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Role of Nine Cysteine Residues in L-serine Deaminase 1 from *Escherichia coli* K-12

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Abstract

Role of Nine Cysteine Residues In L-serine Deaminase 1 from *Escherichia coli* K-12

Ye Man Tang

L-serine deaminase 1 (L-SD1) is one of three enzymes that break down L-serine to produce pyruvate and ammonia in *Escherichia coli* K-12. L-SD1, a 454 amino acid protein encoded by *sdaA* gene, contains nine cysteine residues at the 181, 219, 290, 339, 347, 366, 381, 392, and 453 positions. Blast results show that three cysteines at positions 339, 381 and 392 are in what is then a highly conserved motif is found in most L-SDs that have a Fe-S cluster. The other six cysteine residues are also conserved among some bacterial L-SDs. In this study, all nine cysteine residues have been mutated individually. Assay of enzyme activity both *in vivo* and *in vitro* shows that: (1) eight of nine cysteine residues play an important role in L-SD1 activity; (2) the cysteine at position 181 might be non-essential for L-SD1 activity; (3) cysteines at positions 339, 381 and 392 are essential for L-SD1 activity. This is consistent with the demonstration by Cicchilo and his colleagues that L-SD1 uses a 4Fe-4S cluster for the deamination of L-serine. If so, cysteine residues 339, 381 and 392 are probably essential for this iron-sulfur cluster and ligate to three iron molecules of a 4Fe-4S cluster.

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Introduction

L-serine deaminase (L-SD), also called L-serine dehydratase, is widespread in nature. Alfoldi *et al.* (1968) mentioned in their paper that in the year 1938 L-serine deaminase was discovered as an enzyme breaking down L-serine to produce pyruvate, and ammonia. Consequently, the studies of characteristics, the functions and genetics of L-serine deaminase have been greatly developed. So far, L-SD has been purified and /or partially purified from a variety of eukaryotes and bacteria (Grabowski and Buckel 1991, Tong and Davis 1993, Hofmeister *et al.* 1993, Ogawa *et al.* 2000). L-serine deaminase 1 (L-SD1), one of three forms of L-SD of *Escherichia coli* K-12, is coded by *sdaA* gene. It was purified from *E. coli* K-12 by use of fusion gene (Su *et al.*, 1989, 1993).

Great efforts have also been put into the studying of L-SD. By comparison of the sequences of L-SD1 from *E. coli* K-12 with L-serine deaminase 2 (L-SD2) from *E. coli* K-12, L-SD from *Peptostreptococcus asaccharolyticus* and a putative L-SD from *Haemophilus influenzae*, Hofmeister *et al* (1997) found that L-SD1 has some sequence homology with L-SD with the others. It has been shown that L-SDs from *P. asaccharolyticus, C. sticklandii* and *C. propionicum* are proteins containing iron-sulfur cluster (s) (Grabowski and Buckel 1991, Hofmeister *et al.*, 1994, Zinecker *et al.*, 1998). Therefore, one would speculate L-SD1 from *E. coli* K-12 also contains an Fe-S cluster and then belongs to Fe-S protein family (Hofmeister *et al.*, 1997). Now it has been proved by Cicchillo and his colleagues (2004) that L-SD1 from *E. coli* K-12 contains a

4Fe-4S cluster. They also demonstrate this 4Fe-4S cluster is essential for the enzyme activity (Cicchillo *et al.,* 2004).

Cysteine is the ubiquitous ligand of Fe-S clusters in proteins. L-SD1 from *E. coli* K-12 consists of 454 amino acids, including 9 cysteine residues (Su *et al.,* 1993). Thus, this study is focused on the function(s) of these nine cysteine residues, and demonstrates which cysteine residue is essential for the enzyme activity.

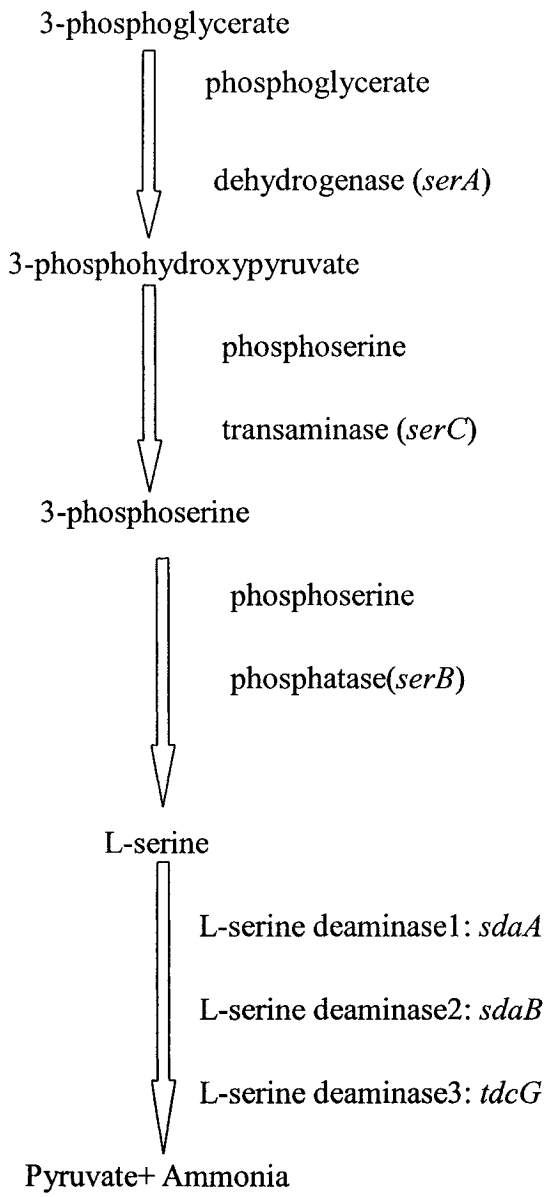
First of all, in this thesis, I will review L-serine metabolism, L-SD and the genes coding for it in *E. coli* K-12. Subsequently, I will introduce cysteine, iron-sulfur cluster, and the relationship between them in L-SD1.

