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The Map Position of the sdaA and sdaB Mutations  
and the Cloning of the ssd Gene  
in Escherichia coli K-12

Daniel J. Dumont

A Thesis

in

The Department

of

Biology

Presented in Partial Fulfillment of the Requirements  
for the Degree of Master of Science at  
Concordia University  
Montréal, Québec, Canada

December 1985

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## ABSTRACT

### The Map Position of the sdaA and sdaB Mutations and the Cloning of the ssd Gene in Escherichia coli K-12

Daniel J. Dumont

In this thesis the map position of two mutations resulting in a deficiency in L-serine deaminase activity have been determined. The wild type gene of a third mutation resulting in an increase in L-serine deaminase activity has been identified on a clone and subcloned.

The sdaA mutation has been located between the lip and gal genes between 15 and 17 minutes and the Escherichia coli K-12 genome. The sdaB mutation has been located to the right of the metE163 at 86.6 minutes on the Escherichia coli K-12 linkage map.

The ssd gene was identified on a cosmid obtained from D. Touati and subcloned to a 3.4 kilo base pair fragment. This has allowed a close determination of the position of ssd, namely between the cpxA and pfkA genes.

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This thesis could never have been completed if it were not for my mother's encouragement and strength during our trying times.

DANIEL J. DUMONT

**Dedication**

**To the Memory of My Father.**

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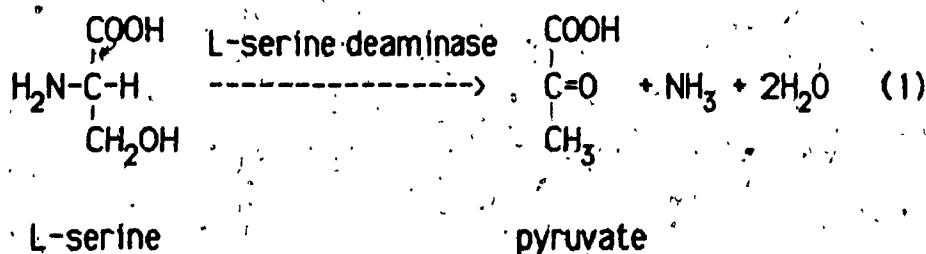
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## Introduction

The enteric bacterium, Escherichia coli K-12, is unable to grow on L-serine as the sole carbon source (27,28), but will use L-serine as a secondary source of carbon when grown with a small amount of glucose (28). The enzymatic activity responsible for degrading L-serine is known as L-serine deaminase (L-SD). It catalyzes the non oxidative-deamination of L-serine to pyruvate:



When strain CU1008 is grown under conditions known to induce L-serine deaminase activity, (e.g. anaerobic growth or growth at 42°C). It is able to use L-serine as the chief source of carbon. Similarly, it can grow with L-serine, glycine and leucine (28). This

may be due to an increased pyruvate supply made available by the higher L-SD levels (26), though other factors may also be involved.

That L-SD activity is absolutely required for growth on L-serine was demonstrated by the characterization of various mutants from strain CU1008 with altered levels of L-SD. Strains that were selected for growth on L-serine as the sole carbon source had L-SD levels which were 7 to 10 times higher than the wild type level (25). These high L-SD levels would presumably deaminate L-serine at a great enough rate to supply enough pyruvate to support growth. Conversely, mutants unable to grow on L-serine, glycine and leucine as carbon sources showed a decreased level of L-SD activity (26).

Although E. coli K-12 cannot use L-serine as sole carbon source, it can use it as an auxiliary source. When E. coli K-12 strain CU1008, was grown with limiting glucose and L-serine, its yield was twice that of cells grown with limiting glucose only (28). This



increase was attributed to the deamination of L-serine which supplies pyruvate for biosynthesis and energy. The fact that this deamination of L-serine is not sufficient to allow E. coli to grow with L-serine as sole carbon source suggests that there may be other factors other than L-SD levels that affect growth on L-serine as the sole carbon source.

#### Factors Influencing L-SD Activity

Unlike other degradative enzymes which are induced by their substrate, i. e. histidases from Klebsiella aerogenes and Salmonella typhimurium and E. coli tryptophanase (10, 35), L-SD is not induced by its substrate, L-serine (24). This is an interesting result, because one would think that synthesis of an enzyme responsible for the degradation of a particular compound would be controlled primarily with reference to changes in the availability of that compound.

The induction of L-SD activity does not follow any such pattern.

L-SD activity is induced in response to DNA damaging agents (23) suggesting that the induction of L-SD is associated with the well studied "SOS system" (17, 38). However it is also induced with these factors: growth at 42°C, anaerobically, upon the addition of 6% ethanol and in the presence of glycine and leucine (29, 28, 24). The variety, and number of inducers raises the question as to whether serine deamination is the result of a single L-serine deaminase, or whether there may other enzymes capable of deaminating L-serine, perhaps even as a side reaction.

## Isolation of Strains with Altered L-SD Levels

### Isolation of L9ssd

Strains which are able to use L-serine as the sole carbon source are readily obtained by plating strain CU1008 onto L-serine (25). The colonies that arise are then tested for some of the characteristics of the ssd mutation which are: a map location near metB, the inability to grow on succinate, high levels of L-SD activity, increased resistance to kanamycin and neomycin, and increased sensitivity to UV irradiation. Strain L9ssd was isolated in this manner and shown to possess the characteristics described above. I have shown that this strain is unable to grow either anaerobically or at 42°C on glucose minimal plates, and that this is due to the ssd mutation. I have subcloned from a larger plasmid and shown that it is located between the cpxA and pfkA genes on a 3.4 kilobase pair (kbp) fragment of the E. coli genome.

### Isolation of MEW128sdaA

While ssd mutants can be directly selected, the isolation of strains deficient in L-SD activity is more involved. The sdaA mutation was obtained from a culture of strain CU1008 that was irradiated with UV radiation. The culture was then plated under conditions in which L-SD activity should be necessary for growth, i.e. media supplemented with L-serine, glycine and leucine (28). Among colonies unable to grow on this medium, some should be deficient in L-SD activity. Strain MEW128sdaA was isolated in this manner. It was unable to grow on SGL, was deficient in L-SD, required thiamine, and was somewhat resistant to bacteriophage T7 (24, 26). In this work, the sdaA mutation is shown to be linked to genes within the 15 to 17 minute region of the E. coli linkage map between lip-9 and gal-1. The experiments used to map this mutation suggest the possibility that the sdaA mutation is suppressible by the amber suppressor, glnV. The various means of suppression, particularly

nonsense suppression, will therefore be discussed later in this introduction.

### Isolation of sdaB

We were unable to take advantage of the Mu::lac operon fusion technology (i.e. Mu::dl (9)), because of the limited use of this phage at high temperatures. The development of a derivative, Mu::dX (4), which could be used at higher temperatures allowed us to use Mu::dX as a mutagenic agent. A review of how Mu::dX was constructed and a description of its properties is presented later in this introduction.

Strain MEW1 was infected with a phage Mu::dX lysate selecting Mu::dX lysogens as antibiotic resistant colonies. These colonies were screened in the same fashion as that described for sdaA, i.e. for the inability to grow on SGL medium.

Strain MEW191 sdaB was obtained in this fashion and was shown to be the result of a single mutation, and to have the same phenotype as MEW128 sdaA, a decreased level of L-SD, an inability to grow on SGL, an increased resistance to phage T7, and a requirement for thiamine (25, 26). In this work, sdaB was differentiated from sdaA by demonstrating it to be linked to chlB, metE and to the ilv gene cluster at 86.5 minutes.

#### Nonsense Suppression

Experiments presented in this thesis indicate the sdaA mutation may be suppressible by an amber suppressor, glnV. This could mean that the sdaA mutation lies within the coding region of the gene and not the regulatory region.

Suppression can be defined as a second lesion within the mutant's genome, which allows the cell to regain a pseudo-parental or

parental phenotype. The additional lesion causing the suppression may be located in the same gene (intragenic suppression) or elsewhere in the genome (extragenic) (33):

An intragenic suppressor is a second mutation within the same gene, and this would allow the protein coded by that gene to regain full or partial biological function.

Extragenic suppression is caused by a mutation in a second gene. Some of these mutations suppress by allowing a functional product to be synthesized from the mutated gene. This means of suppression often involves a mutation within genes that code for proteins which are part of the DNA transcription/translation machinery of the cell. The genes usually affected are those that code for transfer RNAs (tRNA) (33).

Mutations which cause a change of an amino acid specifying codon in messenger RNA (mRNA) into one of three terminator codons, UAG, UAA and UGA, are called nonsense mutations. These mutations lead to premature termination of translation. Suppression of these mutations is usually mediated via mutated tRNA's which have altered anticodons that recognize these termination codons and insert an amino acid, allowing translation to continue. This type of suppression of course is dependent on whether the inserted amino acid allows the protein to perform its biological role. The three types of nonsense mutation are classified by the codons on which they act, amber (UAG), ochre (UAA) and opal (UGA).

The glnV mutation codes for an altered tRNA which suppresses amber mutations by inserting a glutamine residue. The glnV gene is part of a tRNA operon coding for 7 different tRNAs, located at 16 minutes on the E. coli linkage map (22).



Phages Mu, Mu::d1 and Mu::dX

The mutator effect of the temperate bacteriophage Mu first identified in 1963 (34) has been well studied. Phage Mu causes mutations by integrating into the host genome, terminating transcription of that gene and not allowing an intact gene product to be made (7, 14). If the insertion is within an operon, the genes distal to the insertion are also not transcribed due to the premature termination of transcription. Therefore these mutations are considered to be very polar (14).

By fine structure mapping of Mu insertions into the lacZ gene of E. coli, it was shown that Mu insertions were distributed throughout the entire gene, suggesting that the insertions occur randomly (6). This randomness should allow one to obtain Mu mediated mutations within any non-essential gene. Phage Mu can therefore be used as a mutagen. This randomness may not be complete; under conditions that

increase the transcription of a given gene, i.e. induction, the probability of Mu inserting into that gene is decreased (14). The main reason for this is that the translation/transcription machinery of the cell blocks access to the DNA. Usually infections with Mu are done in rich media where the cell's biosynthetic pathways are repressed. Therefore this is not usually a problem (14).

Phage Mu is also considered to be a transposable element (6).

Upon induction of a Mu lysogen, Mu will initiate 100 or more transposition events before encapsidation and lysis occur (11).

The integration of phage Mu into the host does not require all of the Mu DNA information, but only that which is carried on both ends of the Mu genome (8). This fact was used to construct a Mu-lac specialized transducing phage (8). This phage also carried the transposon Tn3 which carries a gene that imparts ampicillin resistance. The phage so constructed, Mu::d1, has the ends of Mu

flanking the lacA,Y,Z genes and the Tn3. The phage retains the ability to insert into the genome virtually at random (8). The lacA,Y,Z genes have their initiation site, but are without their promoter. This truncated lac operon is located close to one end of Mu so that if the prophage is inserted in the proper orientation and close to a promoter, the transcription of these lac genes is governed by that promoter. By recording the levels of  $\beta$ -galactosidase produced by the insert under various environmental conditions, one can study regulation of the gene normally expressed from that promoter.

The defect of Mu::d1 in the design of the experiment is that the Mu::d1 phage does not form stable inserts at temperatures above 30°C. This is because the phage has a temperature sensitive repressor protein, cts, which is responsible for maintaining the phage in the lysogenic state(4).

In order to construct strains which could be studied at 37°C and above which is the temperature used to study L-SD a Mu::d1 derivative, Mu::dX was made, in which the transposon Tn9 (which carries a gene for chloramphenicol resistance) was inserted into the B gene of Mu::d1 (4). Since polar mutations within the B gene of Mu, called X mutations, eliminate this temperature sensitivity (7) and reduce the frequency of secondary transposition (4), strains carrying Mu::dX could be grown at higher temperatures.

Phage Mu lysogens are immune to further infection by Mu. The immunity can be overcome by increasing the multiplicity of infection during infection which then leads to polylysogens (14). This immunity to superinfection is still retained by both phage constructs Mu::d1 and Mu::dX (4). Strains carrying only single inserts, i.e. single mutations, can be obtained if the multiplicity of infection is kept at a low level.

**Materials & Methods:****Media:**

**Minimal Medium:** 5.4 g  $K_2HPO_4$ , 12.6 g  $KH_2PO_4$ , 2 g  $(NH_4)_2SO_4$ , 2 g  $MgSO_4 \cdot 7H_2O$ , and 0.1 g  $CaCl_2$  at pH 7 in 1 liter of glass distilled water (GDW).

**Luria Broth (LB):** 10 g Bacto tryptone, 5g Bacto yeast extract, 5g NaCl in 1 liter of GDW.

**Solid Media:** As above with either 20g/liter Bacto agar or 8g/liter Gelrite (Kelco 63002A).

**Carbon Sources** All the carbon sources were added to a final concentration of 0.2% (w/v) unless indicated otherwise.

**Amino Acid and Vitamin Supplements:** When amino acid supplements were required, they were added to minimal media to the following concentrations:

Histidine	20 $\mu\text{g/ml}$
Isoleucine	50
Valine	50
Leucine	20
Arginine	50
Tryptophan	30
Tyrosine	25
Threonine	50
Proline	50
Methionine	20
Thiamine	5

Special Plates:

Plate lysate plates (LB Ca glu):

LB solid medium supplemented with  $\text{CaCl}_2$  and glucose to a final concentration of 2 mM and 0.1% respectively. Top agar (R-top) for plate lysates contained all the above ingredients with a lower concentration of agar (8 g/L).

**NIV SGL T.T.E.:**

Amount per liter	8g/liter of Geirite agar
------------------	--------------------------

0.02g	isoleucine
-------	------------

0.02g	valine
-------	--------

0.65g	glycine
-------	---------

0.15g	leucine
-------	---------

2.0g	serine
------	--------

0.01g	thiamine
-------	----------

To this was added 1 ml of each of the following solutions per liter.

$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	5 g/L
---	-------

$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.1g/L
---	--------

$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	0.12g/L
---	---------

**NSIV T.T.E.:**

Identical to NIV SGL T.T.E. without glycine and leucine.

