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Study of MEW84 - A Mutant Related to the Post-Translational Modification of L-Serine Deaminase in Escherichia coli K-12

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ABSTRACT

Study of MEW84 - A Mutant Related to the Post-Translational Modification of L-Serine Deaminase in Escherichia coli K-12

Xiao-peng Feng

An Escherichia coli K-12 mutant MEW84 was studied. The corresponding gene, sda84, seems to be involved in the regulation of L-serine deaminase (L-SD), a constitutive enzyme which catalyses the deamination of L-serine to form pyruvate. This mutant shows no detectable L-SD activity in whole-cell assays. However, the L-SD activity in crude cell extracts is restored by incubation with iron and DTT. In addition, this mutant requires thiamine to grow in glucose minimal medium. The characteristics of strain MEW84 resemble those of the previously isolated mutants MEW128 and MEW191, which are involved in the post-translational activating system of L-SD. Strain MEW84 shows an increased sensitivity to L-serine and azaleucine. Analysis of this mutant indicates that the decreased L-SD activity is responsible for the observed L-serine hypersensitivity. However, the increased azaleucine sensitivity is not due to L-SD deficiency.

Genetic mapping experiments indicates that this mutation (sda84) is located at 55 minutes on the E. coli genetic map

and is distinct from any of the previously isolated mutations that affect L-SD activity. A gene complementing sda84 was cloned. However, the Southern hybridization results indicate that the cloned DNA fragment does not contain the corresponding gene mutated in strain MEW84.

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I Introduction

This work attempted to investigate the post-translational control of L-serine deaminase (L-SD) in Escherichia coli K-12 through the study of an L-SD deficient mutant. L-serine deaminase is an enzyme that catalyses the deamination of L-serine to form pyruvic acid and ammonia (Pardee and Prestidge, 1955). This enzyme has been characterized in several microbial species (Newman and Kapoor, 1980). In Clostridium acidi-urici, L-SD is involved in uric acid fermentation (Benziman et al., 1960). In Arthrobacter globiformis (Bridgeland and Jones, 1965) and Diplococcus glycinophilus (Klein and Sagers, 1961), L-SD is required to use glycine as a sole carbon source. It is peculiar that in Escherichia coli K-12 cells grown in glucose minimal medium, this enzyme is produced in a considerable amount (Pardee and Prestidge, 1955), but its metabolic function is not obvious (Newman and Magasanik, 1963).

In E. coli cells, L-serine is synthesized from 3-phosphoglyceric acid (PGA), an intermediate of the Embden Meyerhof pathway (EMP) (Review from Stauffer, 1987). Through the EM pathway, PGA is further converted to form pyruvate. This reaction results in the release of energy in the form of ATP. Alternatively, PGA is used as a precursor of L-serine (Stauffer, 1987). L-serine can then be deaminated by L-SD to produce pyruvate. The production of pyruvate via this alternative pathway does not yield any energy. Consequently

PGA used this way is of no apparent benefit to the cell. Therefore, using L-SD for the sole purpose of deaminating L-serine is energetically wasteful (Ramotar and Newman, 1986). This does not seem to be reasonable, since bacteria have evolved to be energy efficient. It is likely that L-SD has other important function(s) in addition to deaminating L-serine.

Wild type E. coli K-12 can use a great range of different carbon sources. Yet L-serine can not be used as a sole carbon source, unless glycine and leucine are also supplied to the medium (Newman and Walker, 1982b). This is probably due to the inducing effect of these two amino acids on L-SD activity (Pardee and Prestidge, 1955; Newman and Walker, 1982b). Mutants with increased levels of L-SD, or wild type E. coli harbouring multiple copies of the structural gene can grow on serine in the absence of glycine and leucine (Newman et al., 1981; Newman et al., 1982a; Su et al., 1989 and Lin et al., 1990). Under these conditions, L-SD must be converting serine to pyruvate to an extent which would be sufficient to support growth, as we know that pyruvate can be used as a carbon and energy source by E. coli K-12.

Many factors are known to influence L-SD activity in E. coli K-12 cells. Mutations mapping at 6 different genetic loci which confer either increased or decreased L-SD have been isolated in our lab. It has also been observed that the enzyme produced both in vivo or in a crude extract is present

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in an inactive form (Isenberg and Newman, 1974; Newman and Kapoor, 1980). This is not a common phenomenon in bacteria. The inactive form of L-SD can be activated enzymatically *in vivo* or chemically *in vitro* (Newman *et al*., 1985a, Newman *et al*., 1985b). These experimental results suggest that L-serine deaminase is subjected to both transcriptional and post-translational controls. Consequently, L-SD must have an important function other than merely deaminating L-serine in the *E. coli* K-12 cell (Newman *et al*., 1985b).

A detailed investigation into the function of L-SD is proceeding in our lab using mutants that show altered L-SD activities. In this thesis I will present the study of a strain MEW84, which is thought to affect the post-translational modification of L-serine deaminase.

In order to provide insight about this research, the following parts of the introduction will review the experimental evidence related to this topic.

I A The Synthesis, Degradation and Fate of L-serine in
**   E. coli**

L-serine is synthesized from an Embden-Meyerhof pathway (EMP) intermediate, phosphoglyceric acid (PGA), by a three-enzyme biosynthetic pathway. The end-product, serine, controls the flow of carbon through this pathway by inhibiting the first enzyme, phosphoglyceric acid dehydrogenase (PGDH)

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(Stauffer, 1987).

L-serine can be degraded to pyruvate by L-serine deaminase, which is constitutively synthesized by E. coli K-12 cells (Pardee and Prestidge, 1955). The activity of this enzyme is influenced by many factors, but its substrate, L-serine, has no effect on its activity (Isenberg and Newman, 1974; Pardee and Prestidge, 1955).

L-serine is required for a large number of biosynthetic reactions. It serves as a 3-carbon precursor in the synthesis of cysteine, methionine and tryptophan, and as a two-carbon unit for the formation of glycine and purines. Serine is the only source of single-carbon units for biosynthetic and methylation reactions (Stauffer, 1987).

The production, degradation and the fate of L-serine in E. coli cells is outlined in Figure 1.

I B Mutants With Altered L-SD Activity

Six mutants that show altered L-SD activities have been isolated (Table 1). They have been shown to all map at different genetic loci. Amongst them, KEC9(ssd) (Newman et al., 1982a) and MEW26 (rbl) (Lin et al., 1990), which were selected for their ability to use L-serine as a sole carbon source, have elevated L-SD activities. The other four mutants, MEW22 (sdaA) (Su et al., 1989), MEW128 (sda128), MEW191 (sda191) (Newman et al.,1985a) and MEW84 (sda84) (Su, personal

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Table 1   MUTANTS WITH ALTERED L-SD ACTIVITIES

Mutants	Genotype	Phenotype
KEC9	ssd	L-SD , growth on serine, etc.
MEW26	rbl	L-SD , growth on serine, etc.
MEW22	sdaA	No L-SD in vivo or in vitro
MEW128	sda128	L-SD in vivo, no; in vitro, yes
MEW191	sda191	L-SD in vivo, no; in vitro, yes
MEW84	sda84	L-SD in vivo, no; in vitro, yes

4a

Glucose ——→ Phosphoglycerate ——→ Phosphoenolpyruvate ——→ pyruvate

serA
  serC
    serB
                        L-serine

sdaA
(L-SD)

Cysteine   Glycine   Methionine   Tryptophan   C1 units
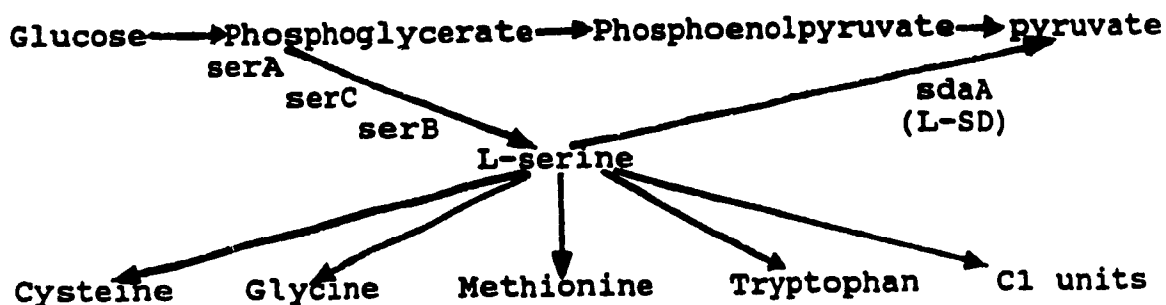
Figure 1. The Production and Fate of L-Serine

The synthesis and fate of L-serine are shown. Genes encoding the serine biosynthetic enzymes are indicated. Also shown is the conversion of L-serine to pyruvate by the sdaA gene product L-serine deaminase, and the conversion of L-serine to other cell components. The gene designations are as follows: serA, 3-phosphoglycerate dehydrogenase; serC, 3-phosphoserine aminotransferase; serB, 3-phosphoserine phosphatase and sdaA, L-serine deaminase.

communication) which were selected for their inability to grow on serine in the presence of glycine and leucine, have lowered L-SD activities as compared with the wild-type strain grown in glucose minimal medium.

L-SD activity in the ssd background (KEC9) is about 6-fold higher than normal. The lesion in ssd also leads to other changes in cell metabolism: it decreases the growth rate and yield of the strain by about 30 %; it makes the strain unable to grow on succinate or α-ketoglutarate as carbon source; the cell is resistant to certain antibiotics and colicin; unable to grow in glucose minimal medium at 42°C or anaerobically, and the cell also has some defect in amino acid transport (Newman et al., 1982a).

The rbl mutation is also pleiotropic. Strain MEW26 (rbl) has a high level of L-SD activity as well as of another amino acid-degrading enzyme, L-threonine dehydrogenase: low levels of 3-phosphoglycerate dehydrogenase activity and end product-inhibited acetohydroxy acid synthase activity. The rbl mutation suppresses the slow growth of a metK strain. It is suggested that the rbl gene product is a regulator of a global response to leucine (Lin et al., 1990).

sdaA has been proven to be the structural gene of L-SD. Strain MEW22, possessing the sdaA mutation, was selected by placmu infection. It can not grow on serine in the presence of glycine and leucine (SGL medium), and has no detectable L-SD activity either in vivo or in vitro (Su et al., 1989).

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Strain MEW128 and MEW191, unable to grow in SGL medium, have no detectable L-SD activity in whole cell assays, but unlike strain MEW22, their L-SD activities in crude cell extracts can be activated with iron and DTT. Strains MEW128 and MEW191 have little metabolic deficit, except for a thiamine auxotrophy as a result of the mutation that causes decreased L-SD activity (Newman et al., 1985a and Newman et al., 1985b).

MEW84, a newly isolated SGL⁻ strain, resembles strain MEW128 and MEW191 in that it possesses no detectable L-SD activity in vivo, its L-SD can be activated in vitro, and it is also thiamine requiring (Su, personal communication). It is this strain that was studied in this work.

I C Environmental Factors That Affect L-SD Activity

L-SD is rapidly induced by certain environmental stresses: exposure to DNA damaging agents, such as UV, nalidixic acid and mitomycin; growth under increased temperature (Newman et al., 1982c); or anaerobic condition; alcohol shock etc. (Newman, personal communication). L-SD activity is also increased by growth in glucose minimal medium supplemented with glycine and leucine (Pardee and Prestidge, 1955; Newman and Walker, 1982b), and by growth in rich medium (Newman et al.,1982c). However, L-SD activity is not induced by its substrate, L-serine (Pardee and Prestidge, 1955;

Isenberg and Newman , 1974).

I D L-Serine Deaminase Might Be Subject to Post-Translational Modification

L-SD is a highly unstable enzyme. When assayed in crude cell extracts, L-SD activity of wild-type cells (MEW1) can not be detected. However an inactive form of L-SD can be activated by incubation with iron and DTT in the presence of oxygen (Newman et al., 1985b; Newman and Walker, 1990). This is also true for the L-SD activity from crude extracts of strains MEW128 and MEW191 (Newman et al., 1985b), which are known to be physiologically devoid of L-SD activity and unable to grow with serine, glycine and leucine as carbon source (Newman et al, 1985a). This suggests that in the E. coli cell, the structural gene of L-SD is transcribed and translated into an inactive protein. The inactive protein may then be activated enzymatically in the parent strain, but not in strain MEW128 or strain MEW191. This might define an activating system of this enzyme encoded by the functional sda128 and sda191 genes. The enzymatic activating function can be mimicked in vitro by certain chemical activators, such as iron and DTT (Newman et al., 1985b).

I E Possible Function of Iron and DTT in Activating or Inactivating Enzymes

It has been reported that the combination of iron and DTT can result in the activation or inactivation of some enzymes (Carter and Sagers, 1972; Punekar and Lardy, 1987; Newman et al., 1985b).

It is known that reactive oxygen species such as super oxide radical anion, hydrogen peroxide, and hydroxyl radical can react with various biomolecules, and might consequently be able to change the state of molecules (Review from Basaga, 1990). These oxidants can be generated during iron cycling between Fe^{++} and Fe^{+++} in the Fenton reaction as shown in the following equations (Walling, 1975):

$$Fe^{++} + O_2 \longrightarrow Fe^{+++} + O_2^{\cdot}$$

$$2O_2^{\cdot} + 2H^{+} \longrightarrow H_2O_2 + O_2$$

$$Fe^{++} + H_2O_2 \longrightarrow Fe^{+++} + OH^{\cdot} + OH^{-}$$
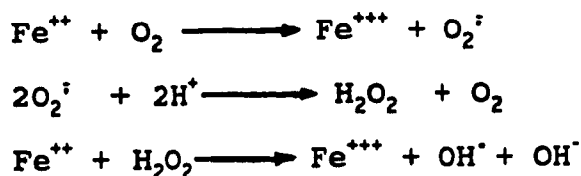
DTT, a reducing agent, can facilitate this cycle by reducing Fe^{+++} to Fe^{++} (Walling, 1975). The cycling can also be catalysed enzymatically (Basaga, 1990). The reactive oxygen species thus generated may react with the enzyme at different sites, resulting in modifications which either activate or inactivate the enzyme. For example, glutamine synthetase of E. coli is modified oxidatively at a histidine residue when incubated with iron and DTT (Kim et al., 1985). Iron and DTT can also cause cleavage or degradation of the biomolecule. Kim et al (1985) have shown that when exposed to iron and DTT, the yeast glutamine synthase was degraded to

smaller fragments in a time dependent manner. Although in the
above two examples iron and DTT cause loss cf enzyme
activities, there are other examples where iron and DTT act
as activators.   L-serine dehydratase (deaminating) from
Clostridium acidi-urici has to be activated by catalytic
amounts of ferrous ion and DTT before it can react with its
substrate (Carter and Sagers, 1972).

I F Examples of Enzymes That are Subject to Post-
        Translational Modification

I F-1 The Activation of E. coli Pyruvate Formate-Lyase (EC
        2.3.1.54)

        Pyruvate formate-lyase is a key enzyme in controlling the
anaerobic metabolism of E. coli cells using glucose as a
carbon source (Review from Knappe, 1987).   This enzyme is
subject to both transcriptional and post-translational control
(Knappe et al., 1984). Synthesis of this enzyme is repressed
during aerobic growth and derepressed in anaerobic condition.
Moreover, the enzyme can be converted post-translationally
between the active form (Ea) and inactive form (Ei) (Knappe
et al., 1984 and Conradt et al., 1984). Upon shifting to
anaerobic condition, the inactive form is activated and
catalyses the conversion of pyruvate to acetyl-CoA and HCOOH.
A free radical component (which originates from the loss of
a hydrogen atom on an unidentified amino acid residue) in the

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resting state of the enzyme is characteristic of the active enzyme, which distinguishes it from Ei. Ea is highly sensitive to dioxygen while Ei is not (Knappe et al., 1984). Introduction of the radical state into the inactive protein is catalysed by an Fe^{++}-dependent constitutive activating enzyme. To fulfil its function, the activating enzyme uses pyruvate as an allosteric effector and is linked to reductive cleavage of S-adenosylmethionine through reduced flavodoxin (Fl), which is generated from NADPH by pyruvate-dependent oxidoreductase (Knappe et al., 1984). The gene encoding the activating enzyme has been cloned and sequenced. The deduced amino acid sequence of this activating enzyme was found to be rich in histidine residues, and three cysteines out of seven are clustered together. These features may be important for the enzyme's function (Rodel et al., 1988 and Plaga et al., 1988). The exact activating mechanism remains unclear. A possible mechanism for the activating process is postulated by Knappe et al. and shown in Figure 2 (Knappe et al., 1984).

That is, the activating enzyme catalyses the formation of an adenosyl-Fe complex [$\overset{CH_2R}{Fe}$]. This complex attacks the Ei molecule and pulls off a hydrogen atom from it, thus leaving an unpaired electron on the pyruvate formate-lyase to form the active form of it. The complex itself undergoes redox reaction to generate the oxidative form of iron Fe^{+++}, and 5'-deoxyadenosine. Fe^{++} is regenerated by reduced flavodoxin, which is in turn regenerated from NADPH and

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AdoMet   Met

CH_2R

[Fe]red ————————————► [Fe]

FlH·

FlH₂   [Fe]ox   E·   E-H
      +
   CH_3R

The general equation is:

Ei + AdoMet + Flavodoxin$_{red}$ ————————►

Ea + 5'-deoxyadenosine + methionine + Flavodoxin$_{ox}$
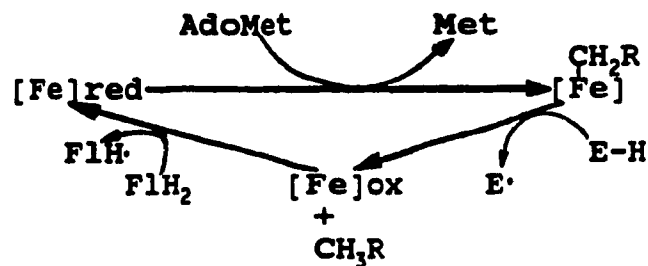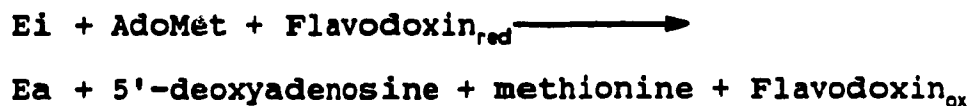
Fig. 2   Catalytic Cycle of the Activating Enzyme for
        Introducing a Free Radical in Pyruvate Formate-
        Lyase (Knappe et al., 1984)

[Fe], iron centre of the activating enzyme at
different redox stages. -CH$_2$R and CH$_3$R, adenosyl (5') and
5'-deoxyadenosine. FlH$_2$ (Flavodoxin$_{red}$) and FlH·
(Flavodoxin$_{ox}$), flavodoxin at reduced state and
oxidized state respectively. E-H and Ei, inactive form of
pyruvate formate-lyase. E· and Ea, active form of pyruvate
formate-lyase. AdoMet, s-adenosylmethionine.

pyruvate-dependent oxidoreductase. This process would depend upon the high adenosyl transfer potential of adenosylmethionine and reducing power of dihydroflavodoxin (Knappe et al., 1984). The function of the radical could be to attack the carbon-carbon bond in pyruvate and cleave it to form acetyl-CoA and formate (Knappe et al., 1984).

I F-2 The Activation of Decarboxylases Using Pyruvate as a Cofactor

Generally, amino acid decarboxylases require pyridoxal 5'-phosphate (PLP) as a coenzyme. However, it has been reported that decarboxylases from some gram -positive bacteria and some eucaryotic sources have pyruvate, rather than PLP, as a covalently bound prosthetic group (van Poelje and Snell, 1990). This group of enzymes have some common features in that they contain two different (alpha and beta) subunits, which are derived from a single precursor (pi) subunit of an inactive proenzyme (van Poelje and Snell, 1990). "During the process of activation, these enzymes are cleaved at a specific site that immediately precedes the amino aci ..n. to yield the alpha and beta subunits of the active enzyme, meanwhile the serine residue is transformed to a pyruvoyl group at the NH_2 terminus of the alpha subunit" (van Poelje and Snell, 1990). Cleavage of the pi chain occurs by serinolysis but not a hydrolytic reaction: the oxygen atom incorporated into the carboxyl terminus of the beta chain does not come from H_2O,

but from the OH-group of the pyruvoyl forming serine residue of the alpha-chain (Recsei et al., 1983).