Part 1. Serine metabolism

L-serine is a neutral amino acid. In *E. coli*, L-serine is involved in the synthesis of cysteine, tryptophan, phospholipids and glycine (Kredich and Tomkins 1966, Raetz and Dowhan 1990, Miles 1995). When *E. coli* cells are grown in glucose medium, formation of L-serine and its metabolites use 15% of carbon assimilated by cells (Stauffer, 1996). However, high levels of serine are toxic to *E. coli* cells (Cosloy *et al.*, 1970). Therefore, maintaining an appropriate level of L-serine is very important for cellular metabolism.

The levels of intracellular serine depend on two pathways for serine biosynthesis and degradation. In *E. coli*, the main pathway of serine synthesis is initiated from 3-phosphoglycerate and involves three steps. First, 3-phosphoglycerate is oxidized to 3-Phosphohydroxypyruvate by 3-phosphoglycerate dehydrogenase coded by *serA* gene.

Figure 1 L-serine metabolism in *E. coli* K-12



This figure adapted from Stauffer, 1987.

Then, 3-phosphoserine aminotransferase (the *serC* gene product) transaminates 3-phosphohydroxypyruvate to form 3-phosphoserine. Finally, the phosphate is removed from 3-phosphoserine by phosphoserine phosphatase, which is coded by *serB* gene, to yield serine (Figure.1).

L-serine can be degraded by both L-serine deaminase (L-SD) and L-threonine deaminase (L-TD) in *E. coli*. The reaction catalyzed by L-SD is pyridoxal 5'-phosphate (PLP) independent (Figure 1). L-SD can directly convert L-serine to pyruvate and ammonia (EC 4.2.1.13) (Pardee and Prestidge, 1955). This reaction results in dehydration as well as deamination of serine. Thus, L-serine deaminase is also called L-serine dehydratase. *E. coli* K-12 possess three kinds of L-SD: L-SD1, L-SD2, and L-SD3 that are coded by *sdaA, sdaB,* and *tdcG* genes respectively. Although the three forms of L-SD have the same function, they are expressed in different conditions (Su *et al.,* 1993, Shao and Newman 1993, Hesslinger *et al.,* 1998).

Degradation of L-serine by L-threonine deaminase also produces pyruvate and ammonia elimination (EC 4.3.1.19). This reaction probably involves the initial elimination of water. L-threonine deaminase, encoded by *ilvA* gene in *E. coli*, is a pyridoxal 5'-phosphate dependent protein (Umbarger and Brown 1957, Hesslinger *et al* 1998). L-threonine deaminase is also involved in the synthesis of L-isoleucine and valine (Neidhardt *et al* 1987). Hence, a strain with deletion of *ilvA* gene cannot grow without L-isoleucine. In this study, we used a strain deleted for the *ilvA* gene to allow us to focus on

the investigation of L-SD and supplemented all media with isoleucine and valine (Newman *et al.*, 1985).

Part 2. Structure, function and activation of L-serine deaminase

2.1. Physiological function of L-serine deaminase

L-serine deaminase exists in all three kingdoms. Serine deaminases from mammalians are mainly located in the liver. Rat liver has, so far, the highest serine deaminase of mammalian livers that were examined. Mammalian L-SD can degrade both L-serine and L-threonine to produce pyruvate and ammonia, whereas bacterial L-SDs are specific to L-serine (Ogawa *et al.*, 2000). In rats, this enzyme plays a key role in gluconeogenesis and the consumption of high-protein diets. Starvation and other treatments can induce the enzyme activity (Snell 1984; Ebara *et al.*, 2001).

Human liver exhibits low serine deaminase activity and the enzymatic properties of human serine deaminase are unknown (Sun *et al.*, 2003). L-SD is involved in photorespiration in plant systems (Bird *et al.*, 1972).

Furthermore, *Campylobacter jejuni* makes use of ammonia by the glutamine synthetase/glutamate: 2-oxoglutarate aminotransferase pathway (Parkhill *et al.*, 2000). As a result, L-SD from *C. jejuni* converts L-serine to provide forms of carbon and nitrogen that can be utilized directly in the central metabolism. This degradation is oxygen-labile and important in the normal physiology of *C. jejuni* (Velayudhan *et al.*, 2004). L-serine degradation catalyzed by L-SD also gives a good nitrogen source for growth of

Mycobacterium tuberculosis and *M smegmatis*, in contrast to *M.* bovis BCG which has not enough expression of L-serine deaminase to use L-serine as a nitrogen source (Chen *et al.*, 2003).

L-serine deaminase, from *E. coli*, has been studied for almost fifty years (Pardee and Prestidge, 1955), but its physiological function is still not clear (Cicchillo *et al.*, 2004). It is thought that L-SD in *E. coli* may possess two functions. While biosynthetic pathways need L-serine, high levels of serine are toxic to *E. coli* and other organisms (Cosloy and McFall, 1970, Hama *et al.*, 1990; Uzan and Danchin, 1978). Nevertheless, the enzyme is produced in significant amounts in *Escherichia coli* K-12 (Pardee and Prestidge, 1955). Therefore, one of the functions of L-SD may be to regulate cellular L-serine levels.

Another function may involve the use of L-serine as a carbon and /or nitrogen source. Normally, *E. coli* K-12 expresses a very low level of L-SD activity. Therefore, it can use pyruvate as the only carbon source but is not able to use L-serine as the sole carbon source. *E. coli* K-12 can only grow with L-serine as the carbon source if a small amount of glycine and leucine are also present in medium. This is due to the ability of glycine and leucine to induce the enzyme activity of L-SD, which in turn converts serine to pyruvate (Newman *et al.*, 1982). Strains with an *ssd* mutation have very high L-SD activity and can grow with serine as the only carbon source (Newman *et al.*, 1982).