**MacConkey Agar Plates:** 40g MacConkey agar/liter

Carbon Source to 0.2%

**Chlorate Medium:**

LB solid medium with 2 g/l of  $\text{KClO}_3$  added after autoclaving.

**Antibiotics:** Added to the growth media to the following final concentrations:

Ampicillin	50 $\mu\text{g/ml}$ (Mu dx), 100 $\mu\text{g/ml}$ (plasmid selection)
Kanamycin	20 $\mu\text{g/ml}$
Tetracycline	10 $\mu\text{g/ml}$
Chloramphenicol	12.5 $\mu\text{g/ml}$ , (170 $\mu\text{g/ml}$ for plasmid amplification.)

Solutions and Buffers:

**MC:** 100 mM  $\text{MgSO}_4$ , 5 mM  $\text{CaCl}_2$

**20X SSC:** 3M NaCl, 0.3 M Na Citrate, pH 7

**TE:** 10 mM Tris-HCl, 1 mM EDTA

**LB Citrate:** LB + 0.5 M Na citrate

**RNAse A:** Was dissolved at 1mg/ml in 10mM Tris-HCl

pH 7.6. The solution was incubated in a boiling water bath for 10 mins. One ml aliquots were stored at  $-20^\circ\text{C}$ .

**Pronase:** Was dissolved at 1 mg/ml in 10 mM Tris-HCl pH 7 incubated at  $37^\circ\text{C}$  for 1.5 hrs. and then stored at  $-20^\circ\text{C}$ .



**Electrophoresis (E) Buffer:**

40 mM Tris OH

20 mM acetic acid

2 mM Na<sub>2</sub> EDTA

pH 8.1

**20X Digest Buffer:**

0.12M Tris-HCl pH7.5

0.12M MgCl<sub>2</sub>

1.5 M NaCl

**Ligation Buffer:**

0.5 M Tris (pH7.4)

0.1 M MgCl<sub>2</sub>

0.1 M Dithiothreitol

10 mM Spermedine

10 mM ATP

1 mg/ml BSA

**20X BSA:** 50 mg/ml Bovine Serum Albumin in Tris-HCl  
pH 7.5

**5X BPB Loading Dye:**

50% Sucrose

0.125% Bromophenol Blue

2.5 strength E Buffer

**MgSO<sub>4</sub>•HEPES:** 10 mM MgSO<sub>4</sub>, 2 mM HEPES, pH 7.5

**CaCl<sub>2</sub>•HEPES:** 50 mM CaCl<sub>2</sub>, 2 mM HEPES, pH 7.5

**Rapid Isolation Buffer:**

5% Sucrose

50 mM Tris- HCl

50 mM EDTA

5% Triton X-100

Filter sterilized and stored at 4°C.

**"Lysis Buffer":** 150 ml

18.75 ml EDTA (0.5M)

7.5 ml Tris-HCl (1M) pH 7.6

4.5 ml Triton X100 10%

Made to 150-ml with GDW, filter

sterilized and stored at 4°C.

**"Resuspension Buffer":** 150 ml

37.5g Sucrose (RNase Free)

7.5 ml Tris-HCl (1M) pH 7.6

120 ml EDTA (0.5M)

Made to 150 ml with GDW, filter

sterilized and stored at 4°C.

**P1 Plate lysates:**

Bacteriophage P1 vir used in this work was grown by plate lysate. Cells were inoculated into 5 ml of Luria Broth (LB) containing 0.15 ml of 1% CaCl<sub>2</sub> (Ca) and incubated at 37°C. Overnight grown cells were subcultured by adding 0.1 ml into fresh LB Ca (5 ml) and allowed to grow at 37°C until they were in the exponential phase. Of these cells 0.2 ml were added to 0.1 ml of phage (diluted to approximately 10<sup>6</sup> phage per ml) in small test tubes and allowed to incubate at 30°C for 20 minutes. Two and a half ml of R-top agar (kept at 46°C) were added to each tube and immediately plated on LB Ca glucose plates. Plates were harvested 24 hours later by adding 2.5 ml LB Ca on to each plate with swirling at every half hour over the next 3 hours. The liquid portion of each plate was collected in small test tubes containing 0.5 ml chloroform (CHCl<sub>3</sub>). Tubes were vortexed vigorously for 30 seconds, left at room temperature for 10 minutes and then centrifuged to precipitate the cell debris. The

supernatant, which contained the phage, was collected and stored at 4°C in tubes containing 0.5 ml  $\text{CHCl}_3$ .

### **Bacteriophage Sensitivity Tests:**

For phage T7, these tests were done in the same manner as Pl vir plate lysates. For phage Mu, cultures to be tested were grown in LB with 2.5 mM  $\text{CaCl}_2$  and 2.5mM  $\text{MgSO}_4$  and plated on the same medium.

### **Bacteriophage Mu cts Lysate:**

The Mu cts lysogen, CU1008 Mu cts was grown overnight in LB with 2.5mM  $\text{CaCl}_2$  and 2.5mM  $\text{MgSO}_4$  at 28°C. It was then subcultured 1/20 into fresh medium, and shaken vigorously at 37 °C. At an optical density at  $A_{550 \text{ nm}} \leq 0.4$ , it was transferred to 43°C for 20 to 30 minutes and then returned to a 37°C shaker. When the cells lysed

after 75 minutes, 0.5 ml of chloroform was added. The tube was vortexed and centrifuged to sediment the cell debris. The supernatant was then removed and stored at 4°C over chloroform.

### **P1 vir Transductions:**

Bacteria grown overnight in 5 ml LB at 37°C in small tubes were centrifuged resuspended in 5 ml MC buffer and starved for 20 minutes by placing them on a rotor at 30°C. Of these cells 0.1 ml were added to, 0.1 ml of various dilutions of the particular phage to be used. Controls contained no phage. The tubes incubated at 30 °C for 20 minutes. Of a 1 M sodium citrate, 0.2 ml was added to each tube to prevent further absorption of P1 phage by chelating the Ca<sup>2+</sup> ions. Three ml of F-top agar kept at 46°C were added to each tube. The contents of the tube were then plated immediately and incubated at 37°C, until colonies appeared.

The results in Table 2 are from two transductions, all other experiments use only one transduction.

### **L-serine deaminase assay:**

The L-serine deaminase assay was based on the production of pyruvate from L-serine according to the method of Pardee and Prestige (28) as modified by Isenberg and Newman (16).

Activity was expressed as nanomoles of keto acid (pyruvate) produced by 0.1 ml of a 100 Klett unit suspension of cells (filter No. 54) in 35 minutes.

### **Large Scale Plasmid Isolation:**

The cultures from which the plasmid DNA was to be prepared were grown overnight in Luria Broth with 0.2% glucose and antibiotic. If the plasmid was amplifiable, chloramphenicol was added to the culture when it reached an approximate optical density of 0.4 at 600 nm. After approximately 14 hours incubation, the culture was centrifuged and the pellet resuspended in 10 ml of resuspension

buffer". To this was added 1.0 ml of a freshly prepared 50 mg/ml lysozyme solution in 10 mM Tris-HCl (pH 7.5) followed by 1.0 ml of RNAase A. The mixture was kept on ice for 15 minutes, after which 4.0 ml of 0.5 M EDTA was added and the incubation on ice continued for an additional 10 minutes. Pronase (1 ml of 20 mg/ml) was added and incubated on ice for another 15 minutes. Fifteen ml of "Lysis Buffer" was added and mixed by inversion gently so as to minimize plasmid shearing. The tubes were then centrifuged at 15,000 rpm at 4°C for 1 hour and the cleared lysate, containing plasmid DNA, was decanted.

#### Isolation of Plasmid DNA in a Cesium Chloride Density Gradient

For each 8 ml of cleared lysate, 7.5 g of Cesium Chloride and 0.3 ml of Ethidium Bromide (5 mg/ml) were added. The sample was centrifuged either 60 hours at 37,000 rpm or for 40 hours at 43,000 rpm in a Beckman Ultracentrifuge. The plasmid DNA was removed by inserting a 20 gauge needle in the appropriate level. The ethidium



bromide was removed from the plasmid DNA by extracting with isopropanol saturated in 40X SSC.

### **Rapid Isolation of Plasmid DNA:**

Ten ml of cells grown either with or without amplification, were centrifuged at 3,000 rpm and resuspended into 600 $\mu$ l of "Rapid Isolation Buffer" with 100 $\mu$ l of 25 % sucrose in 10 mM Tris-HCl (pH8) and 50 $\mu$ l of a 10mg/ml freshly prepared lysozyme solution in 10 mM Tris-HCl (pH8). This mixture was incubated on ice for 1 hour. The tubes were placed in a boiling water bath for 1 minute, cooled on ice for 1 minute and centrifuged for 15 minutes. The resulting mucoid pellet was then removed with a sterile tooth pick. The remaining lysate was extracted once with 45% redistilled phenol, 45% isoamyl alcohol, 10% chloroform solution kept at 4 °C and saturated with TE buffer. The DNA was extracted with ether until the lysate became clear. The DNA precipitated with isopropanol dried and resuspended in TE buffer:

### **Ethanol or Isopropanol Precipitation of DNA:**

To precipitate the DNA from solution, 2.5 volume of absolute ethanol and 0.1 volume of 2.5 M ammonium acetate were added, incubated either in a dry ice ethanol bath for 15 minutes or at  $-70^{\circ}\text{C}$  for at least 1 hour and centrifuged at 13,000 rpm for 15 minutes. The ethanol was drawn off with a Pasteur pipet and the DNA dried in a dessicator under vacuum.

Isopropanol precipitation were carried out in a similar manner using 2 volumes of isopropanol and washing the DNA pellet with cold 70% ethanol.

### **Restriction Endonuclease Digests:**

All digests were performed at  $37^{\circ}\text{C}$  for 4 hours using 20X "Digestion Buffer" and 20X "BSA". The volume of the reactions were adjusted using double distilled deionized water. The reactions were terminated by placing the tubes in a  $65^{\circ}\text{C}$  water bath for 10 minutes

(heat inactivation). The enzymes used were from "Boehinger Mannheim", (Bam HI, Eco RI and Pst I) and BRL (Hind III).

### **Dephosphorylation of Digested DNA Using C. I. A. P. (Calf Intestinal Alkaline Phosphatase)**

One  $\mu\text{g}$  of plasmid DNA was resuspended to 50  $\mu\text{l}$  with 20mM Tris HCl. Then 0.14 units of C.I.A.P. was added and the mixture incubated for 30 minutes at 37°C and then heat inactivated. The mixture was extracted sequentially with, a) phenol, b) phenol : water (1.25 : 1), c) twice with chloroform and c) ether. The DNA was then precipitated with ethanol.

### **Ligation Reactions:**

Ligation mixtures were mixed with 1 to 4 units of T4 DNA ligase from "Boehringer Mannheim" and 10X Linker Ligation Buffer and incubated for 12 to 16 hours at 15°C. The reaction was terminated by heat inactivation.

### Transformation:

Precultures in Luria Broth (LB) were subcultured, grown to a turbidity of 50 Klett units and centrifuged at 4 °C. The pellet was resuspended in 5 ml of  $MgSO_4 \bullet HEPES$ , incubated on ice for 20 minutes, recentrifuged, resuspended in 5 ml of  $CaCl_2 \bullet HEPES$  and incubated on ice for 25 minutes. The cells were again centrifuged at 4°C and resuspended with 1 ml of  $CaCl_2 \bullet HEPES$ . The cells were competent at this stage.

DNA was added to the competent cells which were incubated for an additional 25 minutes on ice. The cell suspensions were transferred to a 37°C for 3 minutes after which 1.5 ml of LB was added and the cells incubated at 37°C for 1.5 hours. After this time, 0.2 ml of these cultures were plated on the appropriate medium. Two controls were plated. One contained DNA, but no cells. The other contained cells, but no DNA. The plates were incubated at 37 °C. Colonies usually were visible after 1 day.

### **Anaerobic Growth:**

Anaerobic growth was attained in 2 fashions, both with the "BBL" gas chamber. In the first method, which was used in testing L9ssd anaerobic growth, the chamber was flushed with nitrogen gas for 15 minutes. The chamber was then sealed and placed at 37°C. This method did not provide complete anaerobiosis, as shown by the anaerobic indicator. The second method used "BBL, Hydrogen + Carbon Dioxide generating envelopes" and provided a more anaerobic environment as shown by the indicator. This method allowed growth of chlorate resistant strains on complex medium, but could not be used for L9ssd tests because the parent strain, CU1008, would not grow on minimal glucose medium under those conditions.

### **Preparation of Colicin E1 (Col E1):**

The procedure for purification of colicin E1 was adapted from reference (31).

## Calculation of Map Distances

### From Cotransduction Frequencies:

The formula used to convert cotransduction frequency to map units is (39):

$$\text{coTD} = (1 - t/d)^3$$

Where, "coTD" is equal to the cotransduction frequency between the two genes, "t", the distance between the 2 genes, and "d" the length of the transducing fragment. For transductions using phage P1 this distance is equal to 2 minutes on the E. coli linkage map.

---

Table 1

## Strains Used in This Thesis

Strain	Relevant Genotype and Characteristics	Source
CU1008	<u>ilvA</u>	L. S. Williams
CU1008 <u>metB</u>	<u>ilvA metB</u>	This Laboratory
MEW1	CU1008 $\Delta$ <u>lac ilvA</u>	(26)
MEW128	CU1008 <u>sdaA ilvA</u>	(26)
MEW191	MEW1 <u>sdaB::Mu::dX ilvA</u>	(26)
MEW191C	MEW1 <u>sdaA, ilvA</u> cured of <u>Mu::dX</u> insert, retaining <u>sdaA</u> mutation	This work
MEW206	MEW1 <u>dps::Mu::dX</u>	(24)
181-34	MEW1 <u>ser::Mu::d1</u>	This Laboratory
CU1008 <u>Mu cts</u>	<u>ilvA, Mu cts</u> lysogen	This Laboratory
CGSC 6092	<u>trxA1, metE70, ilvE720::Tn5,</u>	B. Bachmann
CGSC 4443	<u>chlB2</u>	

Table 1 cont'd.