Histidine decarboxylase from Lactobacillus 30a is the most extensively studied of these enzymes. At its optimum pH, histidine decarboxylase is a hexamer of 208,000 MW, composed of 6 alpha subunits and 6 beta subunits (Recsei et al, 1983; van Poelje and Snell, 1990). This enzyme is originally synthesized as an inactive pro-histidine decarboxylase which is a hexamer of a single pi polypeptide (van Poelje and Snell, 1990). Activation occurs at the ser81-ser82 site of the pi chain to yield a beta chain with ser81 at its -COOH terminus and an alpha subunit with a pyruvoyl residue derived from ser82 at its NH_2- terminus (Recsei et al., 1983). How this activation process proceeds is not totally understood yet.

It was observed that conversion of prohistidine decarboxylase to histidine decarboxylase proceeds optimally in the presence of monovalent cations at neutral pH (Recsei et al., 1983). Monovalent cations may have the ability to stabilize the amide carbonyl oxygen of ser81 as an anion, which would facilitate nucleophilic attack at the amide C atom by the hydroxyl oxygen of ser82 during activation (Recsei et al., 1983).

I G L-Serine Toxicity to E. coli Cells

It is well known that L-serine is toxic to E. coli cells

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(Amos and Cohen, 1954), although this amino acid is indispensable to the cell. Usually L-serine toxicity is released by adding isoleucine to the medium, suggesting that serine might interfere with the isoleucine synthetic pathway (Amos and Cohen, 1954; Uzan and Danchin, 1978). An argument has existed for a long period as to the exact serine inhibition target in this pathway. Recently, Hama et al. (Hama et al., 1990) reported that the serine inhibition site must be located between aspartate and homoserine in the isoleucine biosynthetic pathway. The study in this work supports Hama's observation. For ease of understanding this result, the pathway related to serine toxicity is reviewed below.

I G-1 Common Pathway Leading to the Biosynthesis of Lysine, Methionine, Threonine and Isoleucine

I G-1a Enzymes Involved in This Common Pathway

In E. coli cells, amino acids lysine, methionine, threonine and isoleucine are all derived from the same precursor, aspartate (Fig 3., review from Cohen and Saint-Girons, 1987). Aspartate is derived from the Krebs cycle intermediate oxaloacetate.

Three distinct enzymes, aspartate kinase I, II and III are responsible for the conversion of aspartic acid to aspartyl phosphate. Aspartyl phosphate is then converted to aspartate

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```
                    Oxaloacetate
                         |
                         v
                    Aspartate
                         |,-ATP            Aspartate Kinase I,II,III
                         v
                  Aspartyl Phosphate
                         |
                         |,-NADPH          Aspartate Semialdehyde
                         v                 Dehydrogenase

        Lysine<--Aspartate Semialdehyde
                         |
                         |,-NADPH          Homoserine Dehydrogenase
                         v                 I, II