The L-SD in *P. asaccharolyticus* and *C. propionicum* contain an iron-sulfur complex, and are thought to act through a mechanism analogous to aconitase (Hofmeister

et al., 1993). Aconitase is an enzyme in the tricarboxylic acid cycle. Its apo-enzyme acts as an iron regulation protein (Philpott *et al* 1994). Then it is hypothesized that the L-SD apo-enzyme is involved in the iron metabolism of bacteria. It may be the third function of L-SD in bacteria (Hofmeister *et al.*, 1993).

2.2. Structure of L-serine deaminase

L-SDs have been purified in many organisms. The studies of L-SD using purified proteins help us to comprehend the structure of enzyme more deeply. It is known that rat liver L-SD is a dimer (Ogawa *et al.* 2000, 2002). Sheep liver L-SD, as well as human serine deaminase, is a monomer (Tong and Davis, 1993; Sun *et al.*, 2004). L-SD from *C. jejuni* is probably a homodimer (Velayudhan *et al.* 2004). Hofmeister and his colleagues (1997) purified L-SD from *P. asaccharolyticus* and proved that this enzyme is a heterodimer containing α and β subunits. L-SD from *C. propionicum* is also a heterodimer (Hofmeister *et al.*, 1993). L-SD from *C. acidiurici* contains one subunit of 72KDa (Carter and Sagers, 1972), and the enzyme of *Lactobacillus fermentum* is revealed as a homotetramer with a M_r=150KDa (Farias *et al.* 1991).

In addition, it is also reported that L-threonine deaminase of *E. coli* is a tetramer with M_r=35KDa (Boylan and Dekker, 1981). L-threonine deaminase from *C. propionicum* is a tetramer of identical subunits (M_r=39kDa) (Hofmeister *et al.*, 1993).

It is very fascinating that all of the mammalian L-SDs and L-threonine deaminases from bacteria are PLP-dependent and conserve a glycine-rich sequence GGGGL as well

as the amino acid sequence (G)S(F)K(I)RG. Sequence SAGN also is highly conserved among these L-SDs (Yamada *et al.*, 2003). The general catalytic mechanism of PLP-dependent enzymes is well known (Grabowski *et al.,* 1993). PLP serves as a cofactor to help the removal of the α-proton of the bound amino acid. The mechanism involves transimination and tautomerization to the ketimine which is hydrolyzed to ammonia and pyruvate (Grabowski *et al.,* 1993).

Bacterial L-SDs are independent of PLP. These PLP-independent L-SDs also lack the consensus sequences of PLP-dependent L-SDs. Many L-SDs in bacteria, including *P. asaccharolyticus, C. propionicum, C. acidiurici, Pseudomonas cepacia, L. fermentum and C. jejuni,* contain Fe-S cluster(s) (Hofmeister *et al.* 1993, 1997; Carter and Sagers 1972; Wong *et al.* 1979; Farias *et al.* 1991 and Velaydhan *et al.* 2004). The L-SDs containing Fe-S cluster(s) have similar characteristics: the pure enzymes are yellow-brown; their UV spectrum has one peak at 280nm, two broad shoulders at 300 to 350nm and at 420nm, and the enzyme activity is dependent on the Fe-S cluster(s) (Hofmeister *et al.* 1993, 1997; Carter and Sagers 1972; Wong *et al.* 1979; Farias *et al.* 1991 and Velaydhan *et al.* 2004).

L-SD1 from *E. coli* K-12 was purified as a β-galactosidase fusion protein in 1993 under aerobic condition in our lab. It is a polypeptide of 454 amino acids including nine cysteine residues at positions 181, 219, 290, 339, 347, 366, 381, 392, and 453 (Figure 2) (Su *et al.*, 1993). The amino acid sequence of L-SD1 is 77% identical to L-SD2 and 78% identical to L-SD3 from *E. coli* K-12. Those nine cysteine residues are highly conserved

Figure 2 Sequence of L-serine deaminase1 from *E. coli* K-12

```
MISLFDMFKV GIGPSSSHTV GPMKAGKQFV DDLVEKGLLD SVTRVAVDVY GSLSLTGKGH 60
HTDIAIIMGL AGNEPATVDI DSIPGFIRDV EERERLLLAQ GRHEVDFPRD NGMRFHNGNL 120
PLHENGMQIH AYNGDEVVYS KTYYSIGGGF IVDEEHFGQD AANEVSVPYP FKSATELLAY 180
CNETGYSLSG LAMQNELALH SKKEIDEYFA HVWQTMQACI DRGMNTEGVL PGPLRVPRRA 240
SALRRMLVSS DKLSNDPMNV IDWVNMFALA VNEENAAGGR VVTAPTNGAC GIVPAVLAYY 300
DHFIESVSPD IYTRYFMAAG AIGALYKMNA SISGAEVGCQ GEVGVACSMA AAGLAELLGG 360
SPEQVCVAAE IGMEHNLGLT CDPVAGQVQV PCIERNAIAS VKAINAARMA LRRTSAPRVS 420
LDKVIETMYE TGKDMNAKYR ETSRGGLAIK VQCD                           454
```

C refers to cysteine residue and is shaded grey

Su *et al.*, 1993

in L-SD2 and L-SD3 from this organism. In addition, the amino acid sequence of L-SD1 from *E. coli* K-12 has 30% and 34% homology respectively with the α and β chain of L-SD from *P. asaccharolyticus*. Quite interestingly, four cysteine residues at positions 181, 339, 381, and 392 in L-SD1 from *E. coli* K-12 are also found in L-SD from *P. asaccharolyticus* (Hofmeister *et al.*, 1997). L-SD1 lacks the conserved sequence (G)S(F)K(I)RG and the glycine-rich region that occurs in all PLP-dependent serine deaminases (Grabowski *et al.,* 1993). Consequently, Hofmeister and his colleagues (1997) hypothesized that L-SD1 is a Fe-S cluster enzyme. Now L-SD1 has been purified anaerobically. It is reported that this enzyme contains small amounts of iron and sulfide. Its color is light brown and its UV-visible spectrum shows the characteristic features of 4Fe-4S cluster. In contrast, the reconstituted L-SD1 in absence of dithionite only shows the characteristic features of 3Fe-4S cluster. All of these demonstrate that L-SD1 is an enzyme containing Fe-S cluster (Cicchillo *et al.,* 2004).