Strain	Relevant Genotype and Characteristics	Source
CGSC 6057	<u>ubiF411</u> , <u>asnB::Tn5</u> , <u>nagB2</u> , <u>asnA31</u>	B. Bachmann
CGSC 6403	<u>metE163::Tn10</u>	"
CGSC 4278	<u>gltA6</u> , <u>supE44</u> ( <u>glnV44</u> )	"
CGSC 4286	<u>lip-9</u> , <u>supE44</u> ?	"
CGSC 6054	<u>asnB50::Tn5</u>	"
CGSC 4515	<u>ilvA</u> <sup>+</sup>	"
CGSC 4457	<u>ilvA</u> <sup>+</sup>	"
A179	HfrC <u>trxA::Tn5</u>	(30)
A779	Colicin E1 producing strain	S. Luria
DDA-1	<u>ilv</u> <sup>+</sup> , <u>chlB</u> , <u>metE::Tn10</u> 4443/6403 transductant	This work



Table 1 cont'd.

Plasmid	Relevant Genotype and Characteristics	Source
pST1-2	<u>sodA</u> , <u>cdxA</u> , <u>pfkA</u> , <u>cdh</u> , <u>tpi</u> (Amp <sup>R</sup> )	D. Touati
pST1-5	Deleted for genes given for pST1-2 (Amp <sup>R</sup> )	D. Touati
pDT1-2	<u>sodA</u> (Amp <sup>R</sup> )	(37)
pSC101 (Amp <sup>R</sup> )	(Amp <sup>R</sup> Tet <sup>R</sup> )	M. Dubow
pHC79	(Amp <sup>R</sup> Tet <sup>R</sup> )	D. Touati
pLC16-4	<u>pfkA</u> , <u>cdh</u> , <u>tpi</u> (Col E1 <sup>imm</sup> )	B. Bachmann

Note: "CGSC" represents the Coli Genetic Stock Center number.

## Results:

The experiments reported in this thesis are described in three sections. The first two deal with the mapping strategy used to demonstrate the positions of two heretofore relatively little studied mutations: sdaA and sdaB. These two mutations result in similar phenotypes (26). It is demonstrated here that the sdaA mutation is located in a gene between the lip gene at 14.8 minutes and gal gene at 17 minutes (figure #1). The sdaB mutation is located in a gene to the right of metE at 86.5 minutes (figure #2).

The third section deals with the further characterization and the cloning of the pleiotropic ssd mutation which was located previously at 88 minutes and is now positioned more exactly by cloning, between the cpxA and pfkA gene (figure #3).

### Part 1 Mapping of sdaA

Strain MEW128sdaA is a mutant of Cu1008 which is unable to grow on L-serine, glycine and leucine (SGL) as carbon source. As the result of the single pleiotropic sdaA mutation strain, MEW128sdaA shows very little L-serine deaminase (L-SD) activity and a requirement for thiamine. The experiments that follow localize the sdaA mutation to a gene between the lip (14.8 min.) and gal (17 min.) genes (Figure 1).

#### 1A Demonstration of linkage to gal-1

To localize the sdaA mutation, the ability to grow on SGL was transduced into strain MEW128sdaA using phage grown on strain 4891. Transductants were then tested for the cotransduction of the 4

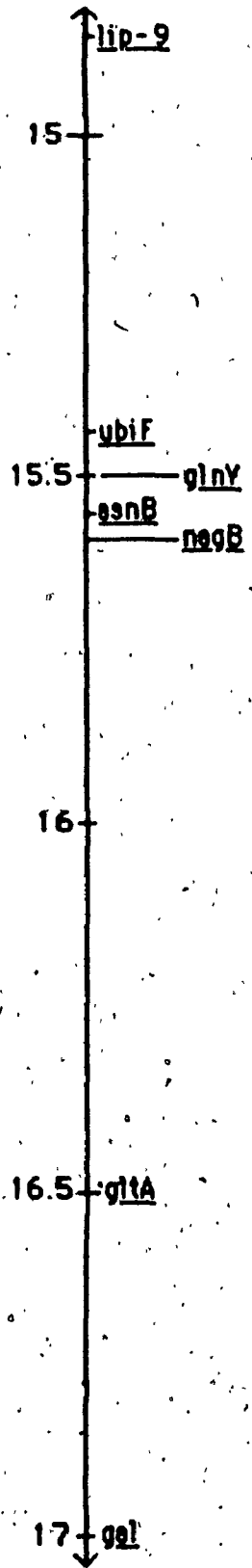


Figure 1 Linkage Map From 15 to 17 minutes  
(from Bachman, 1983)

amino acid requirements and 5 sugar metabolism deficiencies of strain 4891.

Cotransduction of the 4 amino acid requirements was tested by plating transductants on glucose minimal medium. All 591 transductants grew, indicating that none of the 4 mutations were linked by phage P1 to sdaA. Similarly transductants were tested for the ability to use 5 sugars by plating onto MacConkey agar supplemented with each sugar in turn. All transductants were able to use lactose, maltose, mannitol and xylose. However 20 colonies of the 591 tested were unable to use galactose. That is, 3.3% of the SGL<sup>+</sup> transductants also received the gal gene (Table 2). A cotransduction frequency of 3.3%, when transformed to map units, corresponds to a map length of 1.4 minutes. This locates sdaA mutation either at 18.4 or 15.6 minutes on the linkage map.

Table 2

Transduction Frequency of Loci sdaA and arg-6, hisG-1, leuB-6,  
metB-1, gal-1, lacY-1, malA-1, mtl-2, xyI-7

Relevant Host	Genotype Donor	Selected* Marker	Unselected† Markers	Frequency of Unselected Donor Marker	Dist. Min.‡
MEW128	4891	<u>sdaA</u> <sup>+</sup>	<u>gal-1</u> <sup>-</sup>	20/591 (3.3%)	1.4
<u>sdaA</u> <sup>-</sup>	<u>arg</u> <sup>-</sup> <u>his</u> <sup>-</sup>		<u>arg-6</u> <sup>-</sup> <u>hisG-1</u> <sup>-</sup>	0/591 (0.0%)	0.0
	<u>leu</u> <sup>-</sup> <u>met</u> <sup>-</sup>		<u>leuB-6</u> <sup>-</sup> <u>metB-1</u> <sup>-</sup>		
	<u>gal</u> <sup>-</sup> <u>lac</u> <sup>-</sup>		<u>lacY-1</u> <sup>-</sup> <u>malA-1</u> <sup>-</sup>	0/591 (0.0%)	0.0
	<u>mal</u> <sup>-</sup> <u>mtl</u> <sup>-</sup>		<u>mtl-2</u> <sup>-</sup> <u>xyI-7</u> <sup>-</sup>		
	<u>xyI</u> <sup>-</sup>				

\* sdaA<sup>+</sup> scored as the ability to grow on SGL plates supplemented with arginine, histidine, leucine, and methionine.

† arg<sup>-</sup> his<sup>-</sup> leu<sup>-</sup> met<sup>-</sup> scored as the inability to grow on glucose minimal medium, gal<sup>-</sup> lac<sup>-</sup> mal<sup>-</sup> mtl<sup>-</sup> xyI<sup>-</sup> scored as the inability to metabolize galatose, lactose, maltose, mannitol and xylose.

‡ Map distance calculated as described in "Materials & Methods".

### 1B Linkage to gltA

To determine whether sdaA was to the right or to the left of gal-1, linkage between sdaA and another gene in the area was needed. The gene gltA, 16.5 minutes, was selected for this purpose. Mutations in the gltA gene result in a requirement for glutamate (3). Therefore, strain 4278gltA was transduced to glutamate independence with phage grown on strain MEW128sdaA. Transductants were tested for their ability to grow on SGL. Of 158 4278gltA<sup>+</sup> transductants tested, 12 were unable to grow on SGL. This 7.6% cotransduction of sdaA with gltA locates sdaA 1.2 minutes from gltA (Table 3).

Table 3

Transduction Frequency of Loci sdaA and gltA

<u>Relevant Genotype</u>		Selected*	Unselected†	Frequency of Unselected Donor Marker	Dist. Min.‡
Host	Donor	Marker	Markers	Marker	
4278	MEW128	<u>gltA</u> <sup>+</sup>	<u>sdaA</u> <sup>-</sup>	12/158 (7.6%)	1.2
<u>gltA</u> <sup>-</sup>	<u>sdaA</u> <sup>-</sup>				
<u>sdaA</u> <sup>+</sup>	<u>gltA</u> <sup>+</sup>				

\* gltA<sup>+</sup> scored as the ability to grow on glucose minimal medium with uracil.  
 † sdaA<sup>-</sup> scored as the inability to grow on SGL with uracil.  
 ‡ Map distance calculated as in "Materials & Methods".



IC Linkage to lip-9

The sdaA mutation, then, is clearly linked to both gal-1 and gltA. However the difference between the cotransduction frequencies, 3.3% and 7.6%, is small. Moreover the number of transductants scored, particularly in the gltA cross, is relatively small. Therefore one cannot localize sdaA to one side or the other of gal-1. Nonetheless the data fit better with a location to the left (15.3-15.6) than with one to the right (17.7-18.4). This could be clarified with a transduction involving a mutation in a gene even farther to the left of gal-1, in this case the lip gene at 14.8 minutes. Strain 4286 carries a mutation in the lip-9 allele, which results in a requirement for lipotic acid (3).

Strain ~~4286~~lip-9 was transduced to lipoate independence with phage grown on strain MEW128sdaA. Of 66 lip<sup>+</sup> transductants, 48 or 72% were unable to grow on SGL (Table 4). This high cotransduction

Table 4

Transduction Frequency of Loci sdaA and lip-9

Relevant Genotype		Selected* Marker	Unselected <sup>‡</sup> Markers	Frequency of Unselected Donor Marker	Dist. Min. <sup>†</sup>
Host	Donor				
4286	MEW128	<u>lip-9</u> <sup>+</sup>	<u>sdaA</u> <sup>-</sup>	48/66 (72%)	0.2
<u>lip-9</u> <sup>-</sup> <u>sdaA</u> <sup>+</sup>	<u>lip-9</u> <sup>+</sup> <u>sdaA</u> <sup>-</sup>				

\* lip-9<sup>+</sup> scored as the ability to grow on glucose minimal medium with thiamine, histidine, proline, adenine.

‡ sdaA<sup>-</sup> scored as the inability to grow on SGL with thiamine, histidine, proline and adenine.

† Map distance calculated as described in "Material & Methods".

frequency located sdaA at 0.2 minutes from lip-9. If sdaA were to the left of lip-9 at 14.6 minutes, it would be more than 2 minutes away from gal-1, and cotransduction with gal-1 would be extremely rare. It seems then that sdaA must be to the right of lip-9 at about 15 minutes, which agrees reasonably well with the earlier estimates at 15.3 and 15.6.

#### ID Linkage to asnB

The map location of sdaA was further investigated with a transduction to a gene at 15.5 minutes, asnB. The preceding experiments suggest that one should see a very high linkage between sdaA and asnB and this proved to be the case (Table 5).

Strain 6054 asnB::Tn5 is deficient in asnB function due to the presence of a Tn5 transposon in that gene. The Tn5 transposon confers kanamycin resistance making it possible to select for asnB

Table 5

Transduction Frequency of Loci sdaA and asnB

Relevant Genotype		Selected* Marker	Unselected† Markers	Frequency of Unselected	Dist. Min.‡
Host	Donor			Donor Marker	
MEW128	6054	<u>asnB::Tn5</u>	<u>sdaA</u> <sup>+</sup>	20/26 (77 %)	0.2
<u>asnB</u> <sup>+</sup> <u>sdaA</u> <sup>-</sup>	<u>asnB::Tn5</u> <u>sdaA</u> <sup>+</sup>				

\* asnB::Tn5 scored as kanamycin resistant (growth on LB Kan).

† sdaA<sup>+</sup> scored as the ability to grow on SGL.

‡ map distance calculated as described in "Material & Methods".

by transducing strain MEW128 sdaA to kanamycin resistance with phage grown on strain 6054 asnB::Tn5. Of 26 kanamycin resistant transductants, 20 were able to grow on SGL, a cotransduction frequency of 77% with lip-9, corresponding to a map distance of 0.2 minutes.

These results agree reasonably well with the earlier ones and suggest a location to the left of asnB, but to the right of lip-9, at around 15.3 minutes.

#### IE Three Factor Cross #1

To locate sdaA more precisely, it was necessary to carry out a transduction in which it could be localized with respect to two neighboring genes in the same experiment. This could be done using a ubiF mutant, which is unable to grow on succinate and maps at 15.3

Table 6

Transduction Frequency of Loci sdaA, asnB, and ubiF

<u>Relevant Genotype</u>		<u>Selected*</u>	<u>Unselected†</u>	<u>Frequency of Unselected Donor Marker</u>
<u>Host</u>	<u>Donor</u>	<u>Marker</u>	<u>Markers</u>	<u>Marker</u>
MEW128	6057	<u>asnB::Tn5</u>		
			<u>sdaA<sup>-</sup> ubiF<sup>-</sup></u>	104/147 (72%)
<u>asnB<sup>+</sup></u>	<u>asnB::Tn5</u>		<u>sdaA<sup>-</sup> ubiF<sup>+</sup></u>	27/147 (18%)
<u>sdaA<sup>-</sup></u>	<u>sdaA<sup>+</sup></u>		<u>sdaA<sup>+</sup> ubiF<sup>+</sup></u>	16/147 (11%)
<u>ubiF<sup>+</sup></u>	<u>ubiF<sup>-</sup></u>		<u>sdaA<sup>+</sup> ubiF<sup>-</sup></u>	0/147 (0.0%)

\* asnB::Tn5 scored as kanamycin resistant.

† sdaA<sup>-</sup> scored as the inability to grow on SGL, ubiF<sup>-</sup> scored as the inability to grow on succinate.

minutes. Phage were grown on strain 6057 asnB::Tn5, ubiF and used to transduce strain MEW128sdaA to kanamycin resistance. Transductants were then scored for growth on succinate (ubiF) and on SGL (sdaA).

All transductants had of course acquired kanamycin resistance. Of 147 tested, 104 had also acquired ubiF, a linkage of 72% corresponding to a map distance of about 0.2 minutes (Table 6). This agrees with the published location of ubiF about 0.2 minutes from asnB (3).