     Methionine<----Homoserine
                         |
                         v                 Homoserine Kinase
                         |
                         v                 Threonine Synthase
                    Threonine
                         !
                         v
                    Isoleucine
```
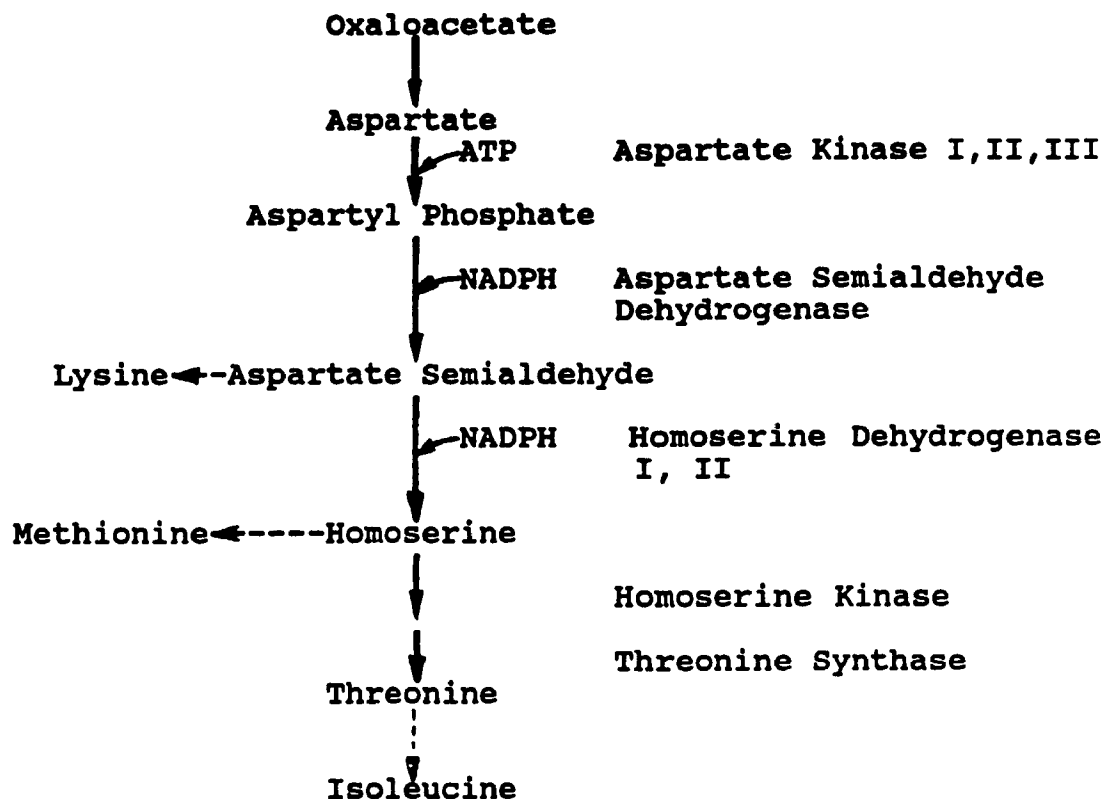
Fig 3. Common Biosynthetic Pathway Leading to the Formation
       of Lysine, Methionine, Threonine and Isoleucine in E.
       coli (Cohen and Saint-Girons, 1987)

       The formation, relevant intermediates and enzymes
        are shown.
       Broken lines indicate multiple steps.

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semialdehyde by aspartate semialdehyde dehydrogenase. The reduction of aspartate semialdehyde to homoserine is catalysed by two distinct homoserine dehydrogenases, homoserine dehydrogenase I and II. Homoserine is then converted to threonine by the actions of homoserine kinase and threonine synthase (Cohen and Saint-Girons, 1987). The activities of aspartate kinase I and homoserine dehydrogenase I are part of the same protein. A second bifunctional protein is responsible for the activities of aspartate kinase II and homoserine dehydrogenase II (Cohen and Saint-Girons, 1987). The activity of the latter is very low in wild-type E. coli cells as compared to the activities of aspartate kinase I - homoserine dehydrogenase I (Hama et al, 1990).

Lysine, methionine and isoleucine are derived from individual intermediates of this common pathway by the functions of specific enzymes (Cohen and Saint-Girons, 1987). It is noteworthy that the overexpression of one of the methionine specific biosynthetic enzymes, cystathionine-β-lyase, confers the ability of E. coli K-12 cells to grow on L-serine as sole carbon source (Brown et al., 1990).

I G-1b Regulation of the Enzymes Involved in This Common Pathway

Synthesis of aspartate kinase - homoserine dehydrogenase I is repressed by threonine plus isoleucine, and its activity is inhibited by threonine (Cohen and Saint-Girons, 1987).

Methionine represses homoserine dehydrogenase II, lysine
inhibits and represses the activity and synthesis of aspartate
kinase III respectively (Cohen and Saint-Girons, 1987).
Cysteine is an inhibitor of the homoserine dehydrogenase
activity in this pathway (Hama et al., 1990).

II Materials and Methods

II A Bacteria

The bacterial strains used, all derivatives of E. coli K-12, are described in Table 1.

II B Growth Media

II B-1 Minimal Medium

Liquid Minimal Medium (±N):

0.527% KH_2PO_4, 1.500% K_2HPO_4, 0.200% $(NH_4)_2SO_4$,

0.020% $MgSO_4$, 0.001% $CaCl_2$.

Solid Minimal Medium:

8 g of gerlite, 5.00 ug of $FeSO_4 \cdot 7H_2O$, 0.10 ug of

$Na_2MoO_4 \cdot 2H_2O$, and 0.12 ug of $MnCl_2 \cdot 4H_2O$ were added to 1

litre of the liquid minimal medium before autoclaving.

50 ug/ml of each of isoleucine and valine, 1 ug/ml of thiamine were added to the minimal media routinely, because MEW1 (△ilvA) and all its derivatives require isoleucine for growth, and some of our mutants require thiamine to grow. The liquid minimal medium before addition of carbon source was termed NIV solution.

Carbon sources were added to the minimal media at the concentration of 2 g/L. Medium containing L-serine as the only carbon source was termed NSIV medium. Medium containing, in addition to 0.2% L-serine, 300 ug/ml glycine and 300 ug/ml

Table 1. E. coli Strains Used

Strain	Relevant Genotype	Source
CU1008	E. coli K-12 ΔilvA	L. S. Williams
MEW1	ΔilvA, Δlac	Newman
MEW84	MEW1 sda84::λplacmu9	Su
MEW128	CU1008 sda128	Newman
MEW191	MEW1 sda191::Mudx	Newman
MEW191C	MEW1 sda191	Newman
MEW22	MEW1 sdaA::λplacmu9	Su
KEC9	CU1008 ssd	Newman
CH22	MEW1 sdaA::Cm^R	Su
CH22/sda84	MEW1 sdaA::Cm^R, sda84::λplacmu	Su
MEW22/ssd	MEW1 sdaA::λplacmu, ssd	This work
MEW84/ssd	MEW1 sda84::λplacmu, ssd	This work
MEW128/ssd	CU1008 sda128, ssd	This work
MEW191/ssd	MEW1 sda191::Mudx, ssd	This work
MEW191C/ssd	MEW1 sda191, ssd	This work
MEW84/sdaA	MEW1 sda84::λplacMu, sdaA::Cm^R	Su
MEW84/rbl	MEW1 sda84::λplacMu, rbl::Tn10	This work
MEW84/rbl/metJ	MEW1 sda84::λplacMu, rbl, metJ	This work
TK4100	MC4100 metJ::Cm^R	Greene
CAG5055	KL16 zed::Tn10 (42')	Singer et al.
CAG8209	KL228 rpoD::Tn10 (63')	Singer et al.
18469	gua-26::Tn10 (54.50')	Singer et al.
18481	zff-208::Tn10 (55.00')	Singer et al.
18480	nadB51::Tn10 (55.75')	Singer et al.
12158	pheA18::Tn10 (56.75')	Singer et al.

leucine was called SGL medium. Strains unable to grow on this medium were termed SGL⁻.

II B-2 Luria Broth

1.0% bactotryptone, 0.5% yeast extract, 0.5% NaCl

To make plates, 1.8% bactoagar was added to the medium before autoclaving.

II B-3 LB Citrate Medium

Luria broth with 0.5 M sodium citrate.

II B-4 Phage Agar Plate for P1 Plate Lysates

1.0% bactotryptone, 0.1% yeast extract, 0.8% NaCl, 1.7% bactoagar.

II B-5 R-Top Agar for P1 Plate Lysates

1.0% bactotryptone, 0.1% yeast extract, 0.8% NaCl, 0.6% bactoagar. Sterilized Ca^{++} and glucose were added to the medium just before use to the final concentrations of 2 mM and 0.1% respectively.

II C Buffers and Solutions:

MC (magnesium sulphate and calcium chloride) buffer used in P1 transduction:

0.100 M $MgSO_4$

0.005 M CaCl2

Phosphate buffer used in whole cell L-SD assay: 50 mM, pH7.5

Glycylglycine buffer used in cell-extracts L-SD assay:

50 mM glycylglycine

pH was adjusted to 8.0 with NaOH

X-gal solution used for selection of Lac[+] clones:

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

Tris acetate and EDTA (TAE) buffer for DNA agarose gel electrophoresis:

Working solution:

0.04 M Tris-acetate, 0.001 M EDTA

Concentrated stock solution (50x):

Per liter:

Tris base	242 g
glacial acetic acid	57.1 ml
0.5 M EDTA (pH8.0)	100 ml

TE (Tris and EDTA used for dissolving DNA (pH 8.0):

10 mM Tris.HCl (pH8.0)

1 mM EDTA (pH 8.0)

Cracking Buffer used for lysing cells (checking plasmids):

Per 100 ml:

2 M Tris.HCl (pH8.0)	2.5 ml
0.2 M EDTA	0.4 ml
10% SDS	10 ml
Sucrose	13.7 g

bromophenol blue   10 mg

Sterilized by filtration.

SSC (sodium chloride and sodium citrate) solution used for

hybridization:

    20x stock solution, per liter:

            NaCl   175.3 g

            Sodium citrate 2H$_2$O   88.2 g

            distilled water   add up to a liter

pH was adjusted to pH 7.0 with concentrated HCl.

II D Antibiotics

    Antibiotics were added to all kinds of media at the

concentration of: kanamycin (Km) 80 ug/ml, ampicillin (Ap) 50

ug/ml, chlororamphenicol (Cm) 20 ug/ml, tetracycline (Tc) 15

ug/ml, streptomycin 100 ug/ml. Specific antibiotic was always

added to the culture of a given strain.

II E Enzyme Assays

II E-1 L-Serine Deaminase Assay
II E-1a Whole Cell L-SD Assay

    The method used was a slight modification of the method

of Pardee and Prestidge by Isenberg and Newman (1974). L-

serine deaminase was determined in the following steps: log-

phase cells were chilled quickly in ice, harvested at 4°C, and

resuspended in phosphate buffer (pH 7.5) to a cell density of OD = 100 at 540 nm. The reaction mixture contained: 0.3 ml of the resuspended cells, 0.02 ml toluene, and 0.1 ml L-serine of 20 mg/ml. The reaction was carried out in 37°C water bath. To stop the reaction, 0.9 ml of 0.0025% 2,4-dinitrophenylhydrazine (dissolved in 4.1% HCl) was added to the reaction mixture, and was allowed to stand at room temperature for 20 minutes. After adding 1.7 ml of 10% NaOH, L-SD was determined by measuring keto acid formation with a Klett Summerson colorimeter at 540 nm. Sodium pyruvate was used as a standard.

One unit of L-SD was the amount of enzyme which catalysed the formation of 1 umol of pyruvate per 0.1 ml cells in 35 min at 37°C.

II E-1b Crude Cell Extracts L-SD Assay

L-SD activity in crude cell extracts was assayed according to the method described by Newman and Walker (1985b).

Preparation of crude-cell extracts: cells were grown in glucose minimal medium overnight at 37°C with shaking. The overnight cultures were subcultured into the same medium and grown to mid-log phase. The log-phase cells were harvested and weighed carefully, and then one gram of cells (wet weight) was suspended in 5 ml of 0.05 M glycylglycine buffer of pH 8.0. Cell suspensions were frozen at -20°C overnight, and

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then the cells were broken with a Sonifer Cell Disruptor #350 (from Branson Sonic Power Co.). After centrifugation at 12,000 r.p.m. for 20 minutes, supernants were collected and were ready to be used.

Reaction mixture: the reaction mixture was composed of 30 ul 10^{-3} M Fe^{+++}, 30 ul 10^{-2} M DTT, 10 ul L-serine of 100 mg/ml, certain amounts of cell extracts, and 0.05 M glycylglycine buffer was added to the mixture to the total volume of 170 ul.

One unit of L-SD was taken to be the amount of enzyme that catalysed the formation of 1 umol of pyruvate in 40 min at 37°C per ug of protein.

II E-2 β-Galactosidase Assay

β-Galactosidase activity was assayed in whole cells by the method of Miller (1972) and expressed in his units.

II F Protein Concentration

Protein content was quantified by Lowry's Protein Assay (Lowry et al, 1951). Bovine serum albumin was used as standard.

II G P1-Mediated Transduction

P1-mediated transduction was performed by the method of

Miller (1972). Overnight cell cultures grown in LB medium at 37°C were harvested and suspended in MC buffer of the same or half volume. Different dilutions of P1 lysates were mixed with 0.1 ml of cells and allowed to stand at 30°C for half an hour. 1 ml of LB citrate was then added to stop the P1 absorption and the mixture was incubated at 37°C for one hour to allow the expression of the transduced phenotype. Transductants were selected on selective agar plates after the transduction mixture was plated and incubated at 37°C for certain times.

II H Mapping by Transduction

Accurate mapping of strain MEW84 was carried out by P1-mediated transduction with the Singer Kits (Singer et al.,1989), containing Tn10 insertions at nearby loci.

II I Isolation of Plasmid

Plasmids larger than 10 Kb were isolated by the alkaline lysis method according to Maniatis et al (1982). Plasmids smaller than 10 Kb were extracted either by the above alkaline lysis method or by the boiling method (Maniatis et al., 1982).

II J Transformation

Transformation was done according to Maniatis et al.(1982) with minor modification.

The overnight culture grown in LB broth at 37°C was subcultured in the same medium to continue to grow to a density of 5×10^7 cells/ml. Cells were then chilled in ice for 30 minutes, and collected by centrifugation at 4°C. Half of the original volume of 0.1 M $MgCl_2$ (kept cold) was added to wash the cells once and the cells were resuspended in 1/20 of the original volume of cold $CaCl_2$ (0.1 M). The cell-Ca^{++} suspension was allowed to sit in ice for 30 minutes. 0.1 ml-0.2 ml of the cells were mixed with 0.1 ul -1 ul plasmids and continued to be kept cold in ice for 30 min. Then the mixture was given a heat-shock at 42°C for 2 minutes, after which the mixture was immediately placed in ice for another 30 minutes. 1 ml LB liquid was added, and the mixture was transferred to a 37°C water bath for 1 hr. Transformants were selected on selective plates.

II L Cloning of sda84 Gene from a Mu Replicon

The usual way to clone a gene involves many steps: genomic DNA has to be isolated, then digested with restriction enzymes and ligated in an adequately digested vector DNA. The vector must be able to replicate in the host cells. Eventually vector DNA bearing the cloned DNA fragment is introduced into host cells. This process is very laborious

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and time consuming. With the construction of the _in vivo_ miniMu cloning system, gene cloning is greatly facilitated.

The miniMu cloning system was constructed by incorporating a plasmid replicon inside a miniMu element, which is deleted for most of its lytic functions but retains its sites for transposition (Pato, 1989 and Croisman _et al_., 1984). Cells lysogenized for the Mu-replicon bacteriophage and a complementing Mucts prophage (which has a temperature sensitive repressor) can be heated to induce transposition. Transposition of this miniMu replicon can occur at many sites on the host chromosomal DNA. If the desired DNA sequence is flanked by two copies of miniMu replicon oriented in the same direction, then the Mu "headful" packaging mechanism might occur to pack the DNA up to 39 Kb from the left side of one of the miniMu replicon through the chromosomal DNA to the second miniMu replicon on the other side. Upon infection to a Mu lysogen cell, recombination can occur between the Mu homologous regions to form a plasmid carrying the host DNA sequence (Groisman _et al_., 1984). Up to now, miniMu replicons having different sizes, selectable drug resistant markers, plasmid replicons and _lac_ fusing elements etc., have been developed (Groisman _et al_., 1984 and Groisman _et al_.,1986). These genetic tools greatly facilitate the cloning procedure and allow the cloning of genomic DNA from 17 Kb to 31 Kb, depending on the Mu replicon used (Groisman _et al_., 1986).

Preparation of Mu replicons: strain Xph43 _sda84_[+] was grow

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overnight in LB medium at 28°C with the supplementation of chlororamphenicol. The overnight culture was diluted 1,000 times into 20 ml LB (in 250 ml flask) with 10 mM MgCl$_2$, and allowed to grow at 28°C to a density about 10^7 cells/ml. The log-phase xph43 $\underline{sda84}^+$ cells were shifted to grow at 42°C to induce transposition and bacteriophage replication (Groisman et al., 1984 and Groisman et al., 1986). The lysates were extracted with chloroform.

Preparation of recipient cells: strain MEW84 $\underline{sda84}^-$ Cm^S was grown in glucose minimal medium overnight at 37° C, with the presence of MgCl$_2$ and CaCl$_2$ in the medium at the concentration of 0.06% and 0.03% respectively. Cells were centrifuged and resuspended in MC buffer.

Infection of MEW84 cells with Mu lysates: the lysates formed were used to transduce strain MEW84 $\underline{sda84}^-$ Cm^S the ability to grow on SGL plate and chlororamphenicol resistance: different amounts of Mu lysates were added to 0.1 ml of the resuspended cells and allowed to absorb at 30°C for about 30 minutes. The absorption mixture was diluted 10 times with LB liquid, and incubated at 28°C with shaking for 2 hrs. Cells were collected by centrifugation, washed 3 times with NIV solution, and plated on SGL plates with the supplementation of both Km and Cm, and incubated at 28°C.

Plasmids pM10 and pM1 were isolated from two colonies growing on SGL plates after two days incubation at 28°C.

The non-vector DNA on pM10 and pM1 were subcloned to

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pBR322 to form pMEP13, pMBH28 and pMBH4 by digesting with different enzymes and ligating to properly cut vector pBR322. Digestion with restriction enzymes and ligation were carried out by the procedures of Maniatis et al (1982). SGL$^+$ colonies were selected at each step. Details of individual experiments are given in the text.

II M Gel-Electrophoresis

DNA agarose gel electrophoresis was carried out by the method described by Maniatis et al. (1982). Usually the agarose concentration used was between the range of 0.7% - 0.9%.

II N Chromosomal DNA Isolation

Chromosomal DNA of strains MEW84 and MEW1 was isolated as described by Silhavy et al.(1984).

II O Hybridization

Hybridization was done according to Southern (1975) with some modifications. The probe used was the 6 Kb HindIII-KpnI fragment isolated from pMEP13 and was labelled with [α-^{32}P]dATP by the random-primer method described by the supplier (Molecular Biology Boehringer Mannheim).

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Isolation of the DNA fragment to be used as probe: plasmid pMEP-13 was totally digested with restriction enzymes KpnI and HindIII, the DNA fragments were separated on a 0.8% agarose gel by electrophoresis using lambda HindIII fragments as a maker. The KpnI-HindIII fragment of pMEP-13 was cut from the gel and cleaned with the Gene Clean kit supplied by Bio/Can Scientific inc., Toronto, and redissolved in TE (pH 8.0) buffer.

Preparation of the DNA to be hybridized: chromosomal DNA from both strains MEW1 and MEW84 were digested completely with restriction enzyme pairs KpnI-HindIII and KpnI-SalI respectively. Restriction fragments were separated on a 0.8% agarose gel by electrophoresis. To denature the DNA, the gel was placed in 0.5 M NaOH for 20 minutes, washed 3 times with distilled water, and soaked in 0.5 M Tris-HCl (pH 8.0) for 20 minutes and washed 3 times with distilled water.

Dehydration of the agarose gel: the gel was transferred with care to 2 sheets of Whatman 3 MM paper which was cut to 2 cm longer and wider than the gel. The gel, together with the Whatman paper, was covered with plastic wrap and dried for 2 hrs at 80°C with the Dual Temperature Slab Gel Dryer Model 1125B, supplied by Bio-Rao Laboratories. The dehydrated gel was placed in a heat-resistant cooking pouch sealed on three edges.

Prehybridization: first, the following solutions were mixed in the order given:

distilled H$_2$O	5.7 ml
20x SSC	3 ml
1% NaPPi	1 ml (autoclaved)
5 mg/ml Heparin	100 ul
20 mg/ml tRNA	100 ul
10% SDS	0.2 ml (autoclaved)

Then, the above mixture was added to the bag, with the bubbles all carefully squeezed out from the bag. After being sealed completely on all its edges, the bag was immersed in a 50°C water bath for 3 hrs.

Labelling the probe: labelling materials used were all included in the Random Primed DNA Labelling Kit purchased from Molecular Biology Boehringer Mannheim, except the [α-^{32}P] was supplied by ICN inc.. The probe DNA was labelled by mixing the DNA with the following solutions:

25 ng denatured probe DNA

3 ul dCTP,dGTP, dTTP

2 ul reaction mixture

1 ul ≙ 50 u Ci [α-^{32}P] dATP, 3000 Ci/mmol

distilled water was added to make up to 19 ul

1 ul Klenow enzyme

This labelling mixture was incubated at 37°C for 30 minutes, and the reaction was stopped by heating to 65°C for 10 min.

Hybridization: the bag containing the prehybridization solution was cut at a corner and the labelling mixture was pipetted into the bag. After bubbles were squeezed out, the

bag was sealed again tightly. Hybridization was carried out at 50°C overnight.

Posthybridization wash: washing solution was made up in the order of:

20x SSC	100 ml
1% NaPPi	100 ml
10% SDS	20 ml
distilled H$_2$O	to a final volume of 2 L

Washing process was carried out with precautions against radio activity. The gel was first washed three times, each for 1 min at room temperature, then 10 min at 50°C, and finally 1 hr at room temperature with gentle shaking.