2.3. *In vitro* activity and activation of L-SD

L-SD activity has been studied in several bacterial species including *E. coli* K-12 (Ogawa 2000). These studies have all shown that the enzymes exist in a partially inactive form when purified under aerobic conditions and the activity is unstable both in crude extracts or purified protein (Newman *et al.*, 1985; Carter and Sager, 1972; Grabowski *et al.*, 1991; Hofmeister *et al.*, 1993; and Zinecker *et al.*, 1998).

However, L-SD can be activated in a variety of ways. For example, L-SD from *C. acidiurici* can be activated with ferrous sulfate and dithiothreitol (DTT) (Carter and Sager, 1972); the L-SDs from *P. asaccharolyticus, C. propionicum,* and *C. sticklandii* could be specifically reactivated by incubation with Fe^{2+} under anaerobic conditions. In addition, all these L-SDs are sensitive to oxygen and specific to L-serine. L-cysteine and D-serine can inhibit the enzyme activity (Grabowski *et al.*, 1991; Hofmeister *et al.*, 1993; Zinecker *et al.*, 1998). The half-life of this enzyme from *C. jejuni* is around 1.5 hours when it was exposed to air. The inactive enzyme can be reactivated by Fe^{2+} and DTT under strict anaerobic conditions. DTT alone or DTT with Fe^{3+}, Cu^{2+}, Mg^{2+}, Mn^{2+}, and Ni^{2+} cannot reactivate the enzyme (Velayudhan *et al.*, 2004). In addition, the L-SDs containing Fe-S cluster(s) lose their spectral features while they are inactive (Hofmeister *et al.*, 1993; Zinecker *et al.*, 1998; and Velayudhan *et al.*, 2004).

L-SD1 from *E. coli* K-12 has been studied previously in our lab. The enzyme activity is unstable in both crude extracts and purified preparations. When it is exposure to air, it will be inactive. However, the inactivated enzyme can be re-activated by incubation with iron and DTT under aerobic conditions in vitro. This enzyme is also specific to L-serine and can be inhibited by L-cysteine and D-serine (Newman *et al.*, 1980; 1985a; Dumont *et al.*, 1986; and Su *et al.*, 1993). L-SD1 in *E. coli* K-12, grown in glucose-minimal medium, can be induced by glycine and/or L-leucine but not its substrate, L-serine. L-SD is also induced by increased growth temperature, by growth in

Luria broth or anaerobic condition, and by alcohol shock (Su *et al.*, 1993). D-serine and L-cysteine can inhibit L-SD (Newman *et al.*, 1982; 1985a; and Su *et al.*, 1993).

Part. 3. Three genes code for L-SD in *E. coli* K-12

There are three genes (*sdaA, sdaB,* and *tdcG) in E. coli* K-12 that code for L-SD. The genes are located at different places in genomic DNA and expressed under different conditions (Su *et al.* 1989, Su and Newman 1990, Shao and Newman 1993, Hesslinger *et al.* 1998).

The *sdaA* gene is 1365 bp. It is located at 40.85 minute and encodes L-SD1. *SdaA* gene can be expressed in cells grown in both Luria broth (LB) and glucose-minimal medium in aerobic and anaerobic conditions (Su *et al.*, 1989; 1991, Shao and Newman 1993). *Ssd* or *lrp* gene products can regulate the transcription of *sdaA* to affect the expression of *sdaA* gene (Newman *et al.*, 1981; Lin *et al.*, 1990). DNA-damaging agents such as: UV, nalidixic acid, and mitomycin can increase the expression of *sdaA* (Newman *et al.*, 1982).

The *sdaB* gene that encodes for L-SD2 is 1368 bp and mapped at 60.1 minute (Su and Newman 1991, Shao and Newman 1993). Its DNA sequences are 73% identical to the sequences of *sda*A.

Both of these two enzymes have very similar enzyme activity (Shao and Newman 1993). Although *sdaA* and *sdaB* are so similar, their expression and regulation are very different. *SdaB* is only expressed in rich medium such as Luria broth (LB), especially in

the absence of glucose in both aerobic and anaerobic condition. This gene is regulated by cAMP and the catabolite activator protein. In contrast to *sdaA*, expression of *sdaB* gene is not subject to the regulation of s*sd* or *lrp* gene products. Furthermore, its expression is not affected by the environmental factors (Shao and Newman 1993, Su *et al.* 1993).

The anaerobically regulated *tdc*ABCDEFG operon of *E. coli* comprises seven genes and is induced anaerobically in the presence of L-threonine and cAMP-CRP (Hobert and Datta 1983, Wu *et al.* 1992). The *tdcG* gene lies at 70.18 minute (Bairoch and Boeckman 1993, Hesslinger *et al.* 1998). It encodes a novel L-serine deaminase named L-SD3 and the enzyme has not yet been characterized. It is only known that *tdcG* is exclusively expressed in anaerobic conditions. This expression is strongly repressed by catabolite. Sequence of L-SD3 is 74% similar to L-SD1 and 71% similar to L-SD2 (Hesslinger *et al.* 1998). In this study, I focused on L-SD1, *sdaA* gene product. Therefore, L-SD2 and L-SD3 will not be considered in any of the work presented in this thesis.