However only 16 strains acquired sdaA<sup>+</sup> with asnB, and none picked up both ubiF and sdaA both from the parent strain. This suggests that sdaA is a considerable distance from asnB on the side away from ubiF, i.e. around 17.5 minutes.

This does not agree with the preceding estimates. The following experiments indicate that the situation is indeed more complicated than expected.

### 1F Three Factor Cross \*2

To investigate this further, a reciprocal cross was done, taking advantage of 2 further mutations carried by strain 6057. In addition to the mutations described earlier, ubiF and asnB, this strain also carried mutations in nagB and asnA. The first, nagB, results in a deficiency in glucosamine metabolism (12). The second, asnA, by itself confers no alteration in the phenotype. However when a cell carries mutations in both asnA and asnB, it is unable to grow without asparagine added to the medium (15).

In this cross, phage grown on strain MEW128sdaA were used to transduce the ability to use N-acetylglucosamine into strain 6057nagB, asnB::Tn5, asnA, ubiF, selecting on plates containing N-acetylglucosamine and asparagine. Transductants were then scored for asparagine requirement, kanamycin resistance and the ability to



use succinate and SGL as carbon sources.

Of the 293 nagB<sup>+</sup> transductants scored, all were asnB<sup>+</sup> as judged by their ability to grow without asparagine (Table 7). All of these were also kanamycin sensitive, indicating that the inserted transposon (Tn5) was lost as expected when the donor gene was acquired. This is in keeping with the close map location reported for these two genes (3). Similarly 275 of the 293 transductants were able to use succinate (i.e. ubiF<sup>+</sup>), also as expected from the reported map positions of nagB and ubiF (3).

However sdaA was transduced with nagB in only 13 of the 293 transductants. This, like the preceding cross, suggests a location well to the right of nagB, 1.3 minutes away, i.e. at 16.9 minutes. It is apparent then that the two crosses with strain 6057 suggests a map position around 17 minutes, and all other crosses suggest a map

Table 7

Transduction Frequency of Loci sdaA, asnB, ubiF and nagB

Relevant Genotype		Selected* Marker	Unselected† Markers	Frequency of Unselected Donor Marker
Host	Donor			
6057	MEW128	<u>nagB</u> <sup>+</sup>	<u>asnB</u> <sup>+</sup> <u>sdaA</u> <sup>+</sup> <u>ubiF</u> <sup>+</sup>	275/293 (95%)
<u>asnB</u> ::Tn5	<u>asnB</u> <sup>+</sup>		<u>asnB</u> <sup>+</sup> <u>sdaA</u> <sup>-</sup> <u>ubiF</u> <sup>+</sup>	4/293 (1.4%)
<u>sdaA</u> <sup>+</sup>	<u>sdaA</u> <sup>-</sup>		<u>asnB</u> <sup>+</sup> <u>sdaA</u> <sup>-</sup> <u>ubiF</u> <sup>-</sup>	9/293 (3%)
<u>asnA</u> <sup>-</sup>	<u>asnA</u> <sup>+</sup>		<u>asnB</u> <sup>+</sup> <u>sdaA</u> <sup>+</sup> <u>ubiF</u> <sup>-</sup>	5/293 (1.7%)
<u>nagB</u> <sup>-</sup>	<u>nagB</u> <sup>+</sup>			
<u>ubiF</u> <sup>-</sup>	<u>ubiF</u> <sup>+</sup>			

\* nagB<sup>+</sup> scored as the ability to grow on N-acetylglucosamine.

† sdaA<sup>-</sup> scored as the inability to grow on SGL, ubiF<sup>-</sup> scored as the inability to grow on succinate, asnB<sup>+</sup> scored as both kanamycin sensitive and asparagine independent.

position nearer 15.5 minutes. This problem can be partially resolved with the hypothesis that sdaA is a suppressible mutation subject to the action of an amber suppressor, glnV, which maps at 15.3 minutes near asnB. This hypothesis is considered in detail in the discussion of this thesis.

## Part 2 Mapping of the sdaB Mutation

Strain MEW191 sdaB::Mu::dX is a derivative of MEW1 in which Mu::dX is inserted into the sdaB gene, resulting in an inability to grow on L-serine, glycine and leucine (SGL) as combined carbon source. This mutation, like the sdaA mutation, is pleiotropic, resulting in a decreased L-SD level, a requirement for thiamine and the inability to grow on SGL medium (26). In this work I have shown sdaB is linked to metE (85.5), chlB (86.4) and the ilv gene cluster (84.5) (figure 2).

2A Lack of Linkage to argG6, hisG1, leuB6,

metB1, lacY1, gal-1, xyl-7, mtP2, malA1

In a transduction similar to the one described for the sdaA mutation in Part 1A, strain MEW191 sdaB::Mu::dX was transduced to growth on SGL with phage grown on strain 4891. None of the 4891

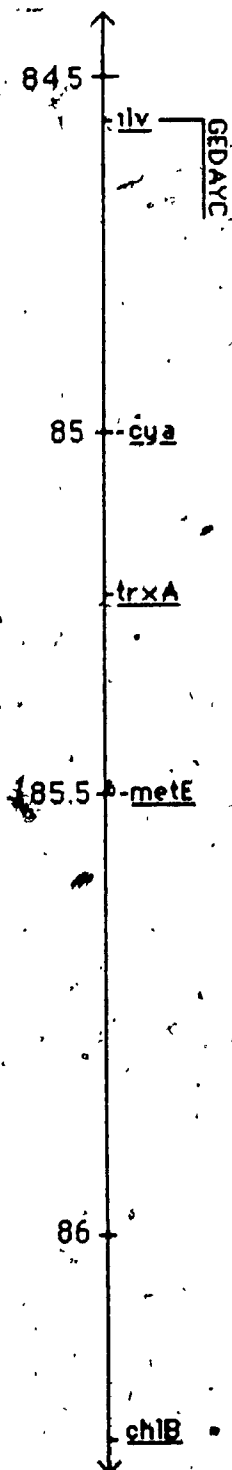


Figure 2 Linkage Map From 84.5 to 86 minutes  
(from Bachman, 1983)

mutations were cotransduced with sdaB in the 70 transductants tested. Thus sdaB is not closely linked to any of these genes.

### 2B Demonstration of Linkage to ilvA

Strain MEW191 sdaB:Mu::dX has a requirement for isoleucine due to a deletion of its ilvA gene. A transduction using strain 4457 as the donor of a functional ilvA gene was done to determine if the sdaB mutation was linked to ilvA (Exp. # 1 Table 8).

Of 81 isoleucine-independent transductants tested, 11 or 13.5% were able to grow on SGL. One would suppose that these strains in acquiring an sdaB<sup>+</sup> allele from the donor, had lost the insert in sdaB. They should therefore be antibiotic sensitive. However 8 of the 11 sdaB<sup>+</sup> strains were in fact antibiotic resistant. Since they were isoleucine independent, it seems that the insert Mu::dX must have moved to another location. The sdaB<sup>-</sup> ilvA<sup>+</sup> transductants were also

tested for antibiotic resistance, and 22 of 70 were sensitive.

In this cross, the controls did not show isoleucine-independent colonies. It seems then that the original population must have consisted of isoleucine-requiring strains, only some of which (8/11; 48/70) were antibiotic-resistant. To be sure that this was not due to some oddity of strain 4457, the same experiment was repeated with strain 4515 as donor (Exp. \*2, Table 8) and the same results were obtained.

Since the cultures for the transductions were grown in Luria broth (LB) without antibiotics, it is possible that  $Mu::dX$  excised from the gene during growth of the culture and inserted elsewhere. If excision were accurate, the resultant cells would be  $sdaB^+$ . If it were inaccurate, the strains would be  $sdaB^-$ . They might be antibiotic-sensitive, if  $Mu::dX$  did not reinsert or antibiotic-resistant if it did.

Table 8

Transduction Frequency of Loci sdaB and ilvA

Exp*	Relevant Genotype Host Donor	Selected* Marker	Unselected* Markers	Frequency of Unselected Donor Marker
1	MEW191	4457	<u>ilvA</u> <sup>+</sup>	<u>sdaB</u> <sup>+</sup> CAmp <sup>R</sup> 8/81 (10%)
	<u>sdaB</u> ::Mu::dX	<u>sdaB</u> <sup>+</sup>		<u>sdaB</u> <sup>+</sup> CAmp <sup>S</sup> 3/81 (4%)
	<u>ilvA</u> <sup>-</sup>	<u>ilvA</u> <sup>+</sup>		<u>sdaB</u> <sup>-</sup> CAmp <sup>R</sup> 48/81 (59%)
				<u>sdaB</u> <sup>-</sup> CAmp <sup>S</sup> 22/81 (27%)
2	MEW191	4515	<u>ilvA</u> <sup>+</sup>	<u>sdaB</u> <sup>+</sup> CAmp <sup>R</sup> 6/103 (6%)
	<u>sdaB</u> ::Mu::dX	<u>sdaB</u> <sup>+</sup>		<u>sdaB</u> <sup>+</sup> CAmp <sup>S</sup> 1/103 (1%)
	<u>ilvA</u> <sup>-</sup>	<u>ilvA</u> <sup>+</sup>		<u>sdaB</u> <sup>-</sup> CAmp <sup>R</sup> 79/103 (77%)
				<u>sdaB</u> <sup>-</sup> CAmp <sup>S</sup> 17/103 (17%)

\* ilvA<sup>+</sup> scored as the ability to grow on glucose minimal medium without isoleucine or valine. † sdaA<sup>-</sup> scored as the inability to grow on SGL, Presence of Mu::dX scored as chloramphenicol and ampicillin resistance (CAmp<sup>R</sup>).



Then, even if sdaB were not linked to ilvA, a percentage of the ilvA<sup>+</sup> transductants would be sdaB<sup>+</sup>, the same percentage as had lost the insert in the original population.

### 2C Isolation of a Partially Cured MEW191

Since the Mu::dX insert is quite unstable, it seemed wise to select a strain which had lost it by incorrect excision. Such a strain - here called partially cured- would still have a mutation in sdaB, but would no longer carry the insert and so would not be subject to its moving around in an uncontrolled fashion.

To obtain such a strain, MEW191 sdaB::Mu::dX was grown subcultured in LB without antibiotics and then plated onto LB again without antibiotics, 3000 colonies were tested for antibiotic resistance. Five colonies were isolated which were unable to grow on plates with ampicillin and chloramphenicol, but still unable to grow

on SGL. They had thus lost the insert without repairing the lesion in sdaB.

Of those 5 strains, 3 were sensitive to both antibiotics. However, 2 were chloramphenicol resistant and ampicillin sensitive. Since all were sensitive to Mu as tested with a Mu cts lysate, all had lost the Mu insert. It seems likely then that the chloramphenicol resistance was due to the Tn9 transposing from Mu::dX into the E. coli genome.

To be sure that the partially cured strain still carried a lesion in sdaB, L-SD was measured on these strains, grown in glucose minimal medium. All 5 had the low level seen in strain MEW191 (Table 9). Strain 191C was used as the host in the following transductions.

Table 9

## L-Serine Deaminase levels of MEW1, MEW191 and Semicured MEW191C

Strain	Antibiotic		Mu	L-SD'
	Ampicillin	Chloramphenicol		
MEW1	S	S	S	19
MEW191	R	R	R	5
MEW191C				
Semicured				
A	S	S	S	5
B	S	S	S	5
C	S	S	S	5
D	S	R	S	5
E	S	R	S	5

'Note: L-SD activity expressed as described in "Material & Methods"

### 2D Three Factor Cross \*1

The use of strain MEW191C as host would bypass all problems due to loss and transposition of the Mu::dX insert. The location of sdaB was further tested using as donor, strain 6092 with a reported genotype of trxA, ilvE::Tn5, metE, metE being located at 85.5 minutes, 1 minute away from ilvE and trxA being close to metE at 85.3 minutes (figure 2). In this transduction, kanamycin-resistant strains were selected, and tested for methionine-independence and for the ability to grow on SGL. The presence of the trxA lesion was not tested.

Of 66 transductants isolated, 49 were metE<sup>-</sup>, i.e. 74% (Table 10). If the insert was located 1 minute away from metE, one would not expect such a high frequency of cotransduction. This suggests that the insert was not intact in ilvE at all, but much closer to metE. Wherever the insertion was, sdaB was cotransduced with it in 9 of 66.

Table 10

Transduction Frequency of Loci sdaB and ilvE and metE

Relevant Genotype		Selected* Marker	Unselected† Markers	Frequency of Unselected Donor Marker
Host	Donor			
MEW191C	6092			
<u>ilvE</u> <sup>+</sup>	<u>ilvE</u> ::Tn5	<u>ilvE</u> ::Tn5	<u>sdaB</u> <sup>+</sup> <u>metE</u> <sup>+</sup>	1/66 (1.5%)
<u>sdaB</u> <sup>-</sup>	<u>sdaB</u> <sup>+</sup>		<u>sdaB</u> <sup>+</sup> <u>metE</u> <sup>-</sup>	8/66 (12%)
<u>metE</u> <sup>+</sup>	<u>metE</u> <sup>-</sup>		<u>sdaB</u> <sup>-</sup> <u>metE</u> <sup>+</sup>	16/66 (24%)
			<u>sdaB</u> <sup>-</sup> <u>metE</u> <sup>-</sup>	41/66 (62%)

\* ilvE::Tn5 scored as kanamycin resistant.

† sdaB<sup>-</sup> scored as the inability to grow on SGL, metE<sup>-</sup> scored as the inability to grow on glucose minimal medium.

transductants. That is, sdaB was linked to an insert which is closely linked to metE.

### 2E- Three Factor Cross #2

The previous cross demonstrated that sdaB was linked to metE and to an insert which was close to metE. To further localize the sdaB mutation, one would like to perform a transduction selecting metE::Tn10 and testing for the cotransduction of genes to the right and to the left of metE. To do this, an appropriate donor had to be constructed. This donor strain DDA-1, was made by transducing strain 4443chlB, to tetracycline resistance with phage grown on strain 6403 metE::Tn10. Strain 4443chlB is resistant to chlorate when grow anaerobically in the presence of potassium chlorate, because of a lesion in its chlB gene. Strain 6403metE::Tn10 has a requirement for methionine due to the transposon Tn10, which carries a gene responsible for tetracycline resistance, inserted within its

metE gene. Strain DDA-1 therefore has the following phenotype metE::Tn10, chlB, i.e. it carries a wild-type ilvA gene to left and a chlB mutation to the right of metE.