II P Testing for Growth

II P-1 Amino Acids used to test Their Effects on Cell Growth

Amino acids were used at the following concentrations unless otherwise stated: L-aspartic acid, 100 ug/ml; glycine, 100 ug/ml; L-histidine, 50 ug/ml; L-leucine, 50 ug/ml; L-lysine, 50 ug/ml; L-methionine, 50 ug/ml; L-proline, 50 ug/ml; L-serine, 100 ug/ml; L-threonine, 80 ug/ml and L-tryptophan, 50 ug/ml.

II P-2 Comparing the Cell Growth by Klett-Colorimeter Readings

Each strain was cultured in aliquots in minimal medium

at 37°C with shaking, with or without the supplementation of certain materials tested. The growth of strains in different media were compared according to the cell density as judged by the Klett readings at about 420 nm (purple filter) unless otherwise noted.

II P-3 Growth Rate Testing

The overnight cultures of cells grown in minimal medium were subcultured in 250 ml side-arm flasks containing 20 ml of corresponding medium to a cell density of OD value = 20 at about 420 nm. To ensure good aeration, all cultures were grown with vigorous shaking in a Gyrotary water bath shaker (New Brunswick Scientific model #G76). Optical density was determined at a time interval of 30 minutes. The doubling time was determined from plots of optical density against time.

III Results

Isolation of MEW84 - A Mutant That Affects Post-Translational Modification of L-Serine Deaminase in E. coli K-12

Wild type E. coli K-12 can not use L-serine as its sole carbon source, but it can use it when glycine and leucine are also added to the growth medium (Newman et al., 1982b). The experiments in this thesis dealt with a mutant strain of E. coli K-12, MEW84, which is unable to grow with the mixture of serine, glycine and leucine as carbon sources. This mutant was isolated by Su Hongsheng, a Ph.D. student in this lab, by λplacmu insertion, and screening for antibiotic-resistant SGL⁻ colonies (Bremer et al., 1984 and Bremer et al., 1985). The mutation from such a strain was transduced to our parent strain MEW1, resulting in strain MEW84 carrying a single insertion (Su Hongsheng, personal communication). The gene into which λplacmu is inserted in strain MEW84 is referred to in this text as sda84.

Details of the isolation of this mutant can be found in Fig.4 (provide by Su Hongsheng with modifications).

When I began working with this strain, it was known that MEW84 showed very low L-SD activity in the whole cell assay. However the L-SD activity in crude extracts of this strain could be activated to some extent on incubation with iron and DTT. This strain needed thiamine to grow in glucose minimal medium (Su Hongsheng, personal communication). The sda84 gene

MEW1

HELPER PHAGE
λplacMu507
(MuA$^+$B$^+$)   λplacMu9

CELLS WASHED

GROWN IN GLUCOSE MEDIUM
FOR 2 HRS TO ALLOW EXPRESSION
OF KANAMYCIN RESISTANCE

SUBCULTURED IN SGL MEDIUM
FOR 1.5HRS

Ap WAS ADDED TO KILL OFF
SGL$^+$ CELLS

PLATE ON LB Km PLATES

SCREEN FOR SGL$^-$ COLONIES
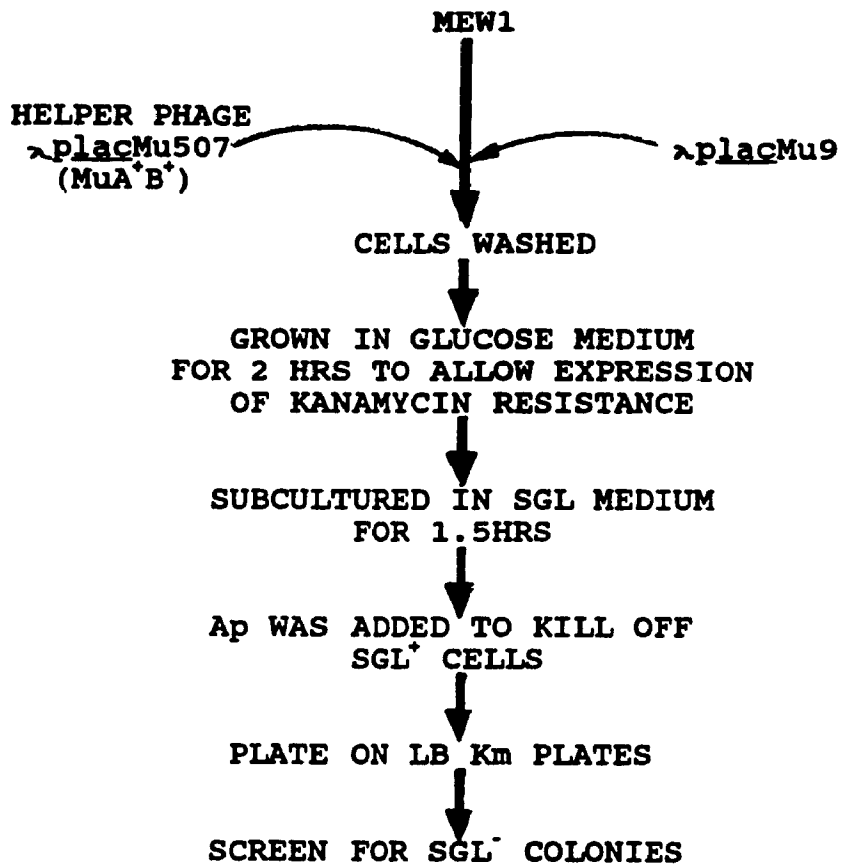
Fig. 4   ISOLATION OF MEW84
(Su Hongsheng, with modifications)

was tentatively located between 45-60 minutes on the E. coli genomic map (Lin Rongtuan, personal communication). To verify these observations, some of these experiments were repeated.

Part 1   Genetic Study of Strain MEW84

III A   Initial Characterization of Strain MEW84

III A-1   L-Serine Deaminase Activity of Strain MEW84

Previously described SGL⁻ strains have made little or no L-serine deaminase 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). Su has showed that strain MEW84 was also deficient in L-serine deaminase under both conditions (Su, personal communication). To verify this, MEW84 was assayed for L-SD activity in whole cell grown in glucose minimal medium with or without the addition of glycine and leucine.   Strain MEW84 showed no significant activity in cells grown in glucose minimal-medium, and very little when grown with the inducers (Table 2).

III A-2   β-Galactosidase Activity of Strain MEW84

The construction of strain MEW84 suggests that it should carry an insert of lacZ in the sda84 gene. If the lacZ insert is in the correct orientation to be transcribed by the sda84 promoter, MEW84 cells grown in glucose-minimal medium might synthesize β-galactosidase.   In fact, they showed a β-

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Table 2. L-SD Activity and β-Galactosidase Activity of
Strain MEW84

Strain	L-SD Activity in Minimal Medium	with Gly & Leu	*β-Gal Activity in Minimal Medium
MEW1	17	82	-
MEW84	1.3	9.6	1300

Cells taken from LB plates were inoculated into glucose minimal medium with or without the addition of 300 ug/ml glycine (Gly) and 300 ug/ml leucine (Leu), and incubated overnight in a 37°C shaker. The overnight cultures were subcultured into the corresponding media. Log phase cells were used for assaying the activities. L-SD (L-serine deaminase) activity is expressed in nmol pyruvate produced per 0.1 ml cells per 35 minutes at 37°C. β-galactosidase activity is expressed in Miller's units.

*β-Gal: β-Galactosidase.

Values are averages of two to three different experiments.

galactosidase activity of 1300 units in glucose minimal medium, a similar result as observed by Su (Su, personal communication), indicating that λplacmu was inserted in the right direction, probably under the control of the sda84 promoter (Table 2).

III A-3   L-SD Activity of MEW84 in Crude Cell Extracts

Previously isolated SGL⁻ mutants MEW128 and MEW191 were deficient in L-SD in the whole cell assay, but their L-SD could be activated with iron and dithiothreitol (DTT) in vitro (Newman et al., 1985a and Newman et al., 1985b). The fact that L-SD could be activated in these strains, but not in the strain (MEW22) with an insertion in the structural gene (sdaA), led to the hypothesis that L-SD was made in an inactive form, and activated in the cell (Newman et al., 1985b). Mutations in strains MEW191 and MEW128 were therefore ascribed to the activating system. To see whether strain MEW84 might also be an activation mutant, L-SD activity was assayed in the crude cell extract with or without the addition of iron and DTT.

The extract incubated with iron and DTT showed much more L-SD activity than the extract without iron and DTT (Fig. 5). Same result was previously obtained by Su (Su, personal communication). This distinguished strain MEW84 from strain MEW22 - the structural gene mutant isolated in the same way, although the L-SD activity of the mutant MEW84 after

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Fig. A   sda84 gene interupted by λplacMu insertion
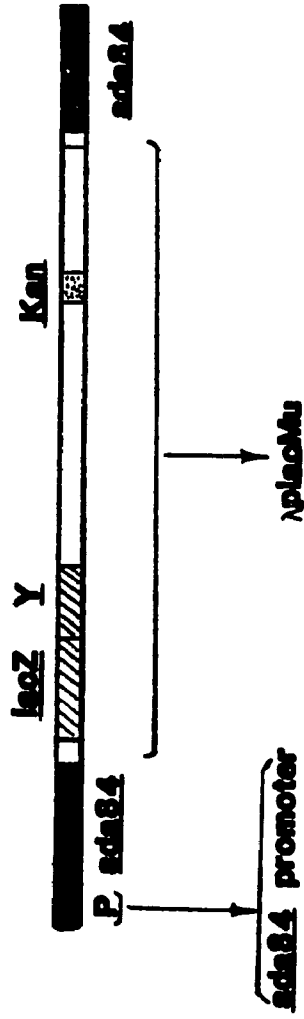
lacZ gene is transcribed from sda84 gene promoter

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Fig. 5. Activation of L-SD by Iron and DTT.

Cells were grown overnight in glucose minimal medium at
37°C, and subcultured into glucose minimal medium
supplemented with 300 ug/ml of glycine and leucine each.
Log phase cells were extracted according to the method
described in Method section. Samples (2 ul) of crude
extracts (approximately 25 mg of protein per ml) were
incubated with or without Fe^{+++} (10^{-3} M) and DTT (10^{-2} M)
for the time indicated.

Curves are drawn according to the data obtained from a
single experiment. Similar results were previously obtained
by Su Hong-sheng(Su, personal communication).

Fig. 5

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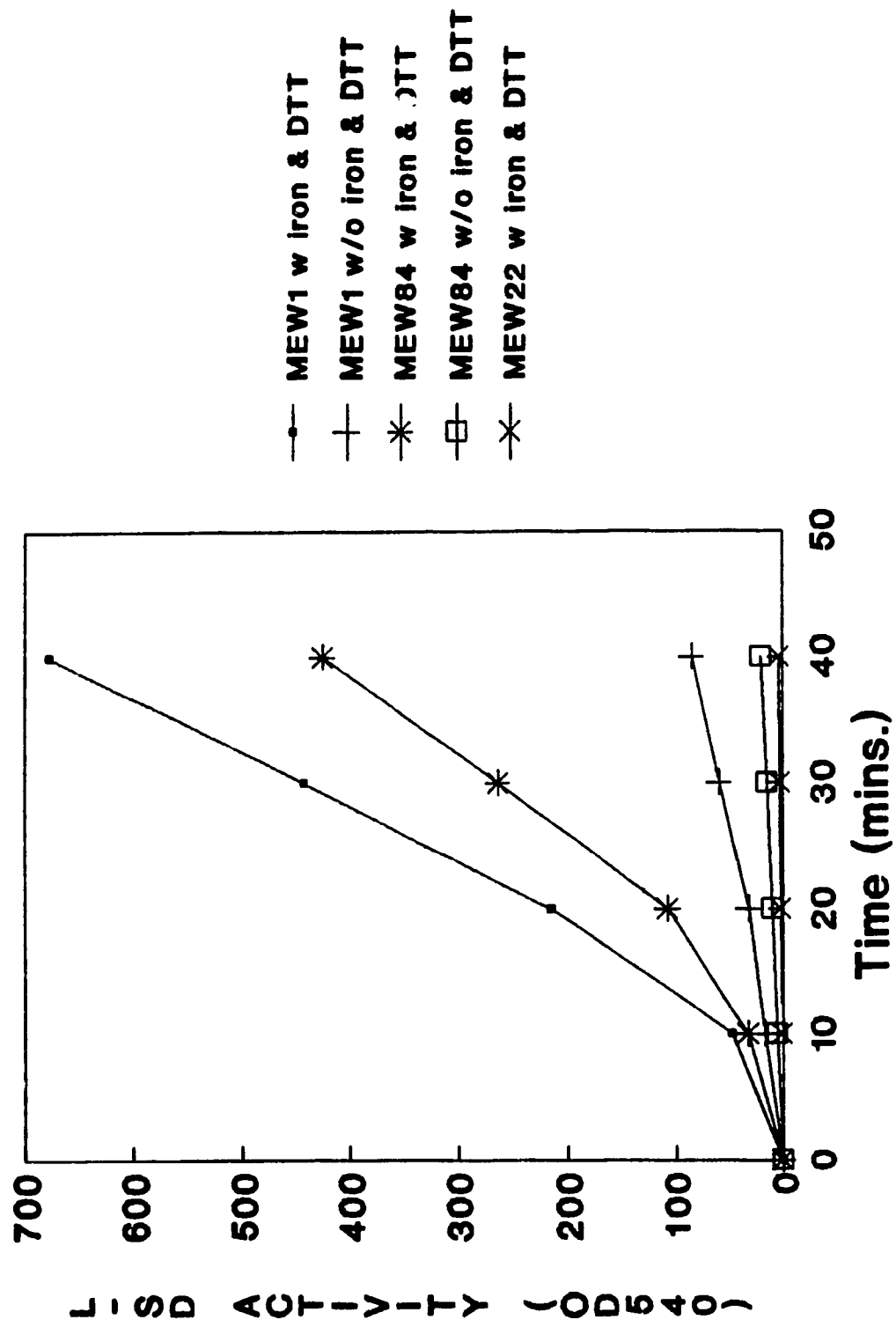
activation was a little lower than the parent strain (Fig. 5).

This, together with the former work, seems to define the existence of an activating system of L-SD, i.e., L-SD is produced in the cell in an inactive form, the inactive protein can be activated by an as yet unknown enzyme reaction(s) which requires the products of functional sda84, sda128 and sda191 genes. The inactive form can also be activated chemically by in vitro incubation with iron and DTT (Newman et al., 1985b).

III A-4 Demonstration of the Requirement of sda84[+] Gene for Activating L-SD

To make sure that the sda84[+] gene is really involved in the activation system, a plasmid carrying the structural gene for L-SD (sdaA) (constructed by Su) was transformed into strains CH22 (sdaA⁻ sda84[+]) and CH22/sda84 (sdaA⁻sda84⁻) and L-SD activities of these strains were assayed. The results in Table 3 show that L-SD activity in strain CH22 carrying the plasmid is much higher than that in double mutant CH22/sda84 carrying the same plasmid. This indicates that in strain CH22, which has the wild type sda84 gene, the L-SD protein is activated, while in CH22/sda84, sda84 gene is mutated and much less active enzyme is produced.

III A-5 Demonstration of a Thiamine-Requirement in Strain MEW84

Table 3. L-SD Activity of Strains Carrying Plasmid psdaA

Strain	Plasmid	L-SD
CH22	none	1
CH22	psdaA	173
CH22/sda84	none	1
CH22/sda84	psdaA	38

Cells were grown in glucose minimal medium overnight in a 37°C shaker, and subcultured into the same medium. Cells were grown to mid-log phase, centrifuged, and resuspended at an optical density of 100 Klett units (#54 filter). L-SD activity is expressed as in Table 2.

Values presented in this Table are from a single experiment.

Su Hongsheng had noticed that strain MEW84 could not grow in glucose minimal medium unless a little amount of LB liquid or thiamine was provided. When I started to work with this strain, I observed that when grown in glucose minimal medium, strain MEW84 grew well on plates supplemented with 1 ug/ml thiamine, but did not grow well on plates without thiamine. To demonstrate clearly that strain MEW84 required thiamine for growth, MEW84 cells grown on LB plates were suspended in minimal medium, diluted, so that about 500-1000 cells were inoculated in or plated on glucose minimal medium with or without the addition of thiamine. Strain MEW84 grew well with thiamine, but not without thiamine.

III A-6 Summary: A Comparison of Strains MEW84, MEW128 and MEW191

With the discovery of strain MEW84, three activation mutants have been isolated. They are similar in that they all have very low L-SD activities in the whole cell assay; their L-SD activities can be activated by iron and DTT in vitro; they all need thiamine to grow in minimal medium. Strains MEW128 and MEW191 have already been differentiated by map locations. To show that strain MEW84 carried a mutation in a gene different from all of the others, I determined its map location, which indeed differs from the above two.

III B Location of the sda84 Gene

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Mapping of the sda84 gene was done by conjugation and transduction. The conjugation experiment was carried out by Lin Rongtuan, using five Hfr strains of the Singer kit (Singer et al., 1989): CAG5051, CAG5052a, CAG5054, CAG5055 and CAG8209 as donors and a streptomycin resistant derivative of strain MEW84 as recipient. The result suggested that sda84 gene might locate between 45-60 minutes on the E. coli genomic map.

The mutation was located more precisely by P1-mediated transduction. P1 phage was grown on a series of strains of the Singer kit (Singer et al., 1989) with Tn10 insertion from 45 minutes to 60 minutes, and used to transduce tetracycline resistance to strain MEW84. The wild type allele of the inserted gene in strain MEW84 was 96% cotransducible with strain 18481 and 35% with 18480. It showed no cotransduction with most of the Tn10 insertions (Table 4). Thus the map location of gene sda84 could be determined according to the formula $X = 1(1- \sqrt[3]{f})$ (Low, 1987), where X is the distance between two genes, 1 is the length of the P1 transducing fragment (two minutes) and f is the frequency of SGL^+ transductants. Using this calculation, sda84 was located around 55 minutes. It was clear that this map location was different from the map positions of the mutation in MEW128 which is at about 15-17 minutes, and the mutation in MEW191 at 86.5 minute (Dumont, 1985).

Examination of the new edition of the E. coli genome

Table 4. Location of sda84 Gene by P1 Mediated Transduction

Donor Strain	Tn10 Map Position	Total	MEW84 Transductants Tc^R	% SGL$^+$ Km^S X-Gal (White)
18469	54.50'	300		0
18481	55.00'	331		96
18480	55.75'	330		35
12158	56.75'	300		0

P1 phage was grown on each of the donor strains. Tn10 was transduced from the donor strains to MEW84 mutant, selecting for tetracycline resistance. Tc resistant transductants were then screened for growth on SGL medium. Linkage between Tn10 and the sda84 mutation was determined by the percentage of SGL$^+$, Km^S, and X-gal white transductants.

(Bachmann, 1990) did not suggest a previously described locus which might be identical to sda84. I conclude that the sda84 gene is a newly discovered gene different from either sda128 or sda191.

III C Cloning of the MEW84 Gene

To understand the activating mechanism of L-serine deaminase, I wished to clone and sequence the sda84 gene, and analyze the sequence with the GenBank and NBRF protein bank to try to deduce something about its function and relatedness to other proteins.

III C-1 In vivo Cloning of sda84 Gene and Determining of Its Restriction Map

Cloning of this gene was conducted by the in vivo cloning system of Groisman (Groisman et al., 1984 and Groisman et al., 1986). The Mu replicon was produced by incubating strain xph43 sda84⁺ at 42°C to induce transposition and bacteriophage replication. The lysates formed were used to transduce to strain MEW84. If one of the miniMu replicons thus formed carried the wild-type allele of sda84, it should be able to transduce to strain MEW84 the ability to grow in SGL medium and chloramphenicol resistance. Transductants were selected on SGL plates supplemented with both kanamycin and chloramphenicol. Two plasmids with the mu replicon and E.

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coli DNA complementing the sda84 mutation were isolated from such transductants, and they were named pM1 and pM10. However, this complementation could only be observed at 28°C.

Digestion with restriction enzymes showed that the nonvector DNA of each plasmid could not be cut with BamHI or EcoRI. Both showed a single HindIII site and a single SalI site. The size of the fragments generated by a variety of enzymes suggest the map shown in Fig. 6. The two plasmids seemed to share a common region of 6-7 Kb but plasmid pM1 carried 3-4 Kb more E. coli DNA at the HindIII end than did pM10 (Fig. 6).

These clones were characterized by their ability to restore growth on SGL medium to MEW84. If they really complement the sda84 gene, they should also restore the L-SD activity of MEW84. To test this, the plasmids were retransformed into MEW84, and L-SD activity was assayed in cells grown both in glucose minimal medium and under inducing conditions, i.e., with the addition of glycine and leucine.

In fact, MEW84 carrying either of these plasmids showed an increase in L-SD to almost the level seen in the parent strain in both conditions (Table 5).

I concluded therefore that plasmids pM1 and pM10 carried a gene which complemented the sda84 mutation.

III C-2 Subcloning of the Putative sda84 Gene

I wished to sequence this gene. However, the nonvector

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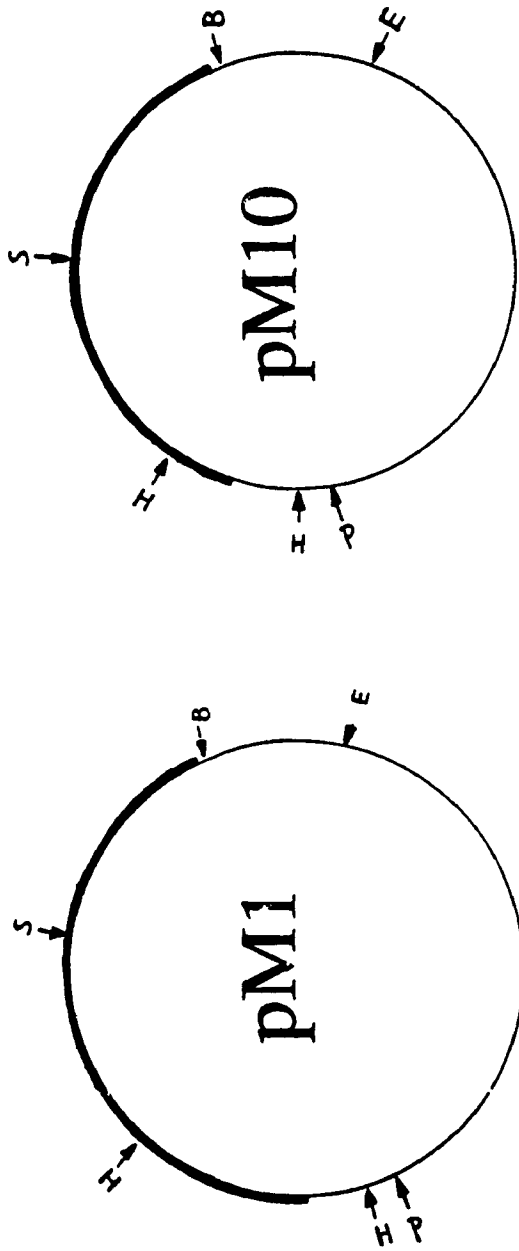
Fig. 6   MiniMu Replicons Obtained by tr; in vivo miniMu
           Cloning Method

Mu DNA: ———— ; non-vector DNA ▬▬▬
H: HindIII; B: BamHI; S: SalI; E: EcoRI and P: PstI.
Enzymes that have no cutting site on the non-vector DNA: PstI
EcoRI and BamHI.
The figure is not drawn to scale. The insert DNA on pM1 is
about 11-12 Kb long and that on pM10 is about 7-8 Kb long.
In both cases the insert DNA fragment between the HindIII
site and the vector BamHI site is about 7.5 Kb long and the
SalI site is located around the middle of this fragment.

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Table 5. L-SD Activity of Strain MEW84 Harbouring pM1 and pM10, and pMEP-13

Host	Plasmid	L-SD Activity in Minimal Medium	With Gly & Leu
MEW1	NONE	17	82
MEW84	NONE	1.3	9.6
MEW84	pM1	14	70
MEW84	pM10	15	70
MEW84	pMEP13	15	72

Cells were grown in glucose minimal medium and in glucose minimal medium supplemented with 300 ug/ml leucine (leu) and 300 ug/ml glycine (gly) overnight in a 37°C shaker. Subcultures were made into the corresponding media. L-SD activity was assayed as in Table 2.

Values for MEW1 and MEW84 without harbouring plasmids and for MEW84 harbouring pM10 represent averages of two different experiments. Values for MEW84 harbouring plasmid pM1 and pMEP-13 are from a single experiment.

DNA fragments on the miniMu replicons were quite long for sequencing, so several attempts at subcloning were made.

III C-2a Attempts to Subclone Fragments From Two-Enzyme Total Digests into pBR322

Total digests of pM10 and pBR322 were carried out with the following pairs of restriction enzymes: 1: EcoRI and PstI; 2: HindIII and BamHI; 3: PstI and SalI; and 4: SalI and BamHI. Each pM10 digest, mixed with the corresponding pBR322 digest, was ligated and used to transform to MEW84. Transformants were selected in two ways - on LB, and more directly, on SGL plates, in both cases with appropriate antibiotics. Plates were incubated at 28°C.

Colonies arose on LB plates from all four experiments within 24 hours. However, on minimal medium, it took 48 hours to see colonies from experiments 1 and 2, and no transformants were ever seen with enzyme mixtures of experiments 3 and 4.

The colonies on LB plates must have carried some plasmids as judged by their antibiotic resistance. If these plasmids carried _sda84_ gene, they should have been able to grow on SGL plates with appropriate antibiotics. This was in fact true for some LB transformants of experiment 1 and 2.

Plasmid DNA was extracted from these transformants, and characterized in two ways: 1) by restriction analysis, and 2) by retransformation into MEW84 and assaying L-SD activity, and growth on SGL plates, in the resulting transformants.

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The EcoRI- PstI subclones from experiment 1, named pMEP-13 (Fig.7, left), did transfer ability to grow on SGL within 48 hours, and to make an L-SD which was active _in vivo_ (Table 5, lane 5). This was not true for the HindIII-BamHI subclones from experiment 2, which were called pMB.

As shown in Fig. 6, both the EcoRI site and the PstI site were on the Mu DNA (the non-vector DNA did not have these two sites). The subcloned plasmid obtained from experiment 1 had the entire insert DNA of the pM10 plasmid. In the case of the experiment 2, about 1 Kb of the non-vector DNA at the HindIII end was lost on total digestion by incubation with HindIII. Since this was the only piece of DNA missing in pMB (at the other end, BamHI cut into the Mu DNA), I concluded that the loss of complementation in pMB was due to the loss of the HindIII site on the cloned chromosomal DNA, in other words, this HindIII site was essential for the L-SD activity.