Part. 4. Cysteine, iron-sulfur clusters and L-serine deaminase in *E. coli* K-12

4.1. Cysteine is a thiol-containing amino acid

Cysteine is a sulfur-containing amino acid. The unique thiol side chain of cysteine is reactive and critically important. The side chain of one cysteine can be bound to another cysteine side chain to form disulfide bridges (CH_2-S-S-CH_2--), which are components of protein structure and act to stabilize the three-dimensional structure of proteins. Cysteine

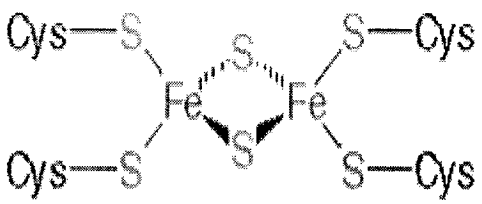
residue can be in the catalytic site of enzymes, can bind iron in Fe-S clusters, or other prosthetic groups, such as Zn, Cu, and hemes that are necessary for enzyme activity. Cysteine is also involved in the metabolism of many essential molecules such as coenzyme A, biotin, and glutathione (Biochemistry, Voet and Voet. 1990).

4.2. Iron-sulfur clusters and iron-sulfur proteins

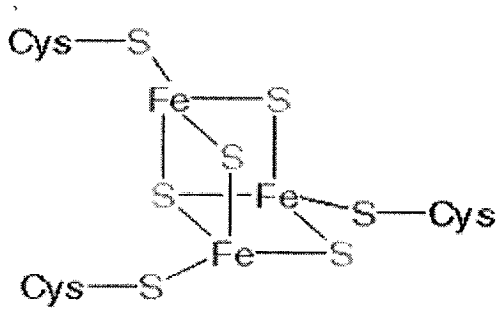
Iron-sulfur (Fe-S) clusters are simple inorganic prosthetic groups that are present in some proteins in all organisms from the most ancient bacteria and archaea to the most advanced eukaryotes (Beinert *et al.* 1997, Beinert 2000). Being the functional elements, iron-sulfur clusters are contained in a variety of proteins classified as iron-sulfur protein(s).

By the mid-1960s, it was already well known that iron-sulfur proteins contain complexes of iron and cysteinate sulfur atoms. Since then, the knowledge of iron–sulfur clusters and iron-sulfur proteins has increased dramatically (Beinert *et al.* 1997, Hewitson *et al.* 2002). The simplest Fe-S proteins, such as rubredoxins, contain a 2Fe-2S cluster with Fe coordinated by four cysteines from the protein. These clusters are also composed of spatially organized assemblies of inorganic iron and sulfide with 2Fe-2S, 3Fe-4S, 4Fe-4S, 7Fe-8S, 8Fe-8S clusters and so on (Figure 3). Among them, 2Fe-2S and 4Fe-4S are the most common cluster types, and they can be converted to each other as well as to a 3Fe-4S cluster in some conditions (Beinert *et al.* 1997, Beinert 2000). For example, Mössbauer Spectroscopy of the FNR (fumarate and nitrate reduction) protein from *E. coli*

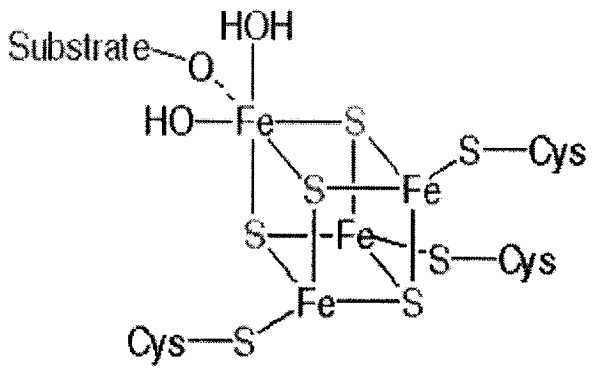
Figure 3 Examples of Fe-S clusters



2Fe-2S cluster



3Fe-4S cluster



4Fe-4S cluster

Figure drawn from http://metallo.scripps.edu/PROMISE/FESMAIN.html#FeS

has shown that the exposure of FNR containing a $[4Fe\text{-}4S]^{2+}$ cluster to O_2, causes a rapid conversion to a $[2Fe\text{-}2S]^{2+}$ cluster both in vivo and in vitro (Khoroshilova *et al.* 1997). When aconitase is exposed to O_2, the 4Fe-4S clusters are converted to 4Fe-3S clusters (Emptage *et al.* 1983). Certainly, these structural conversions of Fe-S clusters are related, according to their biological roles (Beinert *et al.* 1997, Khoroshilova *et al.* 1997, Crack *et al.* 2004).

4.3. Functions of iron-sulfur clusters and Aconitase

4.3.1. Functions of iron-sulfur clusters

Iron-sulfur cluster-containing proteins take part in a number of physiologically important processes, including DNA repair, transcriptional regulation, nucleotide and essential amino acid biosynthesis, and energy metabolism (Flint *et al.* 1996, Beinert *et al.* 1997).