Phage grown on strain DDA-1 were used to transduce MEW191C to tetracycline resistance. All the tetracycline resistant transductants had a requirement for methionine, as tested by their inability to grow on minimal glucose medium supplemented with isoleucine and valine, and their ability to grow when methionine was added. This confirmed that the metE gene was transduced with the transposon inserted within it. Of 165 tetracycline resistant transductants tested, 21 were isoleucine independent, i.e. 13%, which corresponds to a map length of 1 minute between ilvA and metE (Table 11). This agreed with the published distances between these loci (3). Seventy-seven of the 165 tested were able to grow anaerobically in the presence of chorate, chlB<sup>-</sup>, 47%. This corresponds to a distance of 0.4 minutes which is closer than the published distance of 0.7

minutes (3). The difference between the published distance and the one obtained here was not great enough to create any concern considering the relative small number of colonies tested.

The ability to grow on SGL medium, i.e. sdaB<sup>+</sup>, was cotransduced with the metE::Tn10, 19% (30/165, Table 11), which corresponds to a map distance of 0.9 minutes. Since chlB was cotransduced 47% with metE, it must be closer to met than sdaB. Then sdaB must be to the right of chlB, (86.3 minutes) and so cotransduction of sdaB with ilvA, which is located at 84.6 minutes should be rare. This was the case, ilvA was cotransduced with sdaB 4% of the time, which agrees reasonably well with a sdaB map location to the right of chlB.



Table 11

Transduction Frequency of Loci sdaB and ilvE, metE and chlB

Relevant Genotype		Selected*	Unselected†	Frequency of Unselected Donor Marker
Host	Donor	Marker	Markers	
MEW191C	DDA-1	<u>metE</u> ::Tn5	<u>ilvA</u> <sup>+</sup> <u>sdaB</u> <sup>+</sup> <u>chlB</u> <sup>+</sup>	0/165 (0.0%)
<u>sdaB</u> <sup>-</sup>	<u>sdaB</u> <sup>+</sup>	*	<u>ilvA</u> <sup>+</sup> <u>sdaB</u> <sup>+</sup> <u>chlB</u> <sup>-</sup>	2/165 (1.2%)
<u>metE</u> <sup>+</sup>	<u>metE</u> ::Tn5		<u>ilvA</u> <sup>+</sup> <u>sdaB</u> <sup>-</sup> <u>chlB</u> <sup>+</sup>	18/165 (11%)
<u>ilvA</u> <sup>-</sup>	<u>ilvA</u> <sup>+</sup>		<u>ilvA</u> <sup>+</sup> <u>sdaB</u> <sup>-</sup> <u>chlB</u> <sup>-</sup>	1/165 (0.6%)
<u>chlB</u> <sup>+</sup>	<u>chlB</u> <sup>-</sup>		<u>ilvA</u> <sup>-</sup> <u>sdaB</u> <sup>+</sup> <u>chlB</u> <sup>+</sup>	9/165 (5.0%)
			<u>ilvA</u> <sup>-</sup> <u>sdaB</u> <sup>+</sup> <u>chlB</u> <sup>-</sup>	19/165 (12%)
			<u>ilvA</u> <sup>-</sup> <u>sdaB</u> <sup>-</sup> <u>chlB</u> <sup>+</sup>	61/165 (37%)
			<u>ilvA</u> <sup>-</sup> <u>sdaB</u> <sup>-</sup> <u>chlB</u> <sup>-</sup>	55/165 (33%)
Cotransduction Frequencies Between Selected and Unselected Markers				
		<u>metE</u>	<u>ilvA</u>	21 / 165 13%
		<u>metE</u>	<u>chlB</u>	77 / 165 47%
		<u>metE</u>	<u>sdaB</u>	30 / 165 19%

\* metE::Tn5 scored as kanamycin resistant and as methionine requiring.

† sdaB<sup>+</sup> scored as being able to grow on SGL medium, ilvA<sup>+</sup> scored as the ability to grow on glucose minimal medium, chlB<sup>-</sup> scored as the ability to anaerobically on LB + KClO<sub>3</sub> medium.

## 2F Possibility that sdaB was in trxA

Extracts made from strain MEW1.91 sdaB:Mu::dX grown in glucose minimal medium supplemented with thiamine were shown to have no L-SD activity. However when the assay was done in the presence of iron (Fe) and dithiothreitol (DTT), L-SD activity could be obtained (24). This indicated that the strain was deficient in an activating system. Because iron and DTT are strong reducing agents, it would seem that the activation process may involve reduction. One protein which is involved in the reduction of proteins to their active form is thioredoxin. Thioredoxin is involved in the reduction of ribonucleotide, sulfoxide and sulfate in E. coli (13).

For the lytic bacteriophage T7 to replicate in vivo it requires the product of the host trxA gene, thioredoxin (2, 19). Thioredoxin binds to the phage T7 gene 5 product and converts this product to a DNA polymerase (2). Without E. coli thioredoxin, phage T7 is unable

to propagate. Thioredoxin mutants are therefore resistant to phage T7 infection (2, 19).

Thioredoxin is also required for filamentous phage assembly (30). The gene previously identified to be associated with this was called, fip, which was later shown to be an allele of trxA (18).

The thioredoxin gene, trxA, in E. coli is located at 83.5 minutes on the E. coli linkage map (3, 19). This was in the same area where the sdaB mutation was originally thought to be. It seemed possible, then, that sdaB was an allele of trxA.

To determine whether the sdaB mutation conferred phage T7 resistance, strains MEW191sdaB::Mu::dX, MEW191C sdaB, 6092trxA, A-179fip::Tn5, MEW128sdaA, MEW206pds::Mu::dX, 181-34serA::Mu d1 and MEW1 were plated with approximately 2000 phage T7 particles. Strains 6092trxA and A-179fip::Tn5 both carry mutations within trxA

(18). No plaques were formed on these strains. Strains MEW191 sdaB::Mu::dX, MEW191C sdaB and MEW128 sdaA allowed the formation of very few ( $\approx 200$ ) and very small plaques. This was also true however of two strains with phage Mu inserted else where in the genome. Strain 181-34 serA::Mu d1 and strain MEW206 pps::Mu::dX, gave about 300 larger plaques. Only MEW-1, which contained no insertion was completely lysed (Table 12).

It is clear that any Mu insertion, Mu d1 or Mu::dX, confers considerable resistance to phage T7. However it seems that both the sdaA and sdaB mutations confer phage T7 resistance independently, since MEW191C (which has lost the Mu insert) and MEW128 (which had none) are both phage T7 resistant. However neither strain was as resistant as the trxA mutants.

To determine if the increased resistance to phage T7 and the inability to grow on SGL medium was related to a thioredoxin

deficiency, I was lucky to obtain from M. Russel a plasmid, pPFM-5, carrying the trxA gene (30). This plasmid was transformed into strain MEW191C and the ability to grow on SGL medium was screened. Of the 52 tetracycline resistant transformants tested, none were able to grow on SGL. One of these transformants was then also shown to be resistant to phage T7 (Table 12). This would seem to indicate that the sdaB mutation is not within the trxA gene.

To insure that the plasmid, pPMR-5, carried the trxA gene, the plasmid was transformed into strain 6092 trxA. One of the 6092(pPMR-5) transformants was then tested for phage T7 sensitivity as described above. The transformant was completely lysed (Table 12). This was a good indication that the plasmid carried the trxA gene, and sdaB was not at the same locus.

Table 12

Sensitivity to Bacteriophage T7 of Strains 6092, A-179,  
MEW191C, MEW191, MEW191(pPMR-5), MEW128, MEW206, 181-34,  
MEW-1, and 6092(pPMR-5)

<u>Strain</u>	Plaques <sup>‡</sup>	Size <sup>*</sup>
6092 <u>trxA</u>	0	----
A-179 <u>fp::Tn5</u>	0	----
MEW191C <u>sdaB</u>	200	+
MEW191 <u>sdaB::Mu::dX</u>	200	+
MEW191 <u>sdaB::Mu::dX</u> (pPMR-5)	200	+
MEW128 <u>sdaA</u>	200	+
MEW206 <u>pps::Mu::dX</u> ,	300	+++
181-34 <u>serA::Mu d1</u>	300	+++
MEW1	CL	----
6092 <u>trxA</u> (pPMR-5)	CL	----

<sup>‡</sup> CL, Confluent lysis.

<sup>\*</sup> Size of plaques: +, small, 1 mm diameter, +++, 3 mm in diameter.

### Part 3 Cloning of the *ssd* Mutation

The *ssd* mutation has been mapped at 88 minutes between *rha* and *metB* on the *E. coli* linkage map (21). In this work, cloning techniques have been used to locate *ssd* more accurately, between the *cpxA* and *pfkA* genes (figure 3).

#### 3A Further Characterization of the *ssd* Phenotype

Strains carrying the *ssd* mutation differ from the parental strain, CU1008, in many ways. They can use L-serine as the sole carbon source, are unable to use succinate or to grow anaerobically, have a decreased efficiency in the derivation of cell material from glucose, an increase in L-serine deaminase levels, increased resistance to kanamycin and neomycin and a deficiency in proline transport (27). In this work I describe another trait due to this mutation, the inability to grow at 42°C on glucose minimal medium.

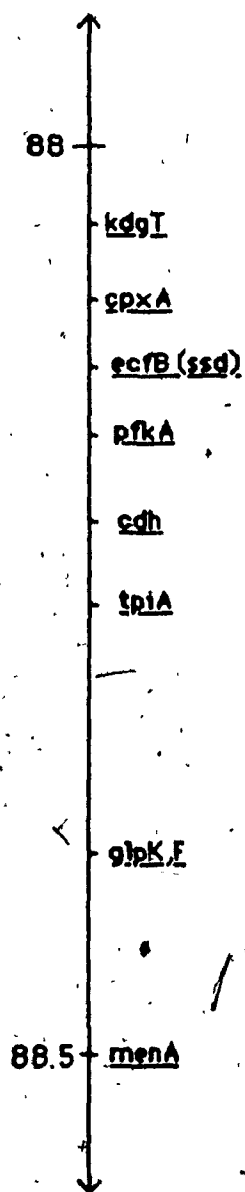


Figure 3 Linkage Map From 88 to 88.5 minutes as revised by Albin & Silverman (1984)



Strain L9~~ssd~~ did not grow on glucose-minimal agar incubated at 42°C, though its parent, strain CU1008 did (Table 13). Both strains were able to grow at 42°C on LB plates.

If this inability to grow at 42°C were due to the ssd mutation, then strains which reverted to ssd<sup>+</sup> phenotype should also be able to grow at 42°C. To test this, succinate-utilizing derivatives of strain L9 were selected, and tested for their ability to grow at higher temperatures.

Of 5 such independent succinate-using derivatives, L9~~ssd~~Succ<sup>+</sup> A-E, four (A-D) regained the ability to grow at 42°C and to grow under anaerobic conditions. They had also lost the ability to grow on L-serine as a carbon source (Table 13). This shows clearly that growth at 42°C can be restored by the same mutation that restored the rest of the ssd phenotype.

Table 13

Characteristics of CU1008, L9<sub>ssd</sub> and L9<sub>ssd</sub> Derivatives Able to Grow on Succinate

<u>Strain</u>	<u>Growth Conditions</u>				Type of Suppression
	42°C	Anaerobic	Succinate	L-serine	
CU1008	G <sup>*</sup>	G	G	NG	---
L9 <sub>ssd</sub>	NG	NG	NG	G	---
L9 <sub>ssd</sub> (Succ <sup>+</sup> )					
A	G	G	G	NG	Intragenic
B	G	G	G	NG	ND
C	G	G	G	NG	ND
D	G	G	G	NG	Intragenic
E	WG	NG	G	G	Extragenic

\* The symbols "G, WG and NG" represent growth, weak growth and no growth at 42°C respectively. "ND", not determined. Anaerobic and 42°C growth were tested on glucose minimal medium.

The fifth succinate-using strain was quite different. It retained much of the ssd mutant phenotype, while acquiring the ability to grow on succinate. Thus it did not grow anaerobically, nor did it grow well at 42°C. It retained the mutant ability to grow with L-serine. Thus the ability to use succinate could be restored in two ways- in strain E, without restoring the entire phenotype, and in strains A-D, with complete restoration.

The mutations which restored succinate utilization to L9ssdSucc<sup>+</sup> A-E could be located either within the ssd gene (intragenic) or in some other gene (extragenic). A strain which carried the extragenic suppressor would still carry the ssd mutant allele, and could donate it in a transduction to a strain carrying a metB mutation, ssd being 33% linked to metB.

To test whether they might carry an extragenic suppressor, phage grown on strains L9ssdSucc<sup>+</sup> A,D and E were used to transduce

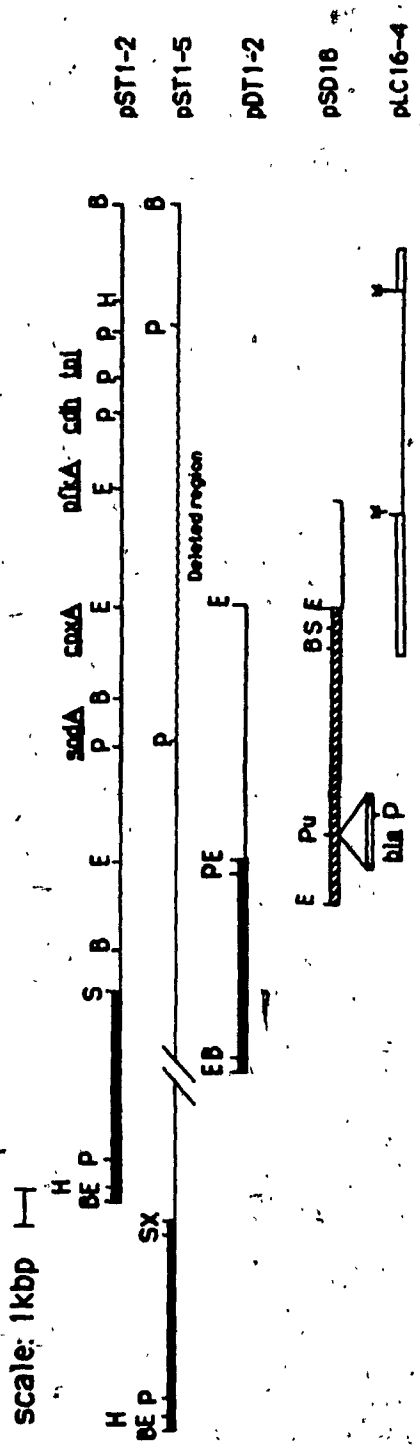
CU1008metB to methionine independence. The transductants were then tested for growth at 42°C. Of 203 such transductants using phage grown on L9ssdSucc<sup>+</sup> E, 54 carried the ssd mutation. However among 75 transductants from phage grown on L9ssdSucc<sup>+</sup> A and 40 from strain D, none showed the ssd phenotype.