A unique SalI site was situated in the middle of the cloned pM10 insert. The non-vector DNA between this SalI site and the PstI site on the Mu DNA was about 4-5 Kb long including the HindIII site. The effort to subclone the non-vector DNA with the enzyme pair PstI and SalI suggested that the SalI site was also indispensable, since no functional subclone was obtained.

III C-2b **Attempt to Subclone From a Mixed Partial/Total Digest**

Fig. 7   Restriction Map of the Subclones from pM10 and pM1

pBR322 DNA:▨▨▨▨; Mu DNA:━━━; non-vector DNA:▄▄▄▄.
H: HindIII; E: EcoRI; P: PstI; C: ClaI; K: KpnI; S: SalI
and B: BamHI.

*Specific cutting site for the non-vector DNA on pMEP-13
and pMBH-28; ** Specific cutting site for the non-vector DNA
on pMB-4.

Enzymes having no restriction site on the non-vector DNA
of pMEP-13 and pMBH-28: XhoI, XbaI, StuI, SmaI, ScaI, EcoRI,
BamHI and PstI.

The figure is not drawn to scale. The non-vector DNA on
pM1 was about 11-12 Kb long and that on pM10 was about 7-8 Kb.

The sizes of the key fragments of the insert DNA on
pMEP13 and pMBH28 are: H-S: 3.5-4.0 Kb, S-K: 3.0-3.5 Kb.

The sizes of the key fragments of the insert DNA on pMBH4
are: K-H: 4.0 kb and H-S: 3.5-4.0 Kb.

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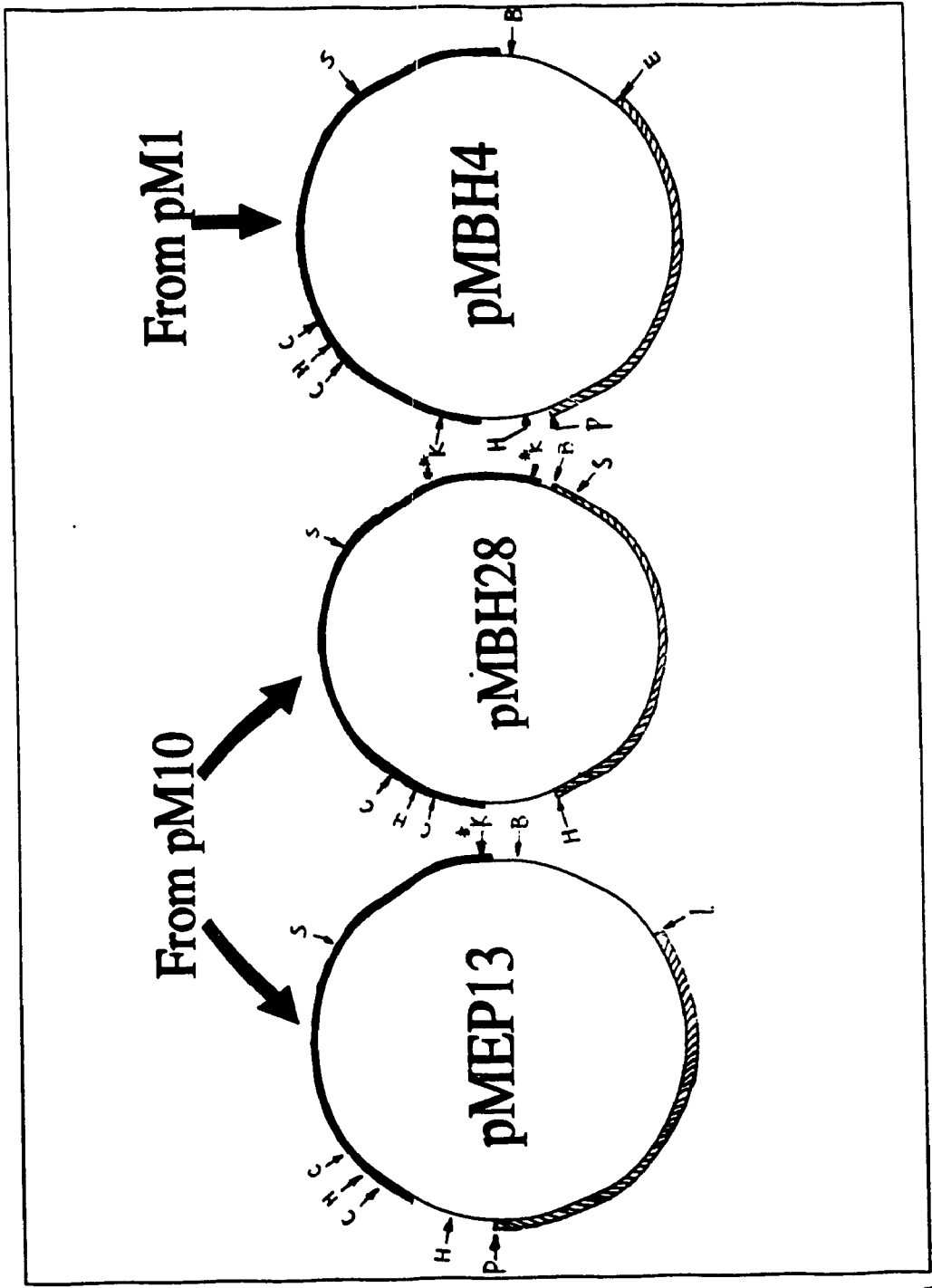
In order to get rid of as much as possible of the Mu DNA in the subclone, I thought that the best enzymes for this purpose would be HindIII and BamHI, whose restriction sites were at the ends of the Mu DNA (Fig. 6). To try to keep the HindIII site on the non-vector DNA intact, I digested pM10 partially with HindIII and totally with BamHI, and ligated with HindIII and BamHI totally digested pBR322. The ligation mixture was transformed into MEW84. Transformants were selected in the same way as stated in III C-2a.

These transformants were able to grow with SGL within 48 hours at 28°C and overnight at 37°C. The plasmid obtained from the transformants, named pMBH-28 (Fig. 7, middle), showed a correct restriction map and had the same ability to restore L-SD activity to MEW84 as pMEP-13 (Table 6). Using the same method as in III A-5, I also showed that this plasmid restored the thiamine independence to strain MEW84.

Further subclone of the insert in pMBH-28 was attempted by digesting pMBH-28 completely with SalI. This would delete the DNA fragment between the SalI site on pBR322 and the SalI site on the non-vector DNA (refer to Fig. 7, middle). I ligated the two SalI sites together. The subclone obtained on LB plates had the correct restriction pattern but totally lost its ability to complement strain MEW84. This again indicated the importance of the sequence between the SalI site and the vector seqence.

The same strategy was used to transfer the chromosomal

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Fig. c   Subclones of pM10
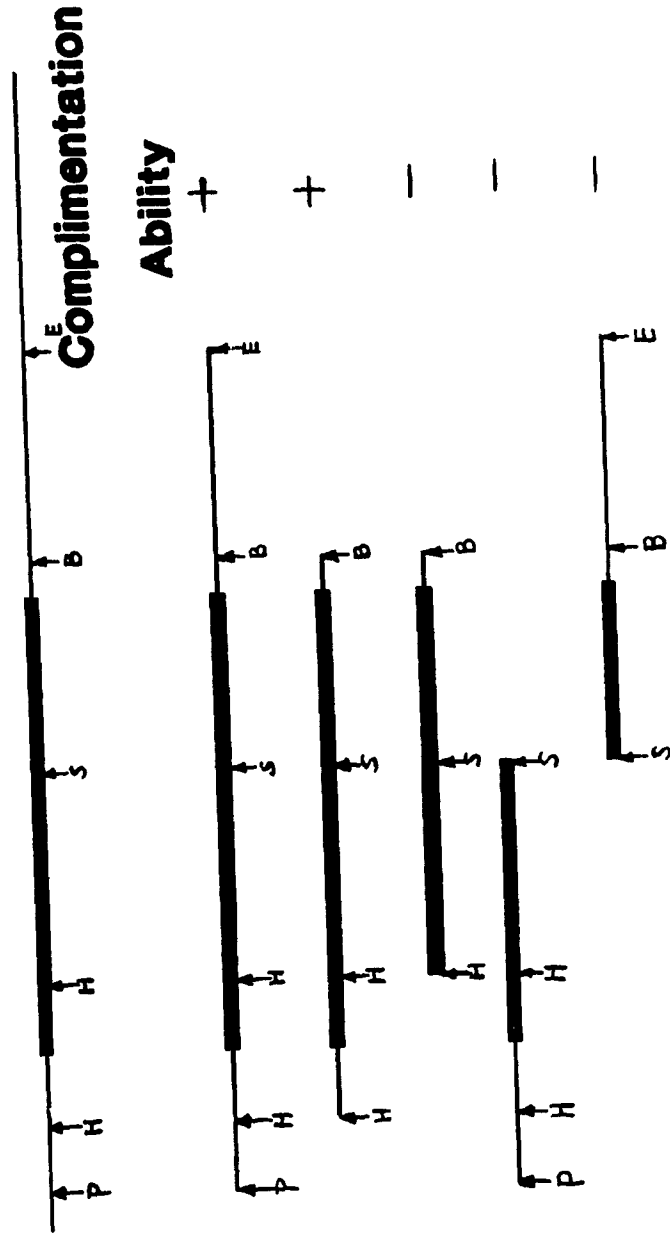
55a

Table 6. L-SD Activity of MEW84 Harbouring pMBH-28

| Host | Plasmid | L-SD Activity in | |
		Minimal Medium	With Gly & Leu
MEW1	pBR322	11	ND
MEW84	pBR322	1	ND
MEW1	pMBH28	10	ND
MEW84	pMBH28	11	76

Cells were cultured the same way as described in Table 5.

L-SD activity was tested and expressed as in Table 2.

Data are from a single experiment. Similar results were obtained in a different experiment.

DNA of pM1 to the pBR322 BamHI-HindIII sites to form pMBH-4
(Fig. 7, right). A detailed restriction mapping of the cloned
fragment was done on these three plasmids and the results in
Fig. 7 showed that plasmid pM10 had a KpnI site at the right
end of its non-vector DNA, which plasmid pM1 did not have.
pM1 had a KpnI site at the left end of its nonvector DNA which
pM10 did not have. Since these two plasmids shared a 6-7 Kb
region of their non-vector DNA, we can speculate that the non-
vector DNA fragments present on pM10 and pM1 were both from
the same chromosomal DNA within a region bordered by the two
KpnI sites (also refer to Fig. 8).

III C-3 Summary: the Cloning and Subcloning of the Putative
      sda84 Gene

By using the minimu in vivo cloning system, two plasmids
pM10 and pM1 were obtained. These two plasmids, shown by
restriction mapping, to share a common non-vector DNA region
of about 7 Kb, restored both the growth on SGL medium and the
L-SD activity of strain MEW84. The non-vector DNA was
transferred from pM10 and pM1 to vector pBR322 by different
strategies to form pMEP-13 and pMBH-28 (from pM10) and pMBH-
4 (from pM1). The study was based on pMEP-13 and pMBH-28
which had the same complementation ability for MEW84 as pM10.

III D Characterization of the Putative Clone by Hybridization

Before sequencing the gene, it is necessary to make sure that the cloned piece contains the wild type allele of the mutated gene in MEW84. This could be tested by Southern hybridization (Southern, 1975). We would expect that the sda84 gene should be in a contiguous stretch of DNA in the parent strain but in two pieces separated by λplacmu in the MEW84 mutant. If the cloned chromosomal DNA actually carries the MEW84 gene, it should hybridize to a corresponding fragment of the chromosomal DNA with the same size from the parent strain. However, this band should be missing in the MEW84 mutant and be replaced by one or two bands of different sizes.

This experiment was done by the following strategy: the probe used was the 5.5-6 Kb HindIII-KpnI fragment isolated from plasmid pMEP-13 (Fig. 7, left and Fig. 8), and labelled with [α-^{32}p] dATP. Chromosomal DNA from both MEW1 and MEW84 were digested completely with two groups of enzymes: HindIII-KpnI; and KpnI-SalI (Fig. 8). There were two reasons for me to use two pairs of enzymes. The fragment obtained by digesting the chromosomal DNA with HindIII-KpnI did not cover all the non-vector DNA cloned on pMEP-13. It left out about 1 Kb non-vector DNA at the left side of the HindIII site. There was therefore a chance, that the λplacmu insertion in MEW84 was inserted in this region, in which case, the results would indicate hybridization to only one band, even though the
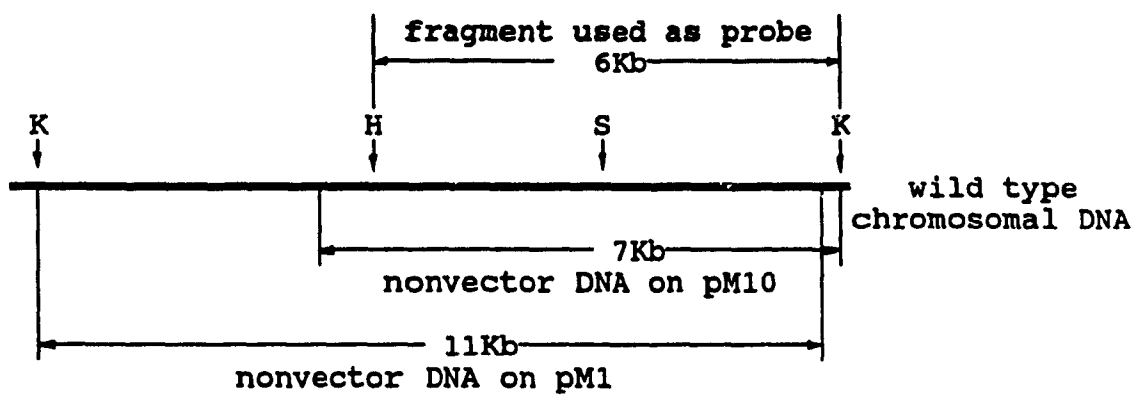
Fig. 8 DNA Fragments Involved in the Hybridization Experiment.

H: HindIII cutting site; S: SalI restriction site;
K: KpnI cutting site.
Figure is not drawn to scale.

correct gene was cloned. In order to avoid this, I wanted to use KpnI to cut the chromosomal DNA, which covered the entire region of the non-vector DNA on pMEP-13. But this piece was too large for the hybridization experiment (about 10-11 Kb), so I used the enzyme SalI, which cuts the KpnI fragment into one 3.5 Kb piece and one 7 Kb piece (Fig. 8). If the λplacmu was inserted at the right side of the SalI site, the 3.5 Kb band would disappear in the MEW84 mutant. If the λplacmu was inserted at the left side of the SalI site in MEW84, then the 7 Kb band should be missing in the hybridization experiment.

The hybridization result was disappointing. The hybridized restriction bands cut with either KpnI-HindIII , or KpnI-SalI did not change in MEW84 as compared with MEW1, indicating that the gene carrying the insertion in MEW84 was not the same gene as was carried on the clone.

This gene could still be sequenced anyway, but I decided against doing this in view of the fact that other aspects was more interesting.

Part 2 Physiological Study of Strain MEW84

III B Growth Rate of MEW84

I noticed that strain MEW84 seemed to grow more slowly than the parent MEW1 in glucose minimal medium. To quantitate this, I grew the strain in different media and followed

turbidity with a Klett Colorimeter #42 filter.

Cells on LB plates were inoculated into glucose minimal medium with or without the addition of L-leucine, since leucine was considered to affect expression of many genes (Lin et al., 1990), and incubated in a 37°C shaker overnight. The overnight cultures were subcultured to a OD420 value of 20 into side-arm flasks containing the same media as the overnight cultures. One more condition was tested: cells from the overnight leucine-free cultures were subcultured in glucose minimal medium supplemented with leucine.

As plotted in Fig. 9, strain MEW84 in glucose minimal medium grew more slowly than strain MEW1 (apparent doubling time 74 min versus 60 min). The parental rate was partially restored by providing the strain with leucine (68 min). The effect of leucine on the growth rate of strain MEW84 showed no difference whether leucine was added at the beginning of the incubation or at the subculture.

III F   Regulation of lacZ Transcription from the sda84 Gene
         Promoter

It is often possible to speculate the function of a gene by determining the conditions that regulate its transcription. In the case of a strain carrying a lacZ gene fused to the promoter of the gene of interest, this can be done by growing the cell in a variety of conditions, and looking for those

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Fig. 9   Growth of Strain MEW84 in Glucose Minimal Medium
   (GMM) With or Without the Supplementation of L-Leucine

        Cells were incubated at 37° C overnight in glucose
minimal medium with and without the supplementation of 112
ug/ml leucine. Log phase cells were subcultured into the
corresponding media in side-armed flasks to a density of
OD420 reading = 20. Incubation was continued in the 37°C
water bath shaker described in Material section, and cells
growth was followed by the Klett readings with a #42 filter
(purple).
        Curves are laterally displaced for ease of reading. The
first point on each curve is taken at the time of inoculation.
        The doubling times (min) of the strains growing in G.M.M.
were: MEW1, 60'; MEW84, 74'. The doubling time in G.M.M.
with leucine were: MEW1: 59'-60'; MEW84, 66'.
        Curves are drawn according to the data obtained from a
single experiment. Similar results were observed in another
experiment under the same condition except that the leucine
concentration supplemented was 300 ug/ml.

OD420

MEW84 in G.M.M.

MEW1 in G.M.M

MEW84 in G.M.M.+Leu

MEW1 in G.M.M.+Leu

- ■
- +
- *
- □

TIME (MIN.)

63
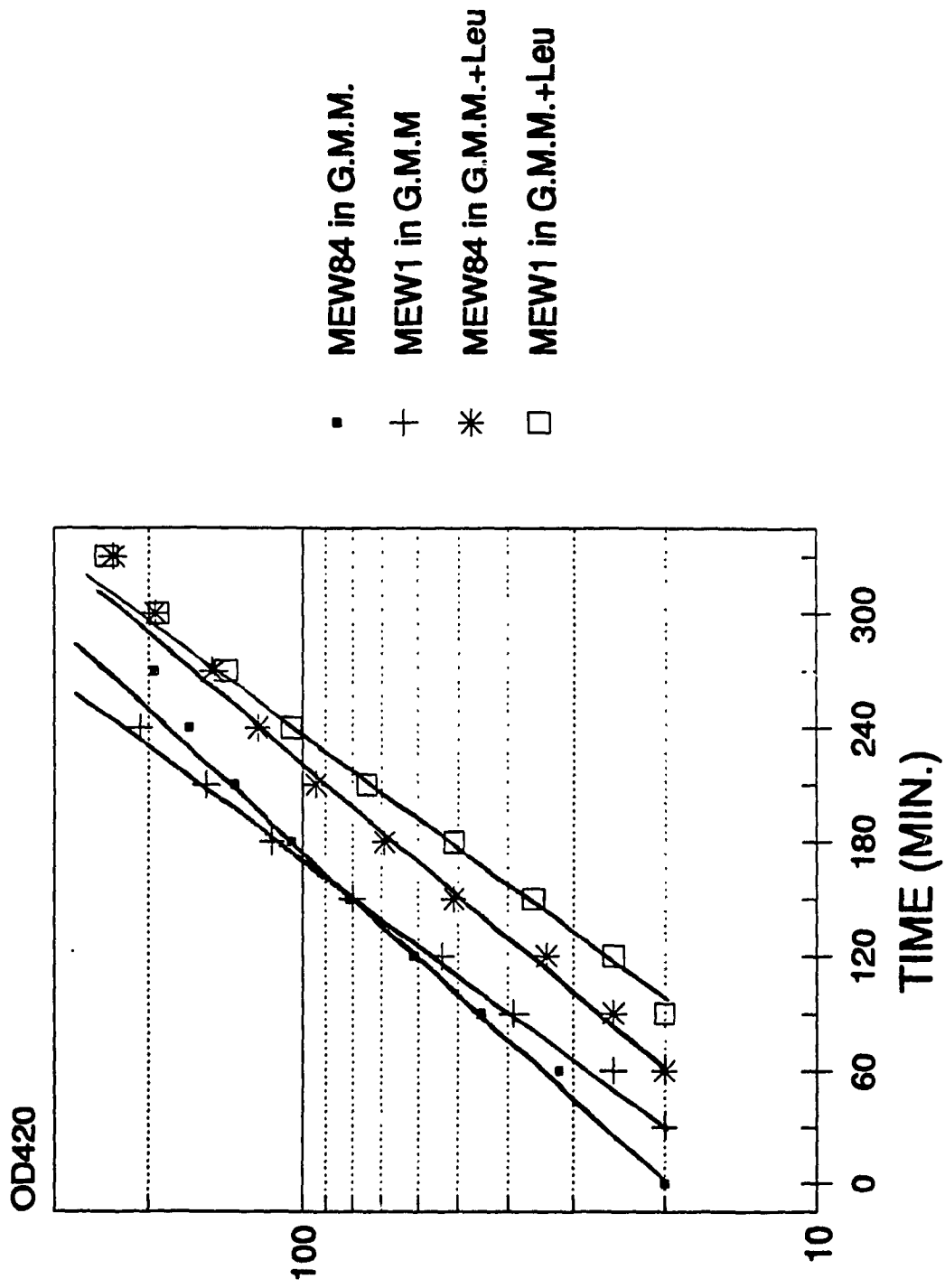
which alter the β-galactosidase activity.

I therefore measured the β-galactosidase activity under a variety of conditions which were known to affect L-SD transcription (Su et al., 1989). In fact, I could find very little alteration in lacZ transcription: β-galactosidase was increased 50% when the cells were grown in glucose-minimal medium with casamino acids, and was nearly doubled when cells were grown in LB. None of the other conditions - addition of various amino acids, use of different carbon sources, altered the enzyme level. β-galactosidase activity was decreased when the cell were grown in glucose minimal medium at 42°C (Table 7 & 8).

III G Construction and Study of the Double Mutant MEW84/ssd

ssd is a highly pleiotropic gene. This mutation increases the L-SD activity to so great an extent that it endows the cell with the ability to grow on L-serine as a sole carbon source, but abolishes the growth of the cell on either succinate or α-ketoglutarate (α-KG) as carbon source (Newman et al., 1982a). It has been proposed that ssd encodes a repressor of the sdaA gene (Newman, personal communication). In order to investigate the functions of the sda84 gene together with the sda128 and sda191 genes, and the relationships between ssd and these three genes, double mutants of MEW84/ssd, MEW128/ssd and MEW191/ssd were

Table 7. β-Galactosidase Activity of MEW84 in Glucose
Minimal Medium Supplemented With Different Amino
Acids or Grown at Different Temperatures

Growth Media	Relative Activity (%)
glucose	100
glucose 42°C	64
glu + gly	86
glu + leu	117
glu + gly leu	93
glu + met	110
glu + casamino acid	143

Cells were grown in glucose minimal medium overnight at 37°C or 42°C, and in glucose minimal medium supplemented with different amino acids overnight at 37°C.   Cells were subcultured into corresponding media and grown at corresponding temperatures.   Log phase cells were used for assaying β-galactosidase activity.   The activity is expressed in Miller's units.

Final concentrations of the supplements: glycine: 300 ug/ml; leucine: 300 ug/ml; methionine: 50 ug/ml; casamino acids: 5%.

Values with glucose or glucose + casamino acid as carbon sources are averages of two different experiments. Value with glucose + leucine as carbon sources is an average of three different experiments. The rest of the data are from a single experiment.

Table 8. β-Galactosidase Activity of Strain MEW84
Grown with Different Carbon Sources

C Source	β-Galactosidase Activity (%)
Glucose	100
Maltose	101
Glycerol	105
Arabinose	103
Lactose	99
LB	172

Cells were grown overnight at 37°C in LB or in minimal media supplemented with different sugars as carbon sources, and subcultured into the corresponding media. β-Galactosidase activity was tested as did in Table 2.

Sugars were supplemented at the final concentration of 2 mg/ml.

The data are from a single experiment except the value with LB as carbon source is an average of three different experiments.

constructed.   To serve as a comparison, a MEW22/ssd double
mutant was also constructed.

III G-1   Construction of the Double Mutants

The facts that the ssd is 33% linked to the metBFJL
operon (Bachmann, 1987) and that an ssd mutant is not able to
grow on α-KG minimal medium (Newman et al., 1982a), enabled
us to construct the double mutants by the following strategy:

A) P1 phage was grown on strain CAG5052, which possesses
a Tn10 insertion in the btuB gene closely linked to metBFJL
genes.   The lysates were used to transduce tetracycline
resistance into strains MEW84, MEW128, MEW191 and MEW22.
Transductants were then screened for methionine dependence by
streaking the transductants to glucose minimal medium plates
with vitamin B$_{12}$ but with or without methionine.   To verify
that the experiment was carried out correctly, I scored the
ratio of the metB⁻ Tc^R transductants compared to the total
tetracycline-resistant colonies (Table 9). Frequency obtained
was in accordance with the map distance between the genes metB
and btuX.

B) Lysates of P1 phage grown on strain KEC9 (ssd) were
used to transduce the metB⁻ strains isolated above to
methionine independence: transductants were selected on
glucose minimal medium plates with B$_{12}$ and screened for their
inability to grow on α-KG minimal medium plates with B$_{12}$.

To make sure that the double mutants carried the ssd

Table 9. Ratios (%) of metB⁻ Transductants Over the Total Tc^R Transductants in Various Experiments With Strain CAG5052 (metB btu::Tn10) as Donor

Recipient Strain	Total Tc^R Transductant	Met Requiring Transductant	% metB/Tc^R
MEW84	318	39	12.26
MEW128	222	33	14.86
MEW191	330	39	11.82
MEW191C	204	25	12.25
MEW22	309	38	12.29

Tc^R transductants were selected on LB Tc plates with the addition of other antibiotics as appropriate. The Tc^R transductants were streaked on glucose minimal medium with or without supplementation by methionine. Methionine-requiring transductants were scored and the ratio of metB⁻ transductants to the total tetracycline resistant transductants is given above.

mutation, P1 phage grown on the putative double mutant MEW22/ssd was used to transduce MEW1metB strain to methionine independence. These methionine non-requiring transductants were scored on α-KG plates supplemented with vitamin B_{12}. It was observed that the percentage of the α-KG$^-$ transductants compared to the total metB$^+$ strains was 29.7%. This was the expected linkage between ssd and metB, indicating that the double mutants constructed were correct.

III G-2   Growth of the Double Mutants on SGL Plates and Their L-SD Activities

The growth of these double mutants on SGL medium was verified by streaking them on SGL plates with the appropriate antibiotics. It was noticed that the double mutant MEW22/ssd showed no growth on SGL plates even after several days incubation at 37°C. Under the same condition, the double mutants MEW84/ssd and MEW191/ssd gave good growth in 24 hrs, whereas the double mutant MEW128/ssd showed some growth in two days. The whole cell L-SD activities of these double mutants are presented in Table 10, these were consistent with their ability to grow on SGL medium. L-SD activities of strains MEW84, MEW128, MEW191, MEW191C, as determined by whole cell assays, were increased in the ssd background. All of these strains grew on SGL medium, whereas the L-SD activity of strain MEW22/ssd, which did not grew on SGL medium, remained unchanged.

Table 10. L-SD and β-Galactosidase Activities of Double Mutants with ssd

Strain	L-SD Activity whole cell	L-SD Activity cell extract	β-Galactosidase Activity
MEW1	16	4	—
KEC9	89	62	—
MEW22	0.8	0.4	42
MEW22/ssd	1.3	0.5	263
MEW84	2.7	1.1	580
MEW84/ssd	37	4.7	388
MEW128	1.3	1.6	—
MEW128/ssd	8.2	6.2	—
MEW191	2.2	1.2	360
MEW191/ssd	16	6.9	350
MEW191C	2.7	1.1	—
MEW191C/ssd	22	5.6	—

β-Galactosidase activity and whole cell L-SD activity assays were done on mid-log phase cultures grown in glucose minimal medium at 37°C, and expressed as in Table 2.

To assay L-SD in extract, cells were grown to mid-log phase, extracts were made in glycylglycine buffer and L-SD was assayed and expressed as in Fig. 5, except that the reaction time of the cells with substrate was 40'.

Values are averages of two to four different experiments.

L-SD activities of the crude cell extracts was also assayed (Table 10). It is clear that the ssd mutation also increased cell-extract L-SD activities of the above mutants (with the exception of strain MEW22). It should be noticed that the increased L-SD activities were all well below the level of that detected in the cell-extract of strain KEC9 (ssd).

III G-3   Effect of the ssd Gene on the Transcription of the sda84, sda191 and sda22 Genes

To determine the effect of the ssd mutation on the transcription of the other genes, β-galactosidase levels from lacZ which was under the control of the sda84, sda191 and sdaA promoters were assayed. Since sda128 is a point mutation, it was not possible to examine its transcription in the same way.

The effect of the ssd mutation on the transcription of the sdaA gene was evident. The ssd mutation increased transcription of sdaA approximately 6-fold (Table 10, line 3, 4; also reported by Su et al, 1989). However, the ssd gene had no significant effect on the transcription of either sda191 or sda84 gene. The ssd mutation did increase L-SD activity in all cases. This was likely to be due to a direct effect of ssd on sdaA, and in any case probably did not involve the sda191 or sda84 gene products.