Fe-S clusters function in electron transfer, sensing, regulation and catalysis (Beinert 2000). The iron can exist in either Fe^{2+} or Fe^{3+} oxidation states, and the sulfur can occur in S^{2-} to S^{6+}, therefore Fe-S clusters acted as prosthetic group can transfer electron(s) from one cluster to another. Hydrogenases are enzymes capable of catalyzing the oxidation of molecular hydrogen or its production from protons and electrons according to the reversible reaction: $H_2 <==> 2H^+ + 2e^-$. The [NiFe]hydrogenase of Desulfovibrio gigas is a typical hydrogenase containing a Ni center and three Fe-S centers, one [3Fe-4S] and two [4Fe-4S] clusters (Guigliarelli *et al.,* 1995). X-ray crystallographic structures of

the [NiFe]hydrogenase of *Desulfovibrio gigas* have also proved it (Fontecilla-Camps *et al.* 1997). The proteins acting as electron transfers include Ferredoxins, Rieske proteins, Rubredoxins and so on (Beinert 1976, Muller *et al.* 1999, Victor *et al.* 2003).

Scientists have renewed interest in the Fe-S proteins in the past ten to fifteen years, since they play important roles in sensing and regulating the levels of O_2, reactive oxygen species (ROS), nitric oxide (NO) and cellular iron. The functions of Fe-S proteins are dependent on Fe-S cluster (Gardner *et al.* 1997, Kiley and Beinert. 1998, Bouton 1999). An example for the case is FNR (FNR stands for fumarate and nitrate reduction). FNR, one of the transcription factors in *E. coli*, is a global regulator controlling the synthesis of up to 125 proteins that are required for the anaerobic metabolism of *E. coli*. Fe-S clusters are key to the functions of FNR as a globe regulator and an O_2 sensor. When Fe-S clusters present as $[4Fe\text{-}4S]^{2+}$ form in FNR, FNR is active. $[4Fe\text{-}4S]^{2+}$ is very sensitive to oxygen. The exposure of FNR containing $[4Fe\text{-}4S]^{2+}$ cluster to O_2, either *in vitro* or in whole cells, $[4Fe\text{-}4S]^{2+}$ cluster will rapidly convert to $[2Fe\text{-}2S]^{2+}$ cluster. This conversion will inactivate FNR. Fe-S clusters also appear to be important for the regulatory functions of the transcription factors IscR and SoxR in *E. coli* (Schwartz *et al.* 2001, Kiley and Beinert 2003). In contrast, loss of $[4Fe\text{-}4S]^{2+}$ cluster is required for the cytoplasmic aconitase function as iron regulatory protein. Fe-S clusters also appear to be important for the regulatory functions of the three transcription factors FNR, IscR and SoxR in *E. coli* (Schwartz *et al.* 2001, Kiley and Beinert 2003).

Non-electron transfer functions of Fe-S cluster also include its role in substrate

binding and catalysis. It is interesting that the electron(s) can be transferred between Fe-S clusters, and be shifted within Fe-S cluster structure, including its ligands. The electrons' shift within Fe-S cluster(s) will cause the polarization of other surrounding and /or attached groups. Then the Fe-S cluster(s) can function as active sites of enzymes to bind to and activate the substrates (Beinert 2000, Kiley and Beinert 2003).

It is also well known that aconitase, one of the well-characterized Fe-S cluster containing proteins, has multiple roles. It catalyses a reaction of the tricarboxylic acid cycle and also serves as the regulator and sensor of iron — iron regulatory protein (Beinert and Kennedy 1993). In the next paragraph I will give detailed explanation of the acotinase.

4.3.2. Aconitase and its functions

Aconitase [citrate (isocitrate) hydratase, EC 4.2.1.3.] reported in 1937 by C. Martius has been found in all organisms (Beinert et al., 1996). In eukaryotes, aconitases are present in both mitochondria and cytoplasm (Philpott *et al.* 1994). The mitochondrial aconitase can catalyze the reversible dehydration of citrate to isocitrate via *cis*-aconitate in the TCA cycle. This step is a non-redox active process. Mitochondrial aconitases have been purified and crystallized from pig heart and beef heart. It is found that the mitochondrial aconitase is a single polypeptide, composing four domains, one hinge-linker and one active site (Figure 4) (Lauble *et al.*1992). The Fe-S cluster is located at the

active site and three cysteine residues in aconitase are ligated to the Fe-S cluster. The function of four domains is not very clear. The first three domains (domain1, domain2 and domain3) are believed to be closely associated with the Fe-S cluster at the active site, while the hinge-linker connects the fourth domain to the other three domains and form an active site cleft. This flexible link allows the fourth domain to bind to either citrate or isocitrate. When substrate exists, a motion along the hinge-linker can make the fourth domain leave from the other three domains and move to the substrate. Then the fourth domain binds to the substrate. This binding causes the substrate to enter the active site (Lauble *et al.*1992, Berneit *et al.* 1996).

Cytosolic aconitase has enzymatic, sensing and regulatory functions. The enzymatic function of the cytosolic aconitase is similar to the mitochondria aconitase. Its sensing and regulatory functions are involved in the uptake, transport, storage and utilization of cellular iron and are associated with the binding of mRNA. Furthermore, all functions of cytosolic aconitase are dependent on the cellular iron level and relation with Fe-S cluster. When the cellular iron level is high, the cytosolic aconitase functions as iron-responsive protein-binding protein (IRP-BP) binding to mitochondria aconitase. There present [4Fe-4S] clusters, as the enzyme is active. If the [4Fe-4S] clusters change to [3Fe-4S] clusters, the enzyme will be inactivated. The mechanisms of this conversion are involved in electron shift within Fe-S clusters. When the cellular iron level is low, the cytosolic aconitase disassembles the [4Fe-4S] clusters and loses its enzymatic function, and only