It is clear from the preceding transductions, that strain L9ssdSucc<sup>+</sup> E carried an extragenic suppressor which restored only part of the phenotype, ability to grow on succinate. Strains L9ssdSucc<sup>+</sup> A, and D however carried secondary mutations in (or very near) the ssd gene. Since these intragenic suppressors restored growth at 42°C as well as the rest of the phenotype, it is evident that growth at 42°C is governed by the ssd gene, as is the rest of the phenotype. Growth at 42°C being particularly easy and rapid to test, it was used in the following cloning experiments to test for complementation of the ssd phenotype.

### 3B *ssd* is not on Plasmid pLC16-4

The plasmid pLC16-4 from the Clark and Carbon collection (9) carries the *E. coli*, *pfkA*, *cdh*, and *tpi* genes and also a plasmid gene imparting colicin E1 immunity (32,36) (figure 4). These three genes are located in the same region of the *E. coli* linkage map as the *ssd* gene. It might therefore also carry the *ssd* gene. To test this, strain L9*ssd* was transformed with plasmid pLC16-4, and colicin E1 immunity was selected. The plasmid containing transformant could not grow at 42°C, nor could it use succinate or glucose anaerobically. It is clear, therefore, that pLC16-4 did not carry the *ssd* gene.

—(Table 14).



Restriction Sites:

- B Bam HI
- S Sal I
- H Hind 3
- E Eco RI
- P Pst I
- Pu Pvu'2
- X Mixed Bam HI, Sau 3a, insensitive to Bam HI
- Y Eco RI + Poly A & T tail, insensitive to Eco RI

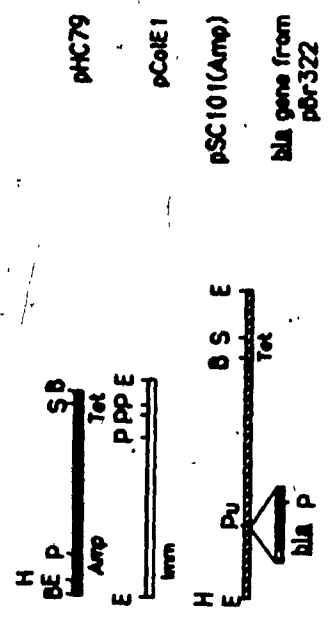


Figure 4 Plasmids used in This Thesis

### 3C Location of *ssd* on Cosmid pST1-2

I was fortunate to obtain a cosmid, pST1-2, from Dr. D. Touati at the Université de Paris VII. This cosmid carried a large amount of *E. coli* DNA, from the left of *sodA* to the right of *tpi* (figure 4).

Cosmid DNA was used to transform L9*ssd*, selecting for ampicillin resistance, determined by a cosmid gene (figure 4). The fact that the cosmid-containing strain showed the parental phenotype indicated that *ssd* is indeed carried on this cosmid. Thus strain L9*ssd*(pST1-2) was able to grow at 42°C, or on succinate, and anaerobically. It also showed a low level of L-SD, and sensitivity to kanamycin and neomycin (Table 14). However strain L9*ssd*(pST1-2) did retain part of the mutant phenotype, namely the ability to grow on L-serine. It is important to note that the cosmid pHC79 alone transformed into L9*ssd*, did not complement (table 14). It seems then that a strain with an *ssd* mutant allele can grow on L-serine, even in

the presence of a copy of the wild-type gene.

### 3D Closer Location of *ssd* by Comparison with Related Cosmids

I also tested one smaller derivative of cosmid pST1-2, and a larger cosmid, deleted for much of the DNA carried by pST1-2, these were kindly provided by Dr. Touati. Cosmid pDT1-2, which carried *sodA* and *cpxA* did not complement (Table 14). The larger cosmid, pST1-5, which carried a long deletion from *sodA* to the right of *tpi* also did not complement.

One can see that this comparison located *ssd* between the Eco RI site on the right of *cpxA* and the Pst I site to the right of *tpi* (figure 4). However plasmid pLC16-4 also was unable to complement. Since that plasmid carries DNA from *pfkA* rightwards, *ssd* must be to the left of *pfkA*. This leaves only a 3.4 kilobase Eco RI fragment between *cpxA* and *pfkA* as the possible site of *ssd*.



Table 14

Characteristics of CU1008, L9ssd and L9ssd plus pCH79, pST1-2, pST1-5, pDT1-2 and pLC16-4

Growth Conditions

Strain	plasmid	Succ	Ser	42°C	Ana.	Kan.	Neo.	L-SD'
CU1008	-----	+	-	+	+	S	S	23
L9ssd	-----	-	+	-	-	R	R	200
L9ssd	pHC79	-	+	-	-	R	R	295
L9ssd	pST1-2	+	+	+	+	S	S	36
L9ssd	pST1-5	-	+	-	-	R	R	261
L9ssd	pDT1-2	-	+	-	-	R	R	ND
L9ssd	pLC16-4	-	+	-	-	R	R	ND

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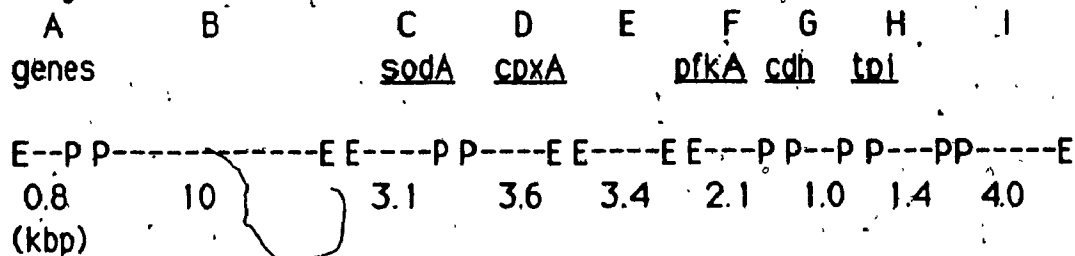
'Note: L-SD expressed as described in "Materials and Methods", "ND", Not determined. "R, S" resistant and sensitive respectively

### 3E Subcloning From pST1-2

The next step in the cloning of ssd was to subclone the 3.4 kbp Eco R1 fragment onto a convenient vector, in this case pSC101(Amp<sup>R</sup>). This involved digesting pSC101(Amp<sup>R</sup>) in such a way as to maximize the possibility of isolating this fragment, ligating with a pSC101(Amp<sup>R</sup>) digest, and transforming into L9ssd.

To do this, pST1-2 was digested with a mixture of restriction endonucleases Pst 1 and Eco R1. If it had been digested only with Eco R1, there would be 4 fragments with two Eco R1 ends. However 3 of those were cut with Pst 1 as well, so that only one fragment with two Eco R1 ends would be formed in a complete digest, this being the fragment carrying ssd.

Fragments:



Now Eco RI cuts produce 5' AATT protruding ends, while Pst I produces a 3' ACGT. These cannot ligate to each other. If a double digest of this kind were ligated with vector fragments produced with only Eco RI cuts, only the 3.4 kbp fragment could ligate. There could in fact also be fragments made by ligating two fragments with an Eco RI cut at one end and a Pst I at the other. However a transformant of this type could be detected readily by the fact that Pst I would cut its DNA.

The vector used in this experiment is a derivative of pSC101 constructed in the laboratory of D. M. Dubow, McGill University, who kindly provided it. pSC101 is a medium copy (3-6) plasmid, with stringently controlled replication. It carries a gene responsible for

tetracycline resistance. The McGill group inserted the bla gene from plasmid pBr322 into the Pvu 2 site of pSC101 using Sac 1 linkers, forming plasmid pSC101(Amp<sup>R</sup>).

The vector pSC101(Amp<sup>R</sup>) was digested to completion with Eco R1 to linearize the plasmid. It was then dephosphorylated with calf intestinal alkaline phosphatase (C.I.A.P.) to remove the 5' terminal phosphates, to minimize reannealing of the vector. The Eco R1/Pst 1 double digest of pST1-2 was then mixed with the digested vector, and ligated.

Strain L9ssd was transformed with this ligation mixture, and ampicillin resistance selected. Of 60 ampicillin resistant colonies, 6 showed the same complemented phenotype as L9ssd(pST1-2). They were able to grow on succinate, on glucose at 42°C and anaerobically, and they also retained the ability to grow on L-serine (figure 5).

One of the six clones was further purified, and plasmid DNA prepared from it. If this plasmid, pSD18, actually carried the 3.4 kbp Eco R1 fragment, this should be released in an Eco R1 digest.

Moreover Pst 1 should not cut this fragment. To test this, pSD18 was cut with Eco R1 in one digest, and with Pst 1 in another. The digests were then subjected to electrophoresis, using 4 standards for size comparison. The first of these was lambda DNA digested with Hind 3 and Eco R1 which gives a well known set of fragments, one of which is 3.5 kbp. The second was pSC101(Amp<sup>R</sup>) linearized by digesting with Pst 1. Two complete digests of the parent plasmid pST1-2 were also run, one with Eco R1 and one with Pst 1.

The complete digestion of pSD18 with Eco R1 produced two fragments. One migrated very slightly farther than the 3.5 kbp fragment of lambda digest. Since the 3.4 kbp fragment of pST1-2 resulting from the Eco R1 digest migrated to the same point, this seemed likely to be the 3.4 kbp fragment of interest. Moreover,

digestion of pSD18 with Pst I produced only the linearized plasmid. The presence of annealed Eco RI/Pst I fragments was therefore excluded.

The second fragment migrated slightly farther than the linearized plasmid, pSC101(Amp<sup>R</sup>). This slight decrease in size suggested that a portion of vector DNA had been lost. Now the vector used coded for resistance to two antibiotics, tetracycline and ampicillin. Since Eco RI does not cut within either gene, one would expect that the pSD18 clone would be tetracycline resistant. However it was not. There may, therefore, have been a small deletion in the tetracycline gene of the vector.

To be sure that the pSD18 DNA carried the ssd gene, it was used to transform L9ssd a second time. Ampicillin resistant transformants were tested on succinate, L-serine, and glucose at 42°C and anaerobically. Of 105 transformants tested, all were able

to grow on L-serine. Seventy-one did not grow at 42°C on succinate, i.e. showed no complementation. Thirty-six were able to grow at 42°C, i.e. were at least partly complemented. However only two of these grew on succinate, and those not well (figure 5).

### 3F Temperature Sensitivity of L9ssd(pSD18) and its Derivatives

The transformants just described had the puzzling phenotype of growing at 42°C, but not growing with succinate, a mixed phenotype not previously seen. To try to clarify this, four colonies which grew at 42°C (\*4, 6, 8, 10) and 4 which did not (\*5, 7, 9, 11) were streaked for single colonies on LB Amp (figure 5, stage 1). Three single colonies from each isolation (4 A, B, C; 5 A, B, C etc.) were then tested for growth at 42°C, and were also themselves streaked for single colonies.





Of 12 colonies tested from the four parents which grew at 42°C, ten grew at 42°C and two (4C, 8B) did not (figure 5, stage 2). Of 12 colonies tested from the four parents which did not grow at 42°C, 3 grew well at 42°C, 2 grew less well, and 7 did not grow. It is clear then that the parent phenotype was not uniformly distributed in the progeny.

The single colonies derived from 4-10 ABC were tested for growth at 42°C. Fifty-five colonies derived from strains originally able to grow at 42°C (i.e. 4, 6, 8, 10) were tested. Eight were able to grow, 13 grew less well, and 34 did not grow at all. That is, the descendants of the colonies growing at 42°C were largely unable to do the same.

Of 66 colonies derived from strains originally unable to grow at 42°C, 9 were able to grow at 42°C, and all the rest were not. Eight of the 9 came from lines in which the ABC stage contained at least

one colony that could grow at 42°C. However one of them, 9B, had no predecessor that grew at 42°C.

It is clear then that growth at 42°C did not follow a clear inheritance pattern. There was a clear accumulation of colonies unable to grow at 42°C. However colonies able to grow were also derived from those unable to grow.

### 3G. Preliminary Study of Stability of pSD18

One could explain an accumulation of cells unable to grow at 42°C by assuming that the complemented cells are at a selective disadvantage. That is, more than one copy of the ssd gene might be metabolically disruptive, even though it complemented the particular defect of the mutant. Strain L9<sub>ssd</sub>(pSD18) might then be both complemented and sick. Cells which lost the plasmid would then be selected.

Plasmid instability was invoked as an explanation during cloning of the neighbouring cpxA (1). The authors isolated plasmid DNA from a clone in which cpxA was complemented, and reintroduced it into their mutant, selecting for antibiotic resistance. Only 5% of the transformants complemented cpxA. The DNA used was a long piece from a Bam H1 site near glpK to another Bam H1 site to the left of cpxA. It thus contained the ssd gene, which might have been responsible for the instability. When they had a subclone which did not carry the 3.4 kbp Eco R1 fragment, instability was no longer seen. The authors did not investigate this further.

Instability of the plasmid was also suggested by the behavior of transformants in liquid media. Among the 121 descendants of 4-11 ABC, 16 were selected for further study under the names, 4B1, 4B2 etc. as described in figure 5 stage 3. When cultures of these were inoculated from glucose ampicillin plates into liquid glucose minimal medium with ampicillin, many did not grow at all, or required 2 days

to produce a sufficiently dense culture for enzyme assay. This suggests that most of the cells inoculated were not ampicillin resistant, i.e. had lost the plasmid.