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III G-4 Summary: The Effect of the **ssd** Gene Product on Strains MEW84, MEW128 and MEW191

An **ssd** mutation increased the L-SD activities of strains MEW84, MEW128 and MEW191. The increased L-SD activities of strains MEW84 and MEW191 were probably a result of the increased transcription of the **sdaA** gene but not **sda84** and **sda191** genes, as judged by the β-galactosidase activities from the promoters of the individual genes in the double mutants with **ssd**. It is not known yet whether the **ssd** mutation has any effect on the transcription of **sda128**, since **sda128** is a point mutation and I was not able to assay its expression by measuring β-galactosidase activity from its promoter.

III H Azaleucine Sensitivity of Strain MEW84

As described earlier, the **sda84** gene was located near 55 minutes on the **E. coli** genomic map. Near this location, there are several genes already known including **purL**, **azl** and **glyA** (Bachmann, 1987). The possibility existed that the **sda84** gene might be identical to one of these genes. Since strain MEW84 did not require purines or glycine to grow, the **sda84** mutation could not be either **purL** or **glyA**. Azaleucine sensitivity was examined by growing the cells in glucose minimal medium at 37°C overnight, and plating 0.1 ml of the overnight cultures on glucose minimal medium plates. Paper discs with a diameter of 6 mm were plated on top of the cell lawn and different

amounts of azaleucine were added to the paper discs. It was surprising to find that MEW84 was very sensitive to azaleucine. The sda84 gene was not likely to be the same as the azl gene, because the mutation in azl is supposed to endow the cell with azaleucine resistance (Bachmann, 1987), whereas the sda84 mutation made the strain more sensitive (see discussion). To see whether the azaleucine sensitivity was due to the decreased L-SD activity, other mutants which have high or low L-SD levels were tested for azaleucine sensitivity in the same way. From Table 11, we can see that strains KEC9 and MEW26 which have high levels of L-SD activity, and strain MEW191 which has low L-SD activity, all showed an increased azaleucine sensitivity, but strain MEW22, which had no detectable L-SD activity, showed the same azaleucine sensitivity as the parent strain. It was evident that the azaleucine sensitivity was not caused by low L-SD. To avoid having to add isoleucine to the medium, strains MEW22, MEW84 and MEW1 were transduced to ilvA⁺, and tested for their azaleucine sensitivity on glucose medium without the addition of isoleucine and valine. The results were consistent with the above observations (Table 12).

III I   Characterization of A Double Mutant Carrying Inserts
        in Both the sda84 Gene and the rbl Gene

A highly pleiotropic E. coli mutant was recently isolated

Table 11. Azaleucine Sensitivity of Strains Grown on Minimal Medium Plates

| Strain | Inhibition Circle (mm) | |
	5ul of 100 mM Azaleucine	50 ul of 100 mM Azaleucine
MEW1	0	10
MEW84	14	34
MEW191C	14	29
MEW26	14	33
KEC9	14	34
MEW22	0	10

Cells were grown overnight in glucose minimal medium at 37°C, 0.1 ml of the cells were plated on glucose minimal plates. Paper discs with a diameter of 6 mm were placed on top of the plates and then different amounts of azaleucine were added onto the paper flakes. The inhibition circle was taken as the diameter of the nongrowth area minus 6 mm.

Data presented in this table are from a single experiment. Similar inhibition pattern was observed for strains MEW84, MEW22 and MEW1 in another experiment with different concentrations of azaleucine (Only strains MEW84, MEW22 and MEW1 were tested in that experiment).

Table 12. Azaleucine Sensitivity of ilvA⁺ Transductants on Minimal Medium Plates Without the Supplementation of Isoleucine and Valine

| Strain | Inhibition Circle (mm) | |
	5 ul of 100 mM Azaleucine	50 ul of 100 mM Azaleucine
MEW1 ilvA⁺	0	22*
MEW84 ilvA⁺	14	32
MEW22 ilvA⁺	0	19*

* the inhibition circles were cloudy.

Cells were grown overnight in +N glucose minimal medium at 37°C overnight. 0.1 ml of the cells were plated on +N glucose minimal medium plates. Azaleucine sensitivity was tested in the same way as done in Table 11.
These data are from a single experiment.

and is being studied in our lab (Lin et al., 1990). It is a rbl::Tn10 insertion mutant mapping near 20 minutes. This mutant, selected for its ability to grow on L-serine as sole carbon source, has an increased transcription of L-SD and L-threonine dehydrogenase, and decreased transcription of serA and ilvIH; it also suppresses the slow growth of a metK strain (Lin et al., 1990). One might wonder whether the sda84 gene was also regulated by this rbl gene. To test this, the following experiments were done.

III I-1 Construction of MEW84/rbl Double Mutant

Construction of the MEW84/rbl double mutant was done by P1 transduction. P1 phage grown on strain MEW26 which carries a Tn10 insertion in the rbl gene was used to transduce tetracycline resistance into MEW84. Antibiotic resistant transductants were selected on LB plates supplemented with both kanamycin and tetracycline, and tested for growth on SGL and glucose minimal media. The transductants showed no growth on SGL plates after the first day, and very little after 48 hrs. Growth on glucose plates was slightly slower. However good growth was observed after two days incubation at 37°C.

It therefore seems that the effect of rbl in increasing sdaA transcription and allowing growth on serine was counteracted by the sda84 mutation, so that the double mutant could not grow on serine with inducers.

Each mutation in the double mutant was transduced back

into the parent strain MEW1 to make sure that the double
mutant carried the expected mutations. To do this, P1 phage
was grown on the double mutant and transduced to MEW1.
Transductants were selected on LB medium with either kanamycin
or tetracycline. Tetracycline would select the mutant with
Tn10 inserted in its genome, and kanamycin would select the
mutant with λplacmu insertion in its genome. After
purification on the same plates as they were originally
selected from, the transductants were tested for their L-SD
activities (Table 13).

I concluded from the results presented in Table 13 that
the double mutant constructed was correct, because 1): the
kanamycin resistant transductants showed a level of L-SD
activity which was expected for MEW84; 2): the tetracycline
resistant transductants showed a level of L-SD activity which
was expected for MEW26.

III I-2   The Observation of the Slow Growth of Strain
          MEW84/rbl

To investigate the relation between sda84 and rbl, I
decided to assay the L-SD and β-galactosidase activities of
the double mutant. Strain MEW84/rbl grew slowly in liquid
glucose minimal medium, and it was impossible to measure its
L-SD and β-galactosidase activities. It seemed that this
strain might be auxotrophic for some compound lacking in
glucose minimal medium. To find out what it needed, the

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Table 13. L-SD Activity of the Transductants from MEW84/rbl to MEW1

	Strain	L-SD Activity
	MEW84	1.3
	MEW26	59
Transductants	Km^R #1	1
from MEW84 to MEW1	Km^R #2	1.8
	Tc^R #1	46
	Tc^R #2	46

Transductants were obtained by using P1 phage lysates of MEW84/rbl to transduce MEW1, and selecting on LB plates containing either kanamycin or tetracycline. L-SD activity was assayed in the usual way. Km^R: kanamycin resistant transductant; Tc^R: tetracycline resistant transductant. Values presented are from a single experiment.

double mutant was cultured in LB liquid medium and glucose minimal medium supplemented with casamino acids and a vitamin mixture respectively at 37°C. The strain grew well in both LB and minimal medium with casamino acids. However, vitamins had no effect on the growth of MEW84/rbl in minimal medium, suggesting that some amino acid(s) must have been required.

Several amino acids or amino acids mixture were added to glucose minimal medium to see if any could enable the mutant to grow faster. An interesting result was observed: methionine alone increased the growth of the double mutant to a great extent. Lysine alone, or glycine and leucine together increased the growth to a lesser extent. Proline, histidine and tryptophan together had little effect. Serine alone, and the fifteen amino acid mixture actually inhibited growth (Table 14). The 15 amino acid mixture included aspartate, asparagine, lysine, arginine, glutamate, glutamine, cystine, tyrosine, phenylalanine, tryptophan, histidine, methionine, proline, alanine and threonine.

To quantitate the effect of methionine on the growth of MEW84 in glucose minimal medium, the growth rate was monitored by using the same method as in III E and the result is presented in Fig. 10 and 11. Methionine increased the growth rate of both the rbl mutant, strain MEW26, and of the double mutant, strain MEW84/rbl.

III I-3 The Effect of rbl Mutation on the L-SD and β-

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Table 14. The Growth of MEW84/rbl in Glucose Minimal Medium Supplemented With Different Amino Acid(s)

| Strain | Cell Density (Klett Reading with #54 Filter) in *G.M.M. supplemented with | | | | | | |
	none	Ser	Lys	ProHis	Met	GlyLeu	Amino Acid Mix
MEW84/rbl	70	4	147	100	270	194	8

Cells taken from LB plate were suspended in NIV solution. Aliquots of cells were inoculated into glucose media with or without the addition of amino acids, and incubated in 37°C shaker overnight. Growth (cell density) was tested by Klett Colorimeter with a filter #54.

*G.M.M.: glucose minimal medium.

Final concentrations of supplements: serine (Ser), lysine (Lys), proline (Pro), histidine (His) tryptophan, methionine (Met) and leucine (Leu) were added at 50 ug/ml each. Glycine (Gly) was added at 100 ug/ml. Amino acid mix was a mixture containing 10 mg/ml of each of the following amino acids: aspartate, asparagine, lysine, arginine, glutamate, glutamine, cystine, tyrosine, phenylalanine, tryptophan, histidine, methionine, proline, alanine and threonine. 0.04 ml of the amino acids mixture was added to 20 ml medium, so the final concentration of the amino acids was 20 ug/ml each.

Data are from a single experiment.

Fig. 10 and 11   Growth of Strains in Glucose Minimal
     Medium (GMM) With (Fig. 10) or Without (Fig.11)
              the Supplementation of Methionine

     Growth was tested the same way as done in Fig. 9, except
that methionine (50 ug/ml) was added to the growth medium
instead of leucine.
     Curves are also plotted the same way as Fig. 9.
     The doubling time of strains grown in G.M.M. were (Fig.
10): MEW84, 72'; MEW26 (rbl), 77' and MEW84/rbl, 84'. The
doubling time of strains grown in G.M.M. supplemented with
methionine were (Fig. 11): MEW84, 65', MEW26 (rbl), 68' and
MEW84/rbl, 68'.
     Data used to plot the curves are from a single
experiment.

Fig.10

Fig.11

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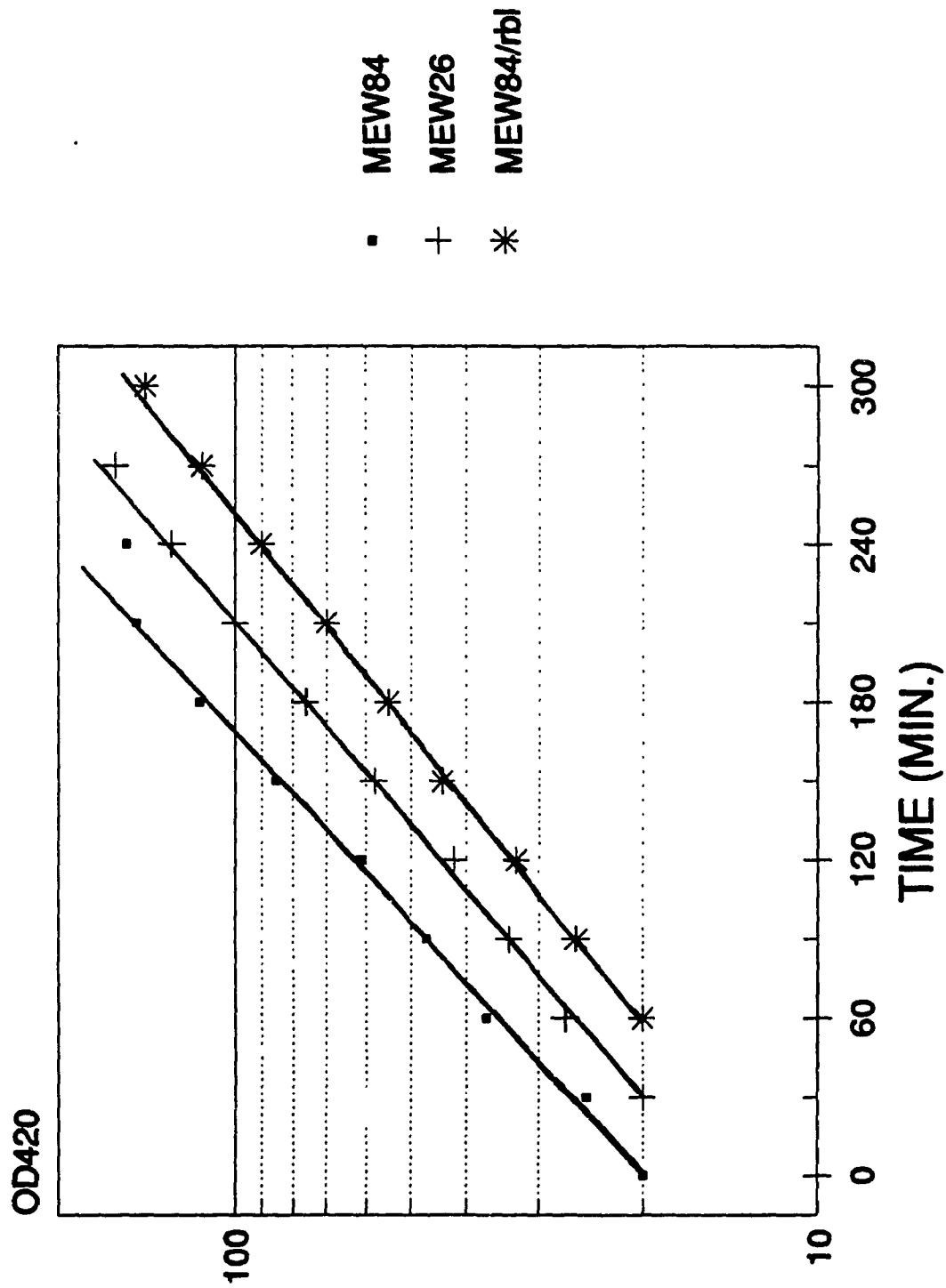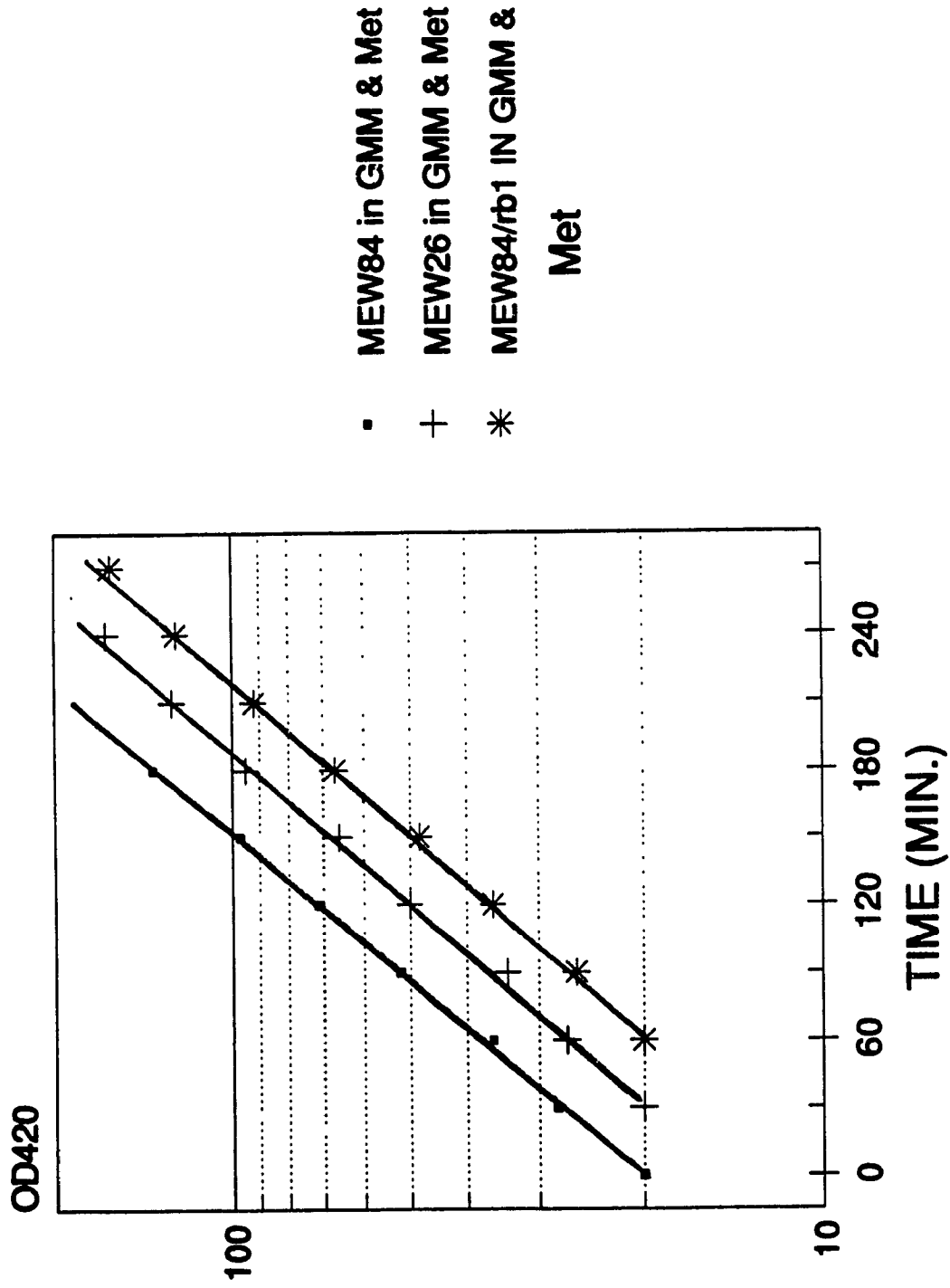
Galactosidase Activities of Strain MEW84

L-serine deaminase and β-galactosidase activities of the double mutant MEW84/rbl were assayed in glucose minimal medium in the presence of 50 ug/ml methionine, since we knew that methionine was able to compensate for the slow growth of this strain. L-SD was increased very slightly in the double mutant as compared to strain MEW84. This may be due to the increased transcription of sdaA by rbl, since rbl had no effect on the transcription of sda84 gene as judged by the β-galactosidase activity (Table 15).

III I-4 Increased Serine Toxicity of Strain MEW84

It is well known that L-serine is toxic to E. coli. This inhibition is released by adding isoleucine to the medium (Amos et al., 1954). Since the strains we use in our lab are all derivatives of MEW1 which is an ilvA deletion mutant, isoleucine and valine are added to all the minimal media and serine toxicity should be avoided. However the MEW84/rbl strain was inhibited by serine in glucose minimal medium supplemented with isoleucine and valine. I wondered then whether the serine toxicity was due to the mutation in the sda84 gene, the rbl gene or the combination of the two mutations. To determine this, strains MEW84 and MEW26 were tested for their serine sensitivity.

MEW84 and MEW26 were inoculated from LB plates to glucose minimal medium with or without the addition of L-serine.

Table 15. L-SD and β-Galactosidase Activities of MEW84 in the Presence of rbl Mutation

| Strain | ** Relative β-Gal Activity (%) | | L-SD Activity |
	Early Log Phase	Late Log Phase	
*MEW84	100	100	1.3
*MEW84/rbl #1	86	71	4.9
*MEW84/rbl #2	86	91	4.1
#MEW84 + Leu	99	120	1.4

*Cells were grown in glucose minimal medium supplemented with 50 ug/ml methionine.

**β-Galactosidase activity relative to value of MEW84 as 100.

#Cells were grown in glucose minimal medium supplemented with 300 ug/ml leucine.

L-SD and β-galactosidase activity were assayed the same way as in Table 2.

Values are from a single experiment.

Cultures were incubated in a 37°C shaker, and growth was followed with the Klett Colorimeter. It was observed that MEW84 was very sensitive to serine, but the growth of MEW26 was actually increased by the addition of serine (Table 16). It was not known whether the deficiency in L-SD made MEW84 serine sensitive or the sda84 mutation also affected the activity of other enzymes which might be related to serine toxicity. To test this, other mutants lacking L-SD activities including the structural gene mutant were cultured in glucose minimal medium to see their reaction to serine. It was observed that all these strains deficient in L-SD were serine sensitive (Table 16). It seemed clear that it was the low L-SD activity which caused the mutants to be serine-sensitive. The internal serine concentration of these strains must be higher than that of the strains which had normal or high L-SD activity. Since serine toxicity was not released by isoleucine, serine may interfere with the biosyntheses of other amino acid(s) as well as isoleucine.

III I-5   Release of Serine Toxicity of MEW84 by Threonine

Although it had long been known that serine was toxic to E. coli cells grown in minimal medium, the target of inhibition was not clear until recently. In their paper, Hama et al. (1990) reported that serine inhibited the activity of homoserine dehydrogenase I, which is very important in the biosynthesis of methionine, threonine and isoleucine, for the

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Table 16. Increased Serine Toxicity of Strains Deficient in L-SD activity and the Release of the Toxicity by Threonine in Glucose Minimal Medium

	Strain	Inc.* Time	glu	Growth (Cell Density at OD540) in: glu+ser	glu+met	glu+serthr	glu+sermet
Exp.1	MEW1	O.N.**	264	300	264	ND	ND
	MEW84	O.N.	224	19	190	ND	ND
Exp.2	MEW84	O.N.	228	2	ND***	218	2
		24hrs	222	24	ND	240	5
	MEW26	O.N.	108	258	244	ND	ND
	MEW26	24hrs	192	256	222	ND	ND
	MEW191	O.N.	52	2	ND	218	2
		24hrs	212	9	ND	214	7
	MEW128	24hrs	50	0	ND	8	0
	MEW22	O.N.	180	0	ND	170	0
		24hrs	315	15	ND	290	0

Cells were inoculated in aliquots into glucose minimal medium with or without the supplementation of amino acids and incubated in a 37°C shaker. Growth was quantitated by Klett Colorimeter with a #54 filter.

Final concentrations of the amino acids: serine (ser): 100 ug/ml, methionine (met): 50 ug/ml, threonine (thr): 80 ug/ml.

*inc time: incubation time; ** O.N.: overnight; ***ND: not determined.

activity of homoserine dehydrogenase II in wild-type E. coli is very low (Cohen and Saint-Girons, 1987; Hama et al., 1990). But a discrepant result was reported before that serine did not inhibit homoserine dehydrogenase I (Patte et al., 1966). To determine what was the case for MEW84, threonine and methionine were tested to see whether they could release the serine toxicity of MEW84. The two amino acids were added respectively to glucose minimal medium supplemented with serine (isoleucine was also added as usual).

As shown in Table 16, threonine released the serine inhibition and methionine actually seemed to enhance the serine toxicity. Later, aspartic acid was also tried and it was observed that this amino acid was not able to reverse the toxicity of serine to MEW84. These results are consistent with the result obtained by Hama et al. The additive effect of serine toxicity by methionine was probably due to the repression of the aspartokinase-homoserine dehydrogenase II by methionine.

III I-6 A Double Mutant MEW84/rbl Could Overcome Serine Inhibition During Purification

Further experiments with the double mutant MEWE84/rbl revealed that the strain lost its serine sensitivity after several purifications on LB solid medium supplemented with both tetracycline and kanamycin (Table 17). Two possibilities

**Table 17. Different Serine Tolerance of Strain MEW84/rbl
After the First and Third Purifications**

| Media | Growth (OD540 values) | | Third Purification |
| | First Purification | | |
	O.N.	24hrs	O.N.
Glu	25	222	36
Glu+Ser	0	142	66
Glu+Met	165	222	202

Strain MEW84/rbl was constructed by using P1 lysate of strain MEW26 to transduce strain MEW84 to tetracycline resistance. Transductants were selected on LB plates with both kanamycin and tetracycline, and purified on the same type of plate. Aliquots of cells were inoculated into different media listed in the table and incubated in a 37°C shaker. Growth was quantitated by Klett readings with a #54 filter.

Serine (ser) was supplemented to a final concentration of 100 ug/ml, Methionine (met) was added to 50 ug/ml, final. O.N.: overnight.

Similar results were observed with another MEW84/rbl transductant obtained in a different transduction experiment.

might be responsible for this: the increased L-SD activity in the double mutant due to the increased transcription of sdaA gene could partially counteract the serine inhibition, though L-SD activity was increased only slightly, not enough for growth on SGL (Table 15). It could also be that the strain acquired some mutation during its purification. For example, the activity of homoserine dehydrogenase II was increased.

III I-7 The Effect of metJ Mutation on the Growth of MEW84/rbl Double Mutant