Figure 4 Structure of mitochondrial aconitase

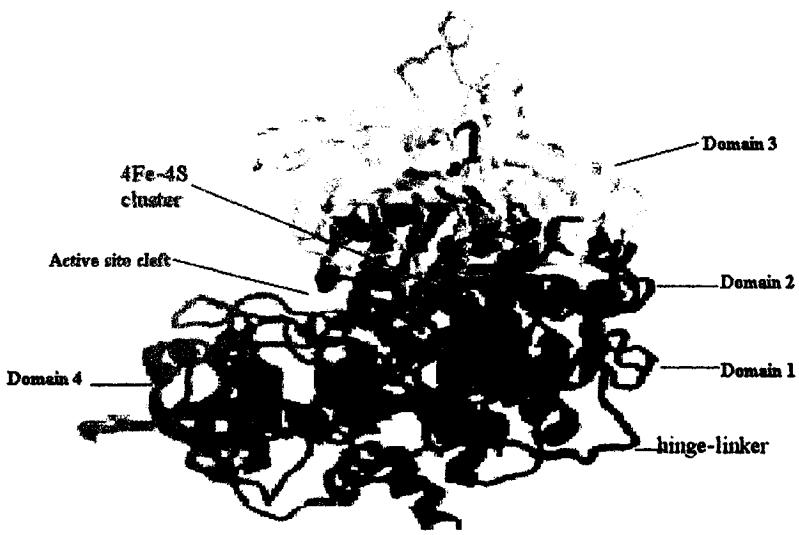


Figure drawn from the structure determined by Lauble *et al.*, 1994.

serves as IRP-BP to bind to the iron responsive elements (IREs), a specific stem-loop in the untranslated regions of mRNA. Because 3' ends of IREs of the mRNA encode transferrin receptor, this site will prevent the degradation of IREs of mRNA. Thus cells will produce more transferrin receptor proteins and transfer more iron into cells to increase the cellular iron concentration. On the other hand, the 5' ends of IREs of mRNA encode the proteins involved in iron store and /or utilization such as ferritin. Binding of cytosolic aconitase (IRP-BP) to the 5' ends of IREs of mRNA will lead to prevention of translation of this mRNA and also induce degradation of the target mRNA. These will reduce the proteins involved in iron store and /or utilization such as ferritin to increase the cellular iron concentration (Philpott *et al.* 1994, Berneit *et al.* 1996). In *E. coli*, there are two isoforms of aconitase, AcnA and AcnB. AcnA is active under conditions of iron and oxidative stress and has been purified. Electron paramagnetic resonance studies of the purified enzyme confirmed that it contains a [4Fe-4S] cluster (Bennett *et al.,* 1995). AcnB acts as the TCA cycle enzyme. AcnA and AcnB can bind to mRNA (Jordan *et al.,* 1999).

4.4. The relation between L-serine deaminase, cysteine and the Fe-S cluster

As described previously, L-SDs from *P. asaccharolyticus* (Grabowski *et al.,* 1991), *C. sticklandii* (Zinecker *et al.,* 1998), *C. propionicum* (Hofmeister et al., 1994), *E. coli* (Cicchillo *et al.,* 2004) and *C. jejuni* (Velaydhan *et al.,* 2004), have shown spectral features of iron-sulfur clusters. The study of electron paramagnetic resonance of L-SD

from *P. asaccharolyticus* has revealed that this enzyme contains a 4Fe-4S cluster at its active site, as does mitochondrial aconitase. In *Klebsiella aerogenes*, *Pseudomonas cepacia*, and *Arthrobacter globiformis*, PLP-independent L-SDs were also found containing Fe-S clusters (Wong *et al.* 1979, Vining *et al.,* 1981, Gannon *et al.* 1977). Four conserved cysteine residues in bacterial L-SDs are proposed to coordinate a [4Fe-4S] cluster (Hofmeister *et al.* 1997, Zinecker *et al.* 1998, Velaydhan *et al.* 2004).

Part 5 The object of this study

L-SD1 of *E. coli* K-12 consists of 454 amino acids, including 9 cysteine residues (Su *et al.,* 1993). It is shown to be an enzyme containing 4Fe-4S cluster (Cicchillo *et al.,* 2004). Cysteine is the common ligand of Fe-S cluster in proteins. Thus, my study will focus on the role of these cysteine residues. I would like to know whether the nine cysteine residues are important for the activity and/or activation of L-SD1.

Spectroscopic techniques including Electron Paramagnetic Resonance, Mössbauer spectroscopy and Electron-Nuclear Double Resonance (ENDOR) and X-ray crystallography have been widely applied to the study of the structures of Fe-S cluster proteins. Site-directed mutagenesis is still one of most common means to demonstrate the chemistry and reaction mechanism of Fe-S proteins (Moulis *et al.* 1996).

In this study, I substituted the nine cysteine residues of L-SD1 from *E. coli* K-12 with other amino acids individually by site-directed mutagenesis via PCR. Then, the wild type L-SD1 and the mutant proteins were overexpressed in a 6×His tag system to get a His-tagged fusion proteins. This 6×His tag is too small to interfere with the structure and function of the recombinant protein. The enzyme activity of the purified proteins could then be studied.

Material and Methods

1. Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Table 1.

2. Media and growth conditions

2.1. Glucose minimal medium

0.54% K_2HPO_4, 1.26% KH_2PO_4, 0.2%$(NH_4)_2SO_4$, 0.2%$MgSO_4.7H_2O$, 0.01%$CaCl_2$, pH 7.0.

Since MEW28 carries a deletion in *ilvA* gene, it requires L-isoleucine for growth. L-isoleucine and L-valine were therefore added to the media at a concentration of 50 μg/ml each unless noted otherwise.

2.2. Luria Broth (LB)

1%Bactotryptone, 0.5%yeast extract, 0.5%NaCl. When making plates, 2% Bactoagar was added to the medium before autoclaving. Sterile D-glucose (as a carbon source) was provided at a concentration of 2 mg/ml after autoclaving.

2.3. Other additions to the medium

Ampicillin was added to a final concentration of 100μg/ml. Isopropylthiogalactoside (IPTG) was added at to a final concentration 0.4 mM.