Similarly when the overnight culture was subcultured into medium with fresh antibiotic, they decreased in density and took a further day to grow. This seems to indicate that the overnight cultures consisted of a few antibiotic-resistant cells, and many antibiotic-sensitive segregants which had lost the plasmid and overgrown the culture. Resistance to ampicillin is mediated by an enzyme which is located in the periplasm and in the medium. Therefore a small proportion of antibiotic-resistant, but otherwise metabolically disturbed cells could destroy enough antibiotic to allow growth of the faster-growing ampicillin sensitive cells (37)

Some clones did grow in liquid medium, and L-SD values are listed for these in Table 15 column D. Of 7 such colonies, 5 were

derived from colonies originally growing at 42°C (4B1, 6B1, 8A2, 10B1, and 10C2). One (7C1) came from a nongrowing parent through a colony which grew at 42°C. However one (5B1) came from a nongrowing lineage.

If the accumulation of cells not growing at 42°C were due to a loss of the plasmid, these cells should be antibiotic sensitive. Conversely, if cells were maintained with ampicillin, nongrowers should not accumulate. The testing described to this point was done on plates up to one week old, stored at 4°C. The ampicillin in these plates might be considerably degraded.

To see if exposure to more ampicillin altered the results, patches isolated at stage 3 were restreaked on freshly made plates. When cultures which had grown on such plates were tested, most grew well at 42°C. However two clones (4B1, 9A2) did not grow and two grew weakly (5A1, 5B1). All cultures grew on succinate and on

L-serine, indicating that they contained both the mutant and wild-type gene, not necessarily however in the same cell.

The culture from fresh ampicillin plates (stage 4) were inoculated into minimal glucose medium with ampicillin and incubated overnight at 37°C. Only one of these grew as well as a usual E. coli, i.e. was ready for subculture in the morning. This strain, 7C1, grew at 42°C, and had slightly elevated L-SD activity (Table 15, column H). Two other cultures reached a high enough density to assay without subculture (i.e. grew more slowly, but still reasonably well). These cultures (10C2, 5B1) had a phenotype similar to that of 7C1. All three were sensitive to kanamycin and neomycin, but able to grow on succinate and on L-serine.

The remaining cultures grew only after 2 to 3 days. All of these cultures grew with succinate and with L-serine, indicating that they, like the first three strains which grew well, still carried both

alleles. Eight strains were sensitive to kanamycin and neomycin. Of these, 7 grew at 42°C, and 1 did not. Four strains were resistant to the antibiotics. Of these, 3 grew at 42°C, and 1 did not.

These results do not provide a clear pattern. It seems that ampicillin sensitive colonies are constantly selected. One might ascribe these to a selection for cells that lost the plasmid. However these should not grow with succinate. It seems most likely that every clone tested so far has been a mixture. As the colony or patch developed, four things might happen. Some cells would integrate the wild-type allele into the host DNA, and some would not. In both cases, some of the cells would lose the plasmid and some would not. Depending on the test medium, one subpopulation would grow on succinate and another on L-serine. The result obtained from any patch would depend on the proportion of cells which resulted from each kind of event.

Table 15

Stage <sup>1</sup> Strain	Testing of L9 <sub>ssd</sub> (pSD18) clones							
	A	B	C	D	E	F	G	H
	1	2	3	L-SD*	4 <sup>†</sup>	Kan	Neo	L-SD*
5A2	-	-	-	DNG	W	R	R	DNG
5B1	-	-	-	209	W	S	S	80
7C1	-	+	+	52	+	S	S	50
7B2	-	-	-	DNG	+	R	R	DNG
9A2	-	-	-	DNG	-	R	R	DNG
9B1	-	-	+	DNG	+	S	S	DNG
11B2	-	+	-	DNG	+	R	R	DNG
11C1	-	W	+	DNG	+	S	S	DNG
4B1	+	+	-	52	-	S	S	DNG
4B2	+	+	W	DNG	+	S	S	DNG
6B1	+	+	+	18	+	S	S	DNG
6B2	+	+	-	DNG	+	S	S	DNG
8A1	+	+	+	DNG	+	S	S	DNG
8A2	+	+	-	290	+	R	R	DNG
10B1	+	+	-	52	+	S	S	DNG
10C2	+	+	+	52	+	S	S	64

<sup>1</sup>Stages as described in the text and in figure 5. \* L-SD activity as described in "Materials and Methods". Symbols: "DNG", did not grow, "S", sensitive, "R", resistant, "+", growth, "W", weak growth, "-" no growth at 42°C, "Kan", and "Neo", kanamycin and neomycin respectively. <sup>†</sup> All the transformants tested at stage 4 grew on succinate and on L-serine.



## Discussion

The work in this thesis has dealt with genetic aspects of mutations affecting L-serine metabolism in *E. coli* K-12. Two mutations which produce as yet indistinguishable phenotypic effects have been shown to result from changes at different loci about 30 minutes apart. A third gene has been subcloned from a well-known plasmid, and a start on the characterization of the clone has been made.

In the first part of this discussion, I consider first the probable exact locations of sdaA and sdaB. The data presented for sdaA localizes it to a region on the *E. coli* linkage map, but does not permit an unequivocal localization. I try to explain this discrepancy in two ways. In section 1 of the discussion, I show that a lot of the data, but not all of it, can be reconciled with one assumption, that the sdaA mutation, as isolated, is suppressible by the glnV-suppressor. In section 2, I show that the data can be reconciled in another way, by

assuming that there is some error in the genotype ascribed to strain 6057. The present understanding of the location of sdaA is summarized in section 3.

In section 4, I give a similar account of the map location of sdaB. This has not been localized accurately, but is clearly in the region of 88 minutes. I discuss evidence that it is different from a nearby gene, trxA, and also from ssd. In section 5, both sdaA and sdaB are differentiated from all other loci known to affect thiamine biosynthesis.

In the second part of this discussion, I discuss the cloning of the ssd gene. In section 1, I discuss the phenotype of the ssd mutant transformed with cosmid pST1-2 and conclude that the ssd gene must be present on the cosmid. In section 2, I discuss its subcloning into a different vector, pSC101(Amp<sup>R</sup>). Because the plasmid isolated, pSD18, did not confer tetracycline resistance, another explanation of its structure is presented.

A preliminary physiological characterization of the plasmid-containing strain L9<sub>ssd</sub>(pSD18) is presented in section 3. The plasmid proved to be very unstable. Reasons for this instability are presented in section 4. In section 5 a possible model of ssd function is presented. The discussion ends with a note of caution as to the actual phenotype of L9(pSD18).

#### **Part 1: Map Location of sdaA and sdaB**

##### **SECTION 1: Is sdaA Suppressible by glnV**

The experiments in this thesis clearly demonstrate that sdaA is linked to mutations from 14.8 to 17 minutes on the E. coli linkage map. However the experiments fall into two groups, one set suggesting a map position around 15.3 minutes, and the other suggesting a location at 17 minutes.

This dilemma can be resolved in 2 ways. In this section of the discussion, I consider the hypothesis that sdaA is at 16.7 minutes and is suppressible by the glnV suppressor. In the following section, I consider the possibility that it is at 15.3 minutes, but there is some as yet unknown problem with strain 6057. Neither hypothesis explains the data completely.

Suppose then that the sdaA allele isolated can be suppressed by glnV, a suppressor gene reported to be present in some of the donor strains and suspected to be present in others. Then the ability to grow on SGL medium could be conferred on an sdaA<sup>-</sup> strain in two ways- either by transducing in sdaA<sup>+</sup> or by transducing in glnV<sup>-</sup>.

Consider first the experiment described in Table 2. Strain 4891 is reported to carry a glnV mutation. The cross then would be fully described as:

Host		Donor
<u>glnV</u> <sup>+</sup> <u>sdaA</u> <sup>-</sup> <u>gal-1</u> <sup>+</sup>	/	<u>glnV</u> <sup>-</sup> <u>sdaA</u> <sup>+</sup> <u>gal-1</u> <sup>-</sup>

Transductants able to grow on-SGL medium would then be of two classes: a) glnV<sup>-</sup> sdaA<sup>+</sup> or <sup>-</sup>, and b) glnV<sup>+</sup> sdaA<sup>+</sup>. Class A would very rarely also carry gal-1 because gal-1 is at 17 minutes, 1.7 minutes away from glnV. Class B might be gal-1, depending only on the distance between sdaA and gal-1. The class A transductants would then make the apparent linkage of sdaA<sup>+</sup> to gal-1 at least 50% lower than the real linkage. Since the experiment suggested a map position 1.4 minutes from gal-1, the real position would then be 0.7 minutes away - or 16.3 - if the probability of transducing glnV is the same as the probability of transducing sdaA<sup>+</sup>.

A similar explanation can be given for the cross with the gltA<sup>-</sup> host (Table 3). In this case, the actual cross was:

Host		Donor
<u>glnV</u> <sup>-</sup> <u>sdaA</u> <sup>+</sup> <u>gltA</u> <sup>-</sup>	/	<u>glnV</u> <sup>+</sup> <u>sdaA</u> <sup>-</sup> <u>gltA</u> <sup>+</sup>

Now gltA is at 16.5 minutes and glnV at 15.5. Therefore most of the strains which received gltA<sup>+</sup> would also have glnV<sup>-</sup>. In fact the only

way of getting an sdaA<sup>-</sup> phenotype would be to bring in all 3 genes from the donor. The observed linkage of 7.6% or 1.2 minutes is the distance between glnV and the further of the other two, and is consistent with sdaA being very closely linked to gltA e.g. at 16.7 minutes, 1.2 minutes away from glnV.

This explanation also requires that strain 6054 asnB::Tn5 carries the glnV-suppressor. If it did, the actual cross reported in Table 4 would be:

Host		Donor
<u>glnV</u> <sup>+</sup>	<u>sdaA</u> <sup>-</sup> <u>asnB</u> <sup>+</sup>	/ <u>glnV</u> <sup>-</sup> <u>sdaA</u> <sup>+</sup> <u>asnB::Tn5</u>

In this case the selection was for kanamycin resistance (asnB::Tn5, 15.5 min.) which would be most often accompanied by glnV<sup>-</sup> which is very close. One would get sdaA<sup>+</sup> only if one had brought in sdaA<sup>+</sup> and had not brought in glnV<sup>-</sup> which must be a very rare event. Thus the 20/26 linkage of sdaA<sup>+</sup> phenotype with asnB does not reflect the position of sdaA, but that of glnV.

The suppressor has been reported to occur in several of the strains discussed above, other than MEW128. However it has not been reported to occur in strain 6054 and 6057. It seems likely that the position determined in the crosses with strain 6057 (16.5-16.8) is correct, and that all other determinations are biased by the action of the glnV suppressor.

The one cross that is not consistent with this is the one reported in Table 3, with strain 4286 as host. If sdaA is 16.8 minutes, then one should almost never bring in sdaA<sup>-</sup> when selecting lip<sup>+</sup>. However 72% linkage of sdaA to lip was in fact seen. The explanation of this is not obvious. One could get this linkage if strain 4286 were itself an sdaA mutant, and carried a glnV mutation. One would then measure linkage of glnV and lip in this cross. The figure of 72% is however high even for this hypothesis.

The hypothesis that the mutation in strain MEW128 is a nonsense mutation suppressible by glnV has not been tested in this

work. It could be tested in the following way. First, one could test all the strains used for their ability to support replication of phage T4 carrying nonsense mutations suppressible by glnV. If the suppressor were found where it is expected, the MEW128/4891 cross could be repeated. Transductants able to grow on SGL medium (SGL<sup>+</sup>) would be used as donors in transductions to strain 6057, selecting naqB. If some of the SGL<sup>+</sup> donors were in fact suppressed sdaA strains (i.e. sdaA<sup>-</sup> glnV<sup>-</sup>), some of the transductants would be unable to grow on SGL medium.

In summary, if the allele of sdaA is suppressible by glnV, and if strains other than 6057 carry the glnV suppressor, then the most probable location of sdaA is at 16.7 minutes.

## SECTION 2: Alternative Localization of sdaA

One comes to a quite different idea of the location of the sdaA gene if one omits all data involving strain 6057. All remaining data



suggests a map location around 15.3 minutes. Thus, Table 1 showed a distance of 1.4 minutes from gal-1 (17 minutes)- for a map position of 15.6. Similarly Table 2 showed a distance of 1.2 minutes from gltA (16.5)- for a map position of 15.3, which is entirely compatible with the preceding. Again, Table 3 showed a distance of 0.2 minutes from lip (14.8 minutes)- which is a little closer than one might expect, and Table 4 showed a distance of 0.2 minutes from asnB (15.6 minutes)-for a map position of 15.4 minutes. A map position around 15.3 minutes then agrees moderately well with all data not involving strain 6057:

If the transposon in strain 6057 were not where it was thought to be, this might account for some of the discrepancy. In fact, the interpretation of experiments on sdaB have given problems due to Tn5 transposition (see below). However, strain 6057 was shown to require asparagine, so that it must carry mutations in both asnA and asnB (15). Moreover all transductants in Table 6 were kanamycin-sensitive, so that the insert must have been close to nagB.

i.e. in asnB as expected. The strain therefore does seem to behave as described. Then if the map position were around 15.3 minutes, the cross in Table 6 should show a large number of sda<sup>-</sup> transductants, and does not.

One might try to get around some of these problems by suggesting that the sdaA phenotype is actually the result of two mutations, both of which are needed to have the sda<sup>-</sup> phenotype expressed. One of these would be near gltA and the other near asnB. The sdaA mutant phenotype has been shown to revert as a single mutation (26). This does not however exclude the possibility that two genes are involved, both of which are necessary for both aspects of the phenotype.

If then the sdaA strain carries mutations 1 and 2 at 16.8 and 15.3 minutes respectively, one could explain the 1.4 map distance of Table 1 as being the distance between these two loci, with the 16.8 mutation being cotransduced with gal-1 at a high frequency.

Whenever the donor strain brought in both genes 1 and 2, it would become sdaA<sup>+</sup>. Linkage to gal-1 would be the linkage from 15.3 to 17 minutes.

