmid. Therefore the expression of *muoMN* should be induced by arabinose.

The cloned *muoMN* and *torCA* region completely compensated for the inability of mutant MEW20b and MEWb1 to grow on SGL medium. Thus clearly indicates that these two genes are involved in the cells ability to grow on SGL medium. Comparison with other strains constructed with insertions in *muoM* and *torA* further demonstrated this.

The cloneing and expression of *glpABC* operon was not successful. Although it should also contain the promoter region, the plasmid harboring the cloned operon failed to compensate for the SGL- phenotype. Nonetheless experimental data suggests that *glpC* was indeed interrupted by λplacMu insertion in the mutant.

The comparison of the functions of the three mutated genes suggests that electron donation maybe part of the process necessary to activate L-SD.

A surprising finding in this thesis is that the SGL combination actually increased L-SD dramatically for both L-SD mutants and their parent strain MEW1. The fact that even an L-SD deficient strain CH22 also showed elevated L-SD in SGL indicates that L-SD #2, which has been considered to be expressed only in LB medium, might also be expressed in these conditions.

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