As stated above, MEW84/rbl grew slowly in glucose minimal medium, and methionine could restore the growth to some extent. One might ask whether the methionine synthetic pathway might be repressed in this double mutant. To test this, a metJ mutation resulting in derepression of the methionine pathway (Cohen and Saint-Girons, 1987) was transduced to MEW84/rbl. This was done by growing phage P1 on strain TK4100 metJ::Cm^R and transducing to MEW84/rbl. Transductants were selected on LB plates with the antibiotics tetracycline, kanamycin and chloramphenicol. Transductants were screened on NSIV, SGL and Glucose minimal medium plates. The triple mutant grew slowly but showed good growth on all these plates after two days incubation at 37°C, indicating that the triple mutant constructed was correct, since the over expression of the metC gene (due to the metJ mutation) would confer on the strain the ability to grow on serine as a sole

carbon source (Brown and Newman, 1990).

The growth of the triple mutant MEW84/rbl/metJ was tested in liquid minimal medium. It was noticed that the slow growth was not compensated by the metJ mutation. Addition of methionine to the medium increased the growth but not much. However, serine greatly increased the growth as well as did serine + threonine (Table 18).

III I-8 Summary: Effects of rbl Mutation on Strain MEW84

The rbl mutation increased the doubling time of strain MEW84 from 74 minutes to 84 minutes in glucose minimal medium, which could be compensated by adding methionine to the growth medium. The rbl mutation also increased the L-SD activity of strain MEW84 which I thought helped the double mutant MEW84/rbl to overcome serine toxicity. Transcription of the sda84 gene was not changed by the rbl mutation.

**Table 18.   Growth of Strain MEW84/rbl/metJ in Glucose
Minimal Medium Supplemented With Different
Amino Acids**

| Strain | Growth (OD540) in Minimal Medium Plus | | | |
	Nothing	Met	SerThr	Ser
MEW84/rbl/metJ	25	85	160	155

Cells were taken from LB plate and suspended in NIV solution. Aliquots were inoculated into glucose minimal medium with or without the addition of amino acids and cells were incubated at 37°C overnight, shaking. The growth was followed by Klett Colorimeter with the filter #54.

Final concentrations of the supplements: Methionine (met): 50 ug/ml; serine (ser): 100 ug/ml; threonine (thr): 80 ug/ml.

Data are from a single experiment.

IV Discussion

IV A Consideration of the Possible Function of the sda84 Gene Product

Strain MEW84, a newly isolated E. coli-K12 mutant, is unable to grow with serine, glycine and leucine as sole carbon and energy source. It carries a λplacmu insertion in the gene named sda84. This mutation caused the strain to lose its normal L-SD activity as judged by a whole cell assay and to lose the ability to grow on SGL medium. However, the L-SD activity of this strain could be recovered in vitro by incubating with iron and DTT. This indicates that the L-SD protein is present in the MEW84 cell, but in an inactive form, which can be activated by the chemical activator iron and DTT. This characteristic of the sda84 mutant clearly indicates that the sda84 gene does not code for L-SD protein, and suggests that this gene has something to do with the activation of the L-SD protein.

Other previously isolated E. coli K-12 mutants have similar properties. Strain MEW191, bearing a MudX insertion in gene sda191, and MEW128, bearing a point mutation in gene sda128, all have similar phenotypes (Newman et al., 1985a; Newman et, al., 1985b) .

The sda84 mutation is not an allele of either sda128 or sda191, since mapping experiments located the sda84 gene precisely at 55 minutes, which was well apart from sda191 at

about 86.5 minutes and sda128 at about 15-17 minutes. Another

phenomenon which distinguishes sda84 from sda128 and sda191

was that plasmids (pM1 and pM10) complementing sda84 were not

able to complement sda128 or sda191.

From the results presented in Table 10, we see that the

ssd mutation increases sdaA transcription about 6-fold (β-

galactosidase activity of strain MEW22/ssd versus that of

strain MEW22), which is in accordance with the extent of

increased L-SD activity observed in the ssd mutant (L-SD

activity of strain KEC9 versus that of strain MEW1). Although

the ssd mutation also raises the L-SD activities of the other

strains tested, even the increased L-SD levels are in all

cases much lower than that of KEC9. The increased L-SD

activity is apparently not due to the function of the sda84

gene and sda191 gene, since the transcription of these genes

is not affected by ssd. The increased L-SD activities are most

likely due to the increased amount of sdaA product. These

observations not only serve as a further evidence that sdaA

gene is the structural gene for L-SD (Su Hongsheng et al.,

1989) and that ssd gene enforces the negative control on the

transcription of sdaA (Newman, personal communication), they

also indicate that the sda84, sda191 and sda128 genes are

crucial for L-SD activity.

These three mutants, MEW84, MEW128 and MEW191, may define

a post-translational activating system of L-SD. Such kind of

mutation seems to occur in a rather high frequency, since

three mutants out of six isolated in our lab are related to the post-translational modification of L-SD protein. It is likely that this activating system involves even more genes in addition to sda84, sda128 and sda191, but the relationship between them is not clear at all. However I will use sda84, sda128 and sda191 genes as representatives to discuss the possible relationships between the genes involved in this system and the possible mechanisms they use to activate L-SD.

Proposed relationships between these genes:

1) Since many enzymes are composed of subunits, it is possible that L-SD activating enzyme is a protein composed of several subunits. In this case, each of the sda84, sda128 and sda191 genes could encode a subunit of this activating protein.

2) One or two of these three genes encode(s) the activating enzyme. The other one (or two) regulate(s) its level. Regulation could be either at the transcriptional level or at the translational level.

3) The activating enzyme itself needs to be modified. That is, one or two of these genes encode(s) the L-SD activating enzyme, which itself is subject to modification by the other gene(s) product(s).

4) This activating enzyme needs cofactor(s) to carry out its function. The cofactor(s) is produced by one or two of the three genes.

5) These genes encode individual enzymes which work in

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succession to activate L-SD. For example, some of the three genes product(s) modify the L-SD proenzyme at a certain site. This modified site would serve as a recognition target for the action of the other activating gene(s) to modify the L-SD further.

If the sda84, sda128 and sda191 genes are involved in post-translational modification of L-SD protein, one would expect the strains to produce as much inactive L-SD protein as the wild-type strain, but they would be unable to activate it. Then addition of Fe^{+++} and DTT should produce the same amount of activity as is seen in the parent strain. This should also be true for the double mutants with ssd, as compared to the ssd parent. However, all the mutant activities are lower than the corresponding parent strain (Table 10). One possible explanation for these results could be that: the L-SD proenzyme is cut at a specific site(s) by products of the activating genes (Recsei et al., 1983). The resulting fragments may be modified further. They might, for instance, be reduced by the function of the activating system (Newman et al, 1985b). Since strain MEW1 and KEC9 both have a functional L-SD activating system, in their cells L-SD protein can be truncated and properly reduced. After the cell extract is made, the L-SD fragments become oxidized gradually by exposure to O_2 . Adding iron and DTT to the extract can restore the reduction state of the L-SD fragments. However, in strain MEW84, MEW128, MEW191 and the double mutants

MEW84/ssd, MEW128/ssd and MEW191/ssd, most of the L-SD proenzyme remains untruncated and the L-SD activities seen in their cell extracts depends on processing and reduction by iron and DTT. This may be a much less specific reaction than the enzymatic activation, and may result in much lower activity levels. Alternatively, the proenzyme may be recognized as being abnormal, and consequently be degraded.

To understand the relationship amongst the three genes, it would be helpful if we mixed the cell extracts of strains MEW84, MEW128 and MEW191, and test the L-SD activities of these mixture to see if there is any complementation. We could also construct double mutants as MEW84/sda128, MEW84/sda191 and MEW128/sda191, and assay the expression of β-galactosidase activity from the promoter of one of these genes.

Some possible mechanisms of activating L-serine deaminase are proposed as follows:

1) The activating system may produce cofactor(s) for L-SD.

To catalyze a reaction, many enzymes need cofactors to complete their function (Jeter et al, 1987; Knappe, 1984). It is not likely that the L-SD activating system produces a cofactor, since none of the mutants required anything other than thiamine for growth, and adding thiamine to the medium did not restore their L-SD activity. One may argue that the L-SD activity needs a higher concentration of the cofactor

than those required for other needs. Then the sda84, sda128 or sda191 mutation would lower the amount of cofactor production to a level which would be sufficient for cell growth but not for L-SD function. Adding vitamins into the medium and assaying the L-SD activity might enable us to answer the question at least for the cofactors related to them, unless the conversion of that vitamin to the related cofactor is in fact blocked in these mutants. Some other molecules could also be required as a cofactor. If the enzyme is under allosteric control, it may need some allosteric effector to change to its functional form (Stryer, 1975). With the mutation of the L-SD activating system, the amount of the cofactor or allosteric effector would be decreased such that the L-SD activity is abolished.

How could the L-SD activating system activate L-SD protein, if it does not encode a cofactor? Iron and DTT are capable of activating many enzymes, but the mechanisms involved are quite varied. From a review of how iron and DTT activate these enzymes we might be able to find some clue as to L-SD activation.

ℓ) The L-SD proenzyme may be activated by changing its redox state.

Ribonucleotide reductase is produced in an oxidized, inactive form in E. coli cells. It is reduced to an active form in vivo by an activating system which is mimicked in vitro by iron and DTT, a well known reducing agent when they

are present together (Holmgren, 1976; Thelander and Reichard, 1979; Reichard and Ehrenberg, 1983). Knappe (Knappe et al., 1984) has reported the post-translational interconversion of pyruvate formate lyase of E. coli between its active and inactive forms. The activated lyase carries an unpaired electron on its surface introduced by an Fe^{++}-dependent activating enzyme, which requires pyruvate as an allosteric effector and S-adenosylmethionine as a cofactor. L-SD might be activated in a similar way. But it is also possible that the activation of L-SD involves an oxidation reaction.

As shown in the introduction, during iron cycling in a Fenton-type reaction, a mixture of ions and free radicals are generated (Walling, 1975). Newman (Newman et al., 1990) has shown that a transitory hydroxyl radical species may be responsible for the activation of L-SD. This was suggested since hydroxyl radical scavengers prevent activation. Hydroxyl radicals are oxidants, and may oxidize the essential amino acid(s) of the L-serine deaminase to active it.

3) The activating system of L-SD may processes the L-SD proenzyme into smaller, active enzymes.

It has been shown by Brawn and Fridovich (1981) and Kim et al (1985) that iron and DTT caused the processing of both DNA and protein into smaller molecules. Iron and DTT might activate L-SD by processing it into smaller fragments. It could be reasoned that inside the cell, the activating system works in a similar way as iron and DTT. Recsei et al (1983)

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has described the activation of L-histidine decarboxylase (histidine carboxyl-lyase E.C.4.1.1.2.2.) of Lactobacillus 30a. The catalytic function of this enzyme depends upon the amide-bound pyruvoyl group at the active site, which the proenzyme lacks. During the activating process, this enzyme is cleaved at the serine81-serine82 bond by serinolysis to produce two fragments, alpha and beta. Both are active enzyme forms. The serine at the N-terminal of the alpha-subunit is converted to a pyruvoyl group (Recsei et al., 1983; van Poelje and Snell, 1990).

From the sequence of the sdaA gene, a protein of 48,000 MW is predicted, with a serine-serine bond in the middle of this protein (Su et al ,1989). It is quite possible that L-SD is activated by the same mechanism. If this is true, we should be able to distinguish the activated protein from the proenzyme by their migration rate on gels.

It is also possible that L-SD is activated by limited proteolysis of the L-SD proenzyme at its end(s) (Stryer, 1975). There have been reports of changes in the kinetics of lipolytic enzymes after limited proteolysis (van Oort et al.,1989; Hilton, et al., 1990). Many toxin proteins are produced as inactive precursors and activated by proteolytic removal of some amino acids either after or before the release of the proteins from the cell (Howard and Buckley, 1985). Although L-SD is neither a lipolytic enzyme nor a toxin, it might still be activated in a similar manner.

To verify this, L-SD activity from cell extracts of E. coli K-12 incubated with or without proteinases could be assayed. Similarly, one might purify the L-SD protein from both the wild type strain and strains deficient in the activating system, and check the molecular weight of the L-SD protein from different sources. Alternatively, the molecular weight of the wild-type L-SD could be determined before and after the incubation with iron and DTT.

4) Activating L-SD by modifying some specific amino acid(s) of L-SD protein.

Proteins can be modified at different sites after they are translated (Stryer, 1975). For example, protein can be modified " by esterification of hydroxyl group, by acylation of N -lysine residues, by breaking-down of cysteine-cysteine bond or catalysing the formation of this bond cross-linked inside or between protein molecules, by amino acylation of N-terminal glutamine and asparagine residues, by glycosylation of amide or hydroxyl groups, by proline isomeration, and aspartate racemization etc." (Stadtman, 1990). L-SD activation might involve a similar mechanism that changes the inactive L-SD conformation to a functional one.

5) The L-SD proenzyme may be bound to some molecule(s) immediately after its formation. The sda84, sda128 and sda191 gene product(s) might detach the bound molecule(s) from the L-SD protein by destroying or neutralizing the molecule(s), releasing a protein with L-SD activity.

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6) In the _in vitro_ study of the activation of L-SD, Newman _et al_. (1990) showed that hydroxyl radicals were needed to activate L-SD.   I would argue that instead of being oxidized by the hydroxyl radical, L-SD protein uses this radical (or some other radicals) to facilitate its function. Inside the cell, radicals can be produced by various processes and reactions. For example, during oxidative respiration, the reduction of oxygen occurs through sequential reactions to produce radical intermediates (Basaga, 1990):

$$O_2 \longrightarrow O_2^{\cdot} \longrightarrow H_2O_2 \longrightarrow OH^{\cdot} + OH^{\cdot} \longrightarrow H_2O$$
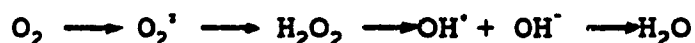
$OH^{\cdot}$ group might interfere with the function of L-SD. Under normal conditions, the $OH^{\cdot}$ group is taken away by the L-SD activating system, but if the activating system is absent, L-SD activity would be greatly afflicted by the presence of the $OH^{\cdot}$ group.

As a summary, the possible functions of sda84 are depicted in Figure 12.

Two further problems may be mentioned. First, does the wild type strain lose activity in extracts because of a change in redox state affecting either L-SD itself, or its cofactor? Alternatively, might the loss in activity be due to the separation of L-SD from its activating system resulting from dilution by making the extract?

Second, the L-SD enzyme does not seem to be very important for E. coli K-12, since strain MEW22, in which the structural gene is inactivated, shows little disadvantage as

sda84 PRODUCT   sda84 PRODUCT   sda84 GENE or PRODUCT

ACTIVATOR   COFACTOR   OTHER GENE OR PRODUCT

sdaA PRODUCT ⟶ ⟶ I-SD
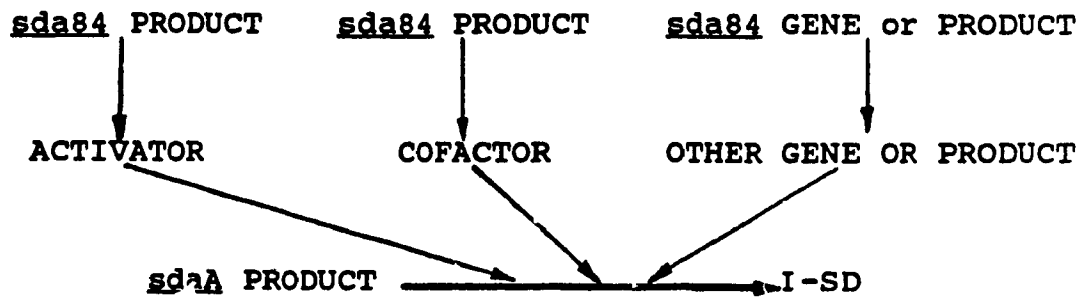
Fig. 12   Possible Mechanism of Activation of L-SD by

         sda84 Gene Product

Possible functions of sda84 gene are postulated.

compared with the parent strain under all the conditions we have tested (growth on glucose minimal medium, UV sensitivity, growth at 42°C, and growth in the L-SD inducer glycine and leucine) (Su et al., 1989). Why then does the cell devote so much emphasis on its regulation, which is effective at both the transcriptional (ssd) and post-translational (sda84, sda128 and sda191) levels?

IV B The Cloned Piece of DNA Might Contain an Operon

By using the in vivo miniMu cloning system (Groisman et al., 1986), two mu replicons pM1 and pM10, were obtained, which, when introduced into the MEW84 strain, could restore the L-SD activity to an extent comparable to the parent strain both in glucose minimal medium and under the inducing condition with glycine and leucine.

Initial restriction enzyme analysis of the two plasmids showed that the two plasmids shared a common region of about 5-7 Kb. Therefore sequences required to complement L-SD activity and restore growth on SGL plates must be in this common region.

The failure to subclone pM10 by total digestion with the enzyme pairs BamHI-HindIII and EcoRI-SalI indicates that the HindIII site and the SalI site on the chromosomal DNA insert are within a region essential for sda84 activity. This was proven by the following two experiments: A: The strategy of

maintaining the HindIII site of the chromosomal DNA by partially digesting pM10 with HindIII and totally digesting with BamHI resulted in a plasmid (pMBH-28) having the same complementing ability as pM10. B: Attempts to delete the DNA fragment between the SalI site on the chromosomal DNA and the SalI site on the vector DNA of plasmid pMBH-28 resulted in a plasmid which lost its ability to complement.

In this case, the DNA fragment between the HindIII-SalI sites and across both sites must be essential for L-SD activity. This encompasses a sequence of at least 4 Kb long. E. coli genes are on average about 1.5 Kb long, taking into account its regulatory region and coding region. The 4 Kb sequence would be enough to hold 2 genes. It is very likely therefore that this may be an operon, but it does not prove that both genes have something to do with L-SD.