This also explains results with strain 6057 as donor. Table 6 shows the results for nagB<sup>+</sup> transductants, most of which would have mutation 2 cotransduced. However mutation 1 would only appear in about 10% of the transductants. In fact only 5% of the transductants showed the sdaA<sup>-</sup> phenotype. However, in the reciprocal experiment in Table 5, asnB is selected and one would expect that mutation 2 would be cotransduced and thus the overwhelming number of transductants would be sdaA<sup>+</sup> and they are not. The 2 mutation hypothesis also cannot explain the high linkage to lip<sup>+</sup> (Table 3).

### SECTION 3: Summary of Evidence on Map Location of sdaA

The gene must be located between 15 and 17 minutes. If it is assumed to be at 15.3 minutes, I cannot account for data involving

strain 6057. If it assumed to be at 16.7 minutes, and suppressible by glnV, I cannot account for data involving strain 4286. The situation cannot be resolved without further experiments.

#### SECTION 4: Map Location of sdaB

The data presented in Table 10 suggest a map position for sdaB around 86.5 minutes, based on the linkage of ilvA with metE. This would place it very close to glnA, and also linked to rha. The position then could be confirmed with crosses involving these markers.

One other mutation influencing synthesis of L-serine deaminase is known to map in this area. This is the ssd mutation which cotransduced with metB about 33%, and is also linked to rha (21). However its position, 88.4 minutes, is far enough from sdaB for them to be clearly distinguished.

The results of earlier experiments (Tables 7 and 9) suggested that sdaB might lie in the region of trxA. The fact that L-serine deaminase (L-SD) must be reduced to be active (24) suggested that a trxA mutation might lead to a loss of L-SD activity, and therefore that sdaB might be an allele of trxA. This was supported by the fact that phage T7 did not grow well on strain MEW191 or MEW128. It did not grow well on other strains carrying unrelated inserts, so that some of the deficit must have been due to the Mu:dx insert itself. However strain MEW128 and MEW191C do not carry an insert. Therefore it must be the mutation itself which decreased T7 synthesis. However strains MEW128 and MEW191C were much less resistant to phage T7 than was a trxA mutant, so that the mechanism of resistance is likely to be different.

In any case, sdaB has been clearly differentiated from trxA in this work. A MEW191 mutant transformed with plasmid carrying trxA did not grow on SGL medium, and was no more sensitive than the MEW191 mutant. The same plasmid restored sensitivity to a trxA

mutant. It is clear then that sdaB is not an allele of trxA and its phage resistance has a different origin.

SECTION 5: Comparison of sdaA,B With Other Mutations Resulting in Thiamine Auxotrophy

Mutations resulting in thiamine auxotrophy have been mapped at four loci: 9.7, 24.6, 46, and 90.4 minutes (26). The map positions now determined for sdaA and sdaB clearly distinguish them both from previous mutations causing thiamine deficiency. This does not exclude the possibility that sdaA and sdaB gene products are involved in thiamine biosynthesis, since only part of the thiamine biosynthetic pathway has been described. However an indirect explanation for thiamine deficiency of these strains has also been given (26).

## Part 2: Cloning of ssd

### SECTION 1: Location of ssd on Cosmid pST1-2

The map location of ssd at 88.4 minutes suggests that it should be located on cosmid pST1-2. This cosmid is known to carry sodA, pfkA, cdh, and tpi (personal communication, Touati) and therefore might very well also carry ssd. That it in fact does is indicated by the fact that transformants of L9ssd(pST1-2) showed an ssd phenotype intermediate between the mutant L9ssd and its wild type parent (CU1008). Transformants were able to grow at 42°C, and at 37°C anaerobically, and on succinate- all characteristics of the ssd strain. However they were able to grow on L-serine, as does L9ssd. Glucose grown cultures showed levels of L-SD much lower than L9ssd, but still higher than the parent strain.

This shows clearly that pST1-2 could complement the ssd mutant phenotype. However the strain carrying this very large cosmid did not grow well under any circumstance, and lost the plasmid (became antibiotic sensitive) very readily. Cultures which maintained the cosmid could be grown only by frequent addition of ampicillin. This instability may be a function not of size, but of the actual nature of the DNA cloned, as is discussed below.

#### SECTION 2: Subcloning of ssd in pSC101(Amp<sup>R</sup>)

The slow-growing strain L9ssd(pST1-2) would have been difficult to characterize physiologically. Instead the gene was subcloned to a smaller portion of the 88 minute region. This was done by digesting pST1-2 DNA with restriction enzymes Pst I and Eco RI, and ligating the mixture to pSC101 (Amp<sup>R</sup>) DNA digested with Eco RI. The ligated mixture was transformed into strain L9ssd and ampicillin resistant transformants selected. One of 6 transformants which grew at 42°C, on succinate and on L-serine, was selected for further



study.

That this transformant had the expected plasmid in it is indicated by the fact that plasmid DNA could be isolated from the transformant, and when cut with Eco RI, produced a fragment of the expected size (3.4KBP). That DNA could be used to transform L9ssd to ampicillin resistance, and transformants were able to grow at 42°C. It seems then that this plasmid carries the expected area of 88 minute DNA.

However this transformant should also have been tetracycline resistant and was not. Indeed direct selection on tetracycline-containing plates produced very few transformants, none of which grew at 42°C. This could be explained in three ways. Two of these explanations use the assumption that the insert is in fact in pSC101(Amp<sup>R</sup>). The insertion into pSC101 (Amp<sup>R</sup>) might be made in such a way that transcription of the tetracycline gene was decreased or abolished. Alternatively the plasmid might be shortened during

selection of the plasmid, with the loss of a portion of the tetracycline gene and the acquisition of tetracycline sensitivity.

There is also a third possibility that the insertion is not in pSC101 (Amp<sup>R</sup>) at all. The pST1-2 plasmid is an insertion of E. coli DNA pHC79 which also carries an ampicillin gene. In the ligation mixture, one would expect fragments of both pHC79 and pSC101 (Amp<sup>R</sup>). The transformant might therefore be a shortened form of pST1-2. It would consist of the Eco R1 fragment joined to pHC79. In this case, it would carry only a small piece of E. coli DNA, an amount equal to the fragment from the end of pHC79 to the first Eco R1 site. This would result in a high copy plasmid rather than a low copy one. In either case, however, the transformant would contain the ssd gene.

The actual nature of the vector pSD18 carried by the transformant could be easily distinguished, though this was not done in this work. The plasmid DNA from the transformant could be digested with Pst I and Eco R1. This should give the 3.4 kbp Eco R1

fragment, and two Eco R1-Pst 1 fragments, one large and one small. The large one would be about 10 kbp from pH79, and only 8 kbp from pSC101(Amp<sup>R</sup>). Conversely, the smaller one should be about 1 kbp from pH79, and 2.2 kbp from pSC101 (Amp<sup>R</sup>). These differences could be easily detected

### SECTION 3: Physiological Characterization of Gene(s) Carried on pSD18

DNA from the first transformant was used to transform L9ssd a second time. The transformants were streaked for single colonies several times on LB ampicillin plates. No stable phenotype could be obtained. Thus, when colonies which grew at 42°C were streaked for single colonies, these descendants often did not grow at 42°C. Similarly when colonies which didn't grow at 42°C were streaked, some of their descendants did grow at 42°C.

It was possible to reduce this variability somewhat by making sure that the LB ampicillin plates were made on the same day they

were used. Colonies isolated on plates made with fresh ampicillin tended to grow when streaked at 42°C. This strongly suggests that growth at 42°C is determined by a gene carried on the plasmid, and that the plasmid is very readily lost from the cell in the absence of a selective pressure.

Suppose that the transformant as first isolated contains an ssd mutation in the host DNA, and an ssd<sup>+</sup> gene on the plasmid. This cell should grow at 42°C as judged by the behavior of L9ssd(pST 1-2), and from the actual screening. This cell could give several kinds of descendants. The simplest would be those which lost the plasmid.

These would be the same as the original L9ssd.

Since L9ssd is a recA<sup>+</sup> strain, the ssd<sup>+</sup> might also recombine into the host DNA. This would produce a wild-type host DNA, but the host would still carry plasmid DNA, which might have ssd mutant genes or ssd wild-type genes or both. This recombinant cell might then lose the plasmid, producing a cell which was more or less

identical to the parent strain, CU1008.

When I plated the transformants on LB ampicillin, only those which contained the plasmid could grow. But as the ampicillin in the plate deteriorated, more and more nonplasmid containing cells might arise. This is true on both LB ampicillin and minimal ampicillin plates. The fact that colonies arose on succinate-ampicillin plates therefore does not mean that the transformant cell originally plated could grow on succinate. It only means that it was able to give rise to a succinate-using descendant.

The situation could be clarified by plating single cells of transformant populations grown with ampicillin onto various media, and testing the nature of the colonies that arose. If many of the cells that grow in the ampicillin culture have lost the plasmid, but are surviving because ampicillin has been destroyed, there should be a great deal of difference in the number of colonies on LB ampicillin

and LB. The ampicillin-sensitive strains, moreover, should breed true, either as L9~~ssd~~ or as CU1008.

Consider also colonies which might arise at 42°C on LB ampicillin or glucose ampicillin plates. Because they grow at 42°C, they must have a copy of the ssd<sup>+</sup> gene either on the plasmid or integrated into the host DNA. At the time of plating they must have a plasmid (Amp<sup>R</sup>), but by the time the colony is formed, many of the cells might have lost the plasmid. That is, anything which is ampicillin resistant at the time of plating could give descendants of a variety of phenotypes.

One difficulty with this kind of explanation is that it predicts that a strain which was incapable of growing at 42°C would have lost the plasmid, and so would not give rise to descendants which grow at 42°C. This was not the case, since cultures of 7B, 7C, 9B, 11B and 11C growing at 42°C were obtained even at stage 4. This is probably due to the fact that the original population is always mixed. If the

number of cells growing at 42°C is small, they could easily be missed on streaking. However only these cells can grow on ampicillin, so that whenever the culture was exposed to ampicillin, it would enrich itself in cells growing at 42°C. If the ampicillin were added freshly, the proportion might become high enough to see on streaking at 42°C.

#### SECTION 4. Possible Explanations for Plasmid Instability

The results presented in this work suggest that the vector carrying ssd, whether pHC79 or pSC101(Amp<sup>R</sup>), is unstable in the cell. pHC79 is normally present in a large number of copies and so is usually thought to be relatively stable. pSC101(Amp<sup>R</sup>) is present in fewer copies, and has its own partition system (20). When cells containing pSC101 were grown for 100 generations without selecting for the plasmid, retention was 100% (20). It is very surprising then that the plasmid is lost from strain L9ssd as quickly as it is.

One might get such results if the presence of extra copies of ssd on a plasmid were itself harmful to the cell. Such a cell might be able to grow at 42°C because it has a functional copy of ssd, but it might be metabolically hindered in other ways. This might be due to the fact that the cell cannot tolerate a mixture of wild-type and mutant ssd products, or that it simply cannot tolerate having several copies. In this case, there might be a strong selection against the plasmid in the absence of ampicillin.

Instability of plasmids carrying DNA from this region was previously described, during experiments designed to clone cpxA (1). These experiments used pBr322 and pBr325 as vectors, so the plasmids should have been in high copy number.

That the cell with the plasmid does not function well is indicated by the fact that subcultures in liquid medium rarely grew well. In most cases, cultures dense enough for enzyme assay were impossible to obtain.



The function of the ssd gene product is unknown. Its phenotype differs from the parental E. coli in two kinds of ways- first, functions which involved L-serine deaminase and L-serine metabolism, and second, a series of membrane functions. The same mutation has been described under the name ecfB, and has been explained as a bioenergetic defect (5). The ecfB mutant is thought to be leaky to protons- it sets up an adequate proton motive force, but it does not translate it efficiently into ATP. The ssd/ecfB product then would be a membrane component involved in translocating protons along the cell surface or back into the cell. No evidence for this mechanism is available. Moreover nothing is known about the relation between the effects on L-SD and the effects on bioenergetics.

Because the function of a single ssd product in the cell is not known, it is exceedingly difficult to imagine what the effect of several copies of it would be. The ssd mutant is a sick cell, growing slowly and having troubles energizing transport and other functions. The cell with many copies, even functional ones, seems to be sick

also. It would be interesting to transfer the clone to CU1008, and see if multiple copies of a functional gene also hinder the cell.

The clone in pSD18 is 3.4 kbp and may carry more than the ssd gene. That plasmid instability is due to this DNA is likely, since it was seen both in this work and in the cloning of cpxA (1). However the instability may be due not to ssd, but to a neighboring gene.

#### SECTION 5: Speculation About ssd Function

The nature of the ssd product is not known. One of the ways of accounting for the phenotype described in this work is the following: Suppose that ssd codes for a regulatory protein, which binds to DNA and controls the rate of synthesis of two or more gene products. One of these would be an operator governing L-SD synthesis. One or more other operators controlling the rate of synthesis from genes coding for product(s) involved in membrane function would also be regulated.

In the ssd mutant, the structure of the product would be altered in such a way that affinity for L-SD operator (\*I) would be increased and the affinity for other operators (\*II) decreased. This would result in high L-SD and decreased membrane function. What, then, might happen if the cell had a mixture of mutant and wild-type products? If the affinity change were great enough, the effect of the mutant product increasing transcription of L-SD might still be seen, even though there were many competing copies of the wild-type product. However, the product(s) of operator(s) \*II should be transcribed at least at their normal rate, and perhaps faster. This increased function might be deleterious.

This kind of hypothesis, though almost entirely speculative, is supported by evidence that ssd function varies according to the fine details of its structure. The phenotype of 5 intragenic revertants showed considerable variation from one to the next, both in L-SD levels and in degree of antibiotic resistance. That is, ssd function is not all-or-none. Slight variations in structure are directly reflected

in enzyme level (25):

#### SECTION 6: A Note on the Complemented Phenotype

When ampicillin-resistant transformants were tested by streaking under various conditions, they were able to grow at 42°C, on succinate and on L-serine. This seemed to indicate that they have a phenotype intermediate between parent and mutant. Indeed the preceding section of the discussion is predicated upon this hypothesis.

However, with the finding of the extreme instability of the plasmid, it has become clear that cells which grow on succinate may not be the same cells that grow on L-serine. It must be concluded then that the phenotype of the plasmid carrying L9ssd is not yet known.

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