IV C The Cloned DNA Does Not Contain the Wild Type Allele of sda84 Gene

If the clone carried the wild type sda84 gene, it should exhibit a different pattern from the parent strain in the hybridization experiment, for we know that the sda84 gene in strain MEW84 is separated into two pieces by placmu insertion, whereas the sda84 gene lies in the parent genome in a contiguous stretch. But unexpectedly, the hybridization bands of MEW84 were exactly the same as MEW1, indicating that

the cloned gene was not the same gene that was interrupted in strain MEW84. Then the question arises: what gene was it on the miniMu replicon that had the ability to complement sda84?

One may think that the cloned sequence might be the structural gene encoding L-SD, since the sdaA gene can complement every mutant isolated so far in our lab that is deficient in L-SD (Su, personal communication). This argument is easily excluded by the evidence that neither pM10 nor pM1 could complement MEW22, which possesses a λplacmu insertion in the structural gene. Moreover, the restriction map of the DNA fragment cloned on pM1 and pM10 was different from that of the sdaA gene.

There are two possibilities as to what the cloned gene could be: A: It could be a structural gene of a product that activates L-SD. In strain MEW84, placmu is inserted in a regulatory gene which regulates the cloned gene. When the gene exists in higher copy number, the cloned gene product is able to function independent of the regulatory gene. B: The cloned gene could encode a protein that has multiple functions. Under normal conditions, this protein performs a function that is not related to L-SD activation, when the conditions change, for example, the enzyme is detached from its cofactor - it can carry on its second function. In this case, the protein encoded by the gene(s) present on the clone is produced in large amount from the gene(s) on the high copy number plasmids, but the cofactor needed for its normal

function can not meet the needs: thus the protein starts its second function - activating L-SD.

The genetic location of the cloned DNA can be found out by the following experiment: one might insert an antibiotic-resistant gene into the cloned gene, by either *in vivo* or *in vitro* methods, and then force the cloned DNA interrupted with the antibiotic-resistance gene into the chromosomal DNA by homologous recombination in *polA* strain and select for double exchange. One would then map its location by the method described in the Methods section, and check to see if the strain is SGL⁻.

IV D   Possible Mechanism for the Increased Azaleucine Sensitivity of Strain MEW84

Strain MEW84 was more sensitive to azaleucine than the parent strain MEW1. This increased azaleucine sensitivity was not due to the deficient L-SD activity in MEW84, since MEW22, the structural gene mutant showed the same response, whereas strain KEC9 and MEW26, which have high level of L-SD activity showed the same azaleucine sensitivity as MEW84.

Azaleucine is an analogue of the amino acid L-leucine. It is a potent growth inhibitor of *E. coli*, because it can compete with leucine to be incorporated into protein, resulting in inactive enzymes. Azaleucine can also mimic leucine in its repressive action on the expression of the

biosynthetic enzymes for leucine, isoleucine and valine
(Umbarger, 1987). Several possible processes can be
postulated to affect azaleucine sensitivity: azaleucine
transport rate, the level of branched-chain amino acid
biosynthetic enzymes, and the degradation rate of leucine in
the cell.

sda84 was mapped around 55 minutes on the E. coli genome
where the azl gene is also located (Bachmann, 1987). The azl
gene is involved in the regulation of the ilv and leu genes
(Dickson et al, 1977; Williams et al.,1976), yet the mechanism
involved is not clear. Mutations in the azl gene that confer
resistance to azaleucine have been isolated (Williams et
al.,1976). These azl[R] mutants were shown to have non-
repressible synthesis of isoleucine, valine and leucine. One
might wonder if this gene encodes a nega'.ve-controlling
protein for the branched amino acids biosynthetic pathway.
Were this the case, mutation in this region might also result
in increased azaleucine sensitivity and sda84 could be allelic
to azl, if the mutation results in a repressor protein
uninducible by the inducer molecule. This does not seem
likely. First, because MEW84 is an insertion mutant, the
function of the sda84 gene is totally abolished. If the sda84
gene is the same as azl, and it encodes a repressor protein,
a lack of this protein can only make the strain azaleucine
resistant and not sensitive as MEW84.

Azaleucine can be transported into the cell by the

branched-chain amino acid (LIV-I) transport system and the aromatic amino acid transport system (Harrison et al., 1975). It has been shown (Quay et al.1977) that a livR mutation leads to increased azaleucine sensitivity, due to the trans-recessive derepression of branched-chain amino acid transport and periplasmic branched-chain amino acid-binding proteins. Although sda84 maps far from livR locus, sda84 may also affect the azaleucine uptake system by an unknown mechanism. For example, the sda84 mutation may alter the energy availability for these transport systems. The experimental observation that MEW84 also had a.. increased serine sensitivity (which will be discussed later) supports this hypothesis, because serine can also be transported into the cell by the Liv-I system (Quay et al., 1977).

Yet the azaleucine toxicity of the cells is not always correlated with the rate and capacity of a strain to uptake azaleucine. It was observed by Quay et al.(1977) that a strain which was 100 times more sensitive to azaleucine than the wild type strain had the same kinetic parameters for azaleucine uptake. It is possible that in strain MEW84, the leucine biosynthetic pathway is somehow slowed down, or the leucine degrading process is speeded up by sda84 mutation, i.e., the cell has a low internal leucine pool. In these cases, the amount of cellular azaleucine compared to leucine could be higher, such that azaleucine has a better chance to be incorporated into proteins even if the azaleucine transport

rate remains unchanged. This idea is supported by the observation that leucine reversed the slow growth of MEW84 partially.

Extracelluar azaleucine can be exchanged with internal leucine through the LIV-I system (Quay et al., 1977). Under normal conditions, the decrease in internal leucine leads to a leucine-limiting signal for the derepression of the branched-chain amino acid biosynthetic enzymes. Presumably intracellular azaleucine does not substitute for leucine in preventing this derepression (Quay et al., 1977). Increased azaleucine sensitivity of MEW84 could then be a consequence of the leucine biosynthetic pathway having a decreased ability to respond to the signal or leucyl-tRNA synthetase. For example, the strain may not able to make enough leucine to compensate for the loss of leucine through the exchange with azaleucine.

IV E Regulation of sda84 Gene

L-SD activity is induced by many factors: growth in rich medium; high temperature; glycine and leucine; and by mutations in the ssd and rbl genes (Newman et al., 1982c; Newman et al., 1982a and Lin et al., 1990).

Rich medium not only causes an increase in L-SD activity level in the parent strain, but also induce L-SD activity in the putative structural gene mutant MEW22. Su Hongsheng (paper

in preparation) has shown that two different L-SD enzymes encoded by sdaA and sdaB are presented in E. coli K-12. The sdaA gene expresses in minimal and in rich medium, whereas the sdaB gene functions only in rich medium.

Transcription of the sdaA gene was increased in LB as shown by the β-galactosidase activity under the promoter of sdaA gene. Su (paper in preparation) has shown that transcription of sdaB, encoding the second L-SD is also turned on in this medium. Transcription of sda84 was also increased under the same condition. The cell does this likely to catch up with the needs of activating two L-serine deaminases. It is also possible that the cell increases sda84 transcription under this condition to carry out some other function, about which we are not clear yet.

Growth at 42°C decreased the transcription of sda84. This contradicts with the observation that L-SD activity is elevated at 42°C. Since the transcription of sdaA was also decreased (Su et al., 1989), it may be that the increased L-SD activity is an indirect consequence of enzyme(s) other than the sdaA gene product. For example, some enzymes are capable of deaminating L-serine to form pyruvate (Brown et al, 1990). Overly high L-SD activity at high temperature could be very harmful to the cell. Consequently the expression of the genes for both the structural and activating system might be repressed to counteract this effect.

The experiments with MEW84 were always done using glucose

as sole carbon and energy source. It is known that many inducible operons are controlled by complementary mechanisms that use cyclic AMP and specific inducers as the signal molecules. In such systems, glucose inhibits the synthesis of cAMP and prevents the expression of these operons (catabolite repression) (Stryer, 1975a). To test whether the sda84 gene is subject to catabolite repression, the expression of sda84 was examined in minimal media supplemented with different carbon sources, by testing the β-galactosidase activity of MEW84 .

These different carbon sources had little effect on the transcription of the sda84 gene, suggesting that there is no repression of sda84 transcription by glucose.

The effects on sda84 transcription are all minor and may not be very significant. The greatest effect was observed in LB, but even then only a 2-fold difference was noted. So I am not really surprised that other things do not have much effect.

IV F Possible Mechanism of the Increased L-Serine Sensitivity of MEW84 and Its Release by Threonine

E. coli cells are subject to serine inhibition (Amos and Cohen, 1954). It is also known that serine interferes with the isoleucine biosynthetic pathway and usually this inhibition can be released by the addition of isoleucine to

the medium (Amos and Cohen, 1954; Uzan and Danchin, 1978).
Strain MEW84 showed an increased serine sensitivity which
could not be reversed by the presence of isoleucine and valine
in the medium.   It was reported (Cosloy and McFall, 1970)
that mutations at different genetic loci could result in
increased serine toxicity. Serine toxicity, perhaps involves
different mechanisms.

Serine toxicity in strain MEW84 probably results from a
deficiency in L-SD activity, since all strains in our lab
lacking L-SD showed the same sensitivity as MEW84.   Strains
with normal (MEW1) or higher L-SD activities (MEW26) had no
trouble at all in growing in the presence of 100 ug/ml L-
serine. This is further supported by the observation that the
MEW84/rbl double mutant which had a slightly higher L-SD
activity than the MEW84 strain, due to the increased
transcription of the sdaA gene caused by the rbl mutation,
was able to overcome the increased serine toxicity.

Threonine but not aspartic acid was able to reverse the
hyper-serine sensitivity of MEW84, suggesting that the serine
inhibition site occurs before threonine and after aspartic
acid in the branched-chain amino acid biosynthetic pathway,
probably the homoserine dehydrogenase I, as observed by Hama
et al. (Hama et al., 1990).   If serine toxicity depends on
inhibition of homoserine dehydrogenase I, one would expect
that overcoming serine toxicity would require isoleucine,
threonine and methionine, all of which are derived from

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homoserine. However, overcoming toxicity of serine to the wild-type cell with its usual L-SD activity requires only isoleucine. Similarly, even the hypersensitive MEW84 needs threonine and isoleucine but not methionine. The following is one possible explanation:

In the branched-chain amino acid biosynthetic pathway, aspartate semialdehyde is converted to homoserine by two enzymes: homoserine dehydrogenase I and homoserine dehydrogenase II (Cohen and Saint-Girons, 1987). In E. coli, the former enzyme activity is very important because the activity of homoserine dehydrogenase II is low (Hama et al., 1990). If serine inhibition is not complete, homoserine dehydrogenase II, together with the residual activity of the homoserine dehydrogenase I could synthesize some homoserine from aspartate semialdehyde. Minimal amounts of methionine and threonine would thus be synthesized to maintain the cells' growth. Homoserine succinyltransferase, which is the first enzyme to convert homoserine to methionine, seems to have a better ability to compete for homoserine than the homoserine kinase, which leads to the formation of threonine. Therefore most of the homoserine goes into the synthesis of methionine, and less is used for the synthesis of threonine (refer to introduction). L-serine is also a substrate for threonine deaminase (Isenberg and Newman, 1974), which is the first enzyme to convert threonine to isoleucine. L-serine therefore competes with threonine to occupy the active site of threonine

deaminase (This was not the case for the strains used in this study, since the strains in our lab were all Δ ilvA). This effect of serine, together with its inhibition of homoserine dehydrogenase I, would prevent the synthesis of isoleucine from threonine under these conditions. So once the cell is limited in homoserine production, the necessity for the three amino acids in the cell would be in the order of: isoleucine, threonine, methionine. In cells with normal L-SD activity, serine inhibition is relieved by degrading exogenous serine with L-SD, and so only isoleucine would be necessary.

The experimental observation that exogenous methionine had an additive effect on serine inhibition must be due to the fact that homoserine dehydrogenase II is subject to methionine inhibition (Cohen and Saint-Girons, 1987).

IV G Effect of rbl Mutation on Strain MEW84

The double mutant MEW84/rbl grew more slowly in glucose minimal medium than either MEW84 or MEW26, and this slow growth was compensated by adding methionine or serine and threonine to the medium. Serine alone inhibited the growth of MEW84/rbl before it was purified. Inhibition by serine could be overcome as a result of purification. The latter phenomenon is a consequence of the combination of the sda84 and rbl genes.

rbl is a highly pleiotropic gene that affects the

activity of a number of enzymes involved in different metabolic pathways (Lin et al., 1990). MEW26 (rbl) has increased L-SD activity and decreased transcription of serA (Lin et al., 1990). We can postulate that the size of the serine pool inside this cell must be very small. In fact, serine alone could restore the slow growth of MEW26. Methionine alone also had a considerable effect. This is not hard to conceptualize if we consider the metabolic relationship between serine and methionine. As for the double mutant, serine production is still limiting (due to the effect of rbl on serA). However, the extra serine supplied externally could not be extensively broken down, due to the lowered L-SD activity by sda84 mutation, so that it inhibits the cell growth. Thus the MEW84/rbl grew normally only in the presence of both serine and threonine, although the double mutant could partially overcome serine toxicity.

V Summary

Strain MEW84, together with strains MEW128 and MEW191, seemed all related to the post-translational activation of the proenzyme of L-serine deaminase, since they all showed no L-SD activities in whole cell assay, however their L-SD activities in crude cell-extract could be activated by iron and DTT. Differentiated by their map locations, these three mutants must compose a complex system for L-SD activation, and more undiscovered genes might be involved in this system. The relationship among these genes and the L-SD activating mechanism are not understood yet. It is very likely that this L-SD activating system is related to the thiamine biosynthetic pathway, since strains MEW84, MEW128 and MEW191 all required thiamine to grow in glucose minimal medium.

In order to understand the activating mechanism of L-SD, I wished to clone and sequence the sda84 gene. A DNA fragment complementing the growth on SGL medium, restoring the L-SD activity and thiamine independence of strain MEW84 was obtained on miniMU replicon by using the miniMu in vivo cloning technique. This DNA fragment was subsequently subcloned to plasmid pBR322. However, the Southern hybridization analysis indicated that the cloned DNA piece did not contain the corresponing sda84 gene. Since the cloned gene was not able to complement any other L-SD deficient mutants in our lab except strain MEW84, it should be a gene

unique to strain MEW84.

L-SD activity is subject to many controls. However, the transcription of gene sda84 was little influenced by the factors known to affect L-SD activcity. By testing the β-galactosidase activities from the promotor of the sda84 gene, it was known that: the transcription of the sda84 gene was not changed by glycine and leucine when grown in glucose minimal medium; decreased slightly by growth in glucose minimal medium at elevated temperature or by either ssd or rbl mutation; increased less than one-fold when grown in rich medium, as compared to the 13-fold enhancement of the transcription of the L-SD structural gene under the same conditions. To test whether the transcription of the sda84 gene was under catabolite repression, the expression of the sda84 gene was examined in minimal medium supplemented with different carbon sources. The β-galactosidase activities obtained suggested that the sda84 gene was not subjected to catabolite repression.

Gene sda84 located near gene azl and strain MEW84 showed more sensitivity to azaleucine than the parent strain. The background information of azl mutation suggested that sda84 gene was not equal to azl gene. By comparing the azaleucine sensitivity of strain MEW84 with other strains devoid of L-SD activities, it was clear that the low L-SD activity in strain MEW84 was not responsible for the increased azaleucine sensitivity of this strain.

Strain MEW84 was highly sensitive to L-serine when grown in glucose minimal medium. This serine hypersensitivity was apparently caused by the decreased L-SD activity in strain MEW84, since all the L-SD deficient strains had the same phenotype.

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