Table 1 Bacterial Strains and Plasmids

Strains	Genotype and /or relevant characteristic	Reference/Source
CU1008	*E. coli* K-12 Δ*ilvA*	L. S. Williams
MEW1	CU1008 Δ*lac*	Newman *et al.*, 1985
MEW28(CH22)	MEW1 *sdaA*::cm	Su *et al.*1991.
JM109	*F′ traD36 proA⁺B⁺ lacIᵠ Δ(lacZ)M15/ Δ(lac-proAB) glnV44 e14⁻ gyrA96 recA1 relA1 endA1 thi hsdR17*	Yanish-Perron, C. *et al.*1985
BL21 (DE3)	F⁻ *ompT hsdS*$_B$ ($_{rB}^-$ $_{mB}^-$) *gal dcm* (DE3)	Studier *et al.* 1990
Plasmids		
pMES22(p*sdaA*)	pBR322 carrying a 2.6 Kb fragment of *sdaA* gene	Su *et al.*, 1989
p*sdaA*/T7-5 (p*sdaA*H6)	*sdaA* gene from HB101 was cloned into pT7-5 vector carrying a 6xHis-tag pMttA2 H6/T7-5.	Dr. Weiner's Lab
p181	p*sdaA* with a new *BsiW*I site by site-directed mutagenesis at amino acid 181 of L-SD1	This study
p219	p*sdaA* with a new *Nhe*l site by site-directed mutagenesis at amino acid 219 of L-SD1	This study
p290	p*sdaA* with a new *Sac*II site by site-directed mutagenesis at amino acid 290 of L-SD1	This study
p339	p*sdaA* with a new *ApaL*I site by site-directed mutagenesis at amino acid 339 of L-SD1	This study
p347	p*sdaA* with a new *Nhe*I site by site-directed mutagenesis at amino acid 347 of L-SD1	This study
p366	p*sdaA* with a new *Afe*I site by site-directed mutagenesis at amino acid 366 of L-SD1	This study
p381	p*sdaA* with a new *Eag*I site by site-directed mutagenesis at amino acid 381of L-SD1	This study
p392	p*sdaA* with a new *NgoM*IV site by site-directed mutagenesis at amino acid 392 of L-SD1	This study

p453	p*sdaA* with a new *BstN*I site by site-directed mutagenesis at amino acid 453 of L-SD1	This study
PCHsdaA	*sdaA* gene from *E. coli* K-12 was cloned into p*sdaA*H6 at *Eco*RI-*Xho*I site	This study
pCH181	p181 was cloned into C-terminal His-tag vector p*sdaA*H6 at *Eco*RI-*Xho*I site	This study
pCH219	p219 was cloned into C-terminal His-tag vector p*sdaA*H6 at *Eco*RI-*Xho*I site	This study
pCH290	p290 was cloned into C-terminal His-tag vector p*sdaA*H6 at *Eco*RI-*Xho*I site	This study
pCH339	p339 was cloned into C-terminal His-tag vector p*sdaA*H6 at *Eco*RI-*Xho*I site	This study
pCH347	p347 was cloned into C-terminal His-tag vector p*sdaA*H6 at *Eco*RI-*Xho*I site	This study
pCH366	p366 was cloned into C-terminal His-tag vector p*sdaA*H6 at *Eco*RI-*Xho*I site	This study
pCH381	p381 was cloned into C-terminal His-tag vector p*sdaA*H6 at *Eco*RI-*Xho*I site	This study
pCH392	p392 was cloned into C-terminal His- tag vector p*sdaA*H6 at *Eco*RI-*Xho*I site	This study
pCH453	p453 was cloned into C-terminal His-tag vector p*sdaA*H6 at *Eco*RI-*Xho*I site	This study

3. Buffers and solutions

3.1. SOC buffer for electro-transformation

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

3.2. TBE (Tris-borate and EDTA) buffer for DNA agarose gel electrophoresis

Concentration of stock solution (5×): 0.45 M Tris-borate, 0.01 M EDTA (pH 8.0)

3.3. Protein crude extract and purification buffers:

1× HEPES buffer: 50mM HEPES pH 7.9

1×Binding buffer: 20mM Tris, 5mM Imidazole, 0.5M NaCl, pH 7.9

1×Wash buffer: 20mM Tris, 100mM Imidazole, 0.5M NaCl, pH 7.9

1×Elution buffer: 20mM Tris, 800mM Imidazole, 0.5M NaCl, pH 7.9

1×Charge buffer: 50mM NiSO4,

3.4. DNPH solution for L-SD activity assays of crude extracts and purified protein

0.25% 2,4-dinitrophenyhydrazine(DNPH) dissolved in 4.1% HCl.

3.5. Phosphate buffer for whole cell L-SD assay

79.1g K_2HPO_4, 6.26g KH_2PO_4 dissolved in 1 liter distilled water (pH 7.8). (Sambrook and Russell, 2001).

4. Chemicals

General chemicals in this study were from Fisher or Sigma. Restriction enzymes, pfu DNA polymerase and DNA modifying enzymes were purchased from MBI Fermentas (Montreal, Canada) and New England Biolabs. Oligonucleotides were purchased from Biocorp (Montreal, Canada).

5. Enzyme assay

5.1 *In vivo* L-SD1 activity assay (whole cell L-SD1 activity assay)

L-SD1 activity was assayed as described in toluene-treated whole cells (Isenberg and Newman, 1974, Pardee and Prestidge, 1955). In this study, cells were grown in glucose minimal medium and harvested at an absorbance of 0.8 (OD_{600}). KPO_4 buffer (pH 7.8) was used to adjust the cells to 100 Klett units using a Klett-Summerson colorimeter equipped with a 540 filter. The reaction mixture was prepared by sequential addition of the following reagents: 100 µl L-serine (20 mg/ml), 300ul cells in phosphate buffer and 2 µl toluene. This mixture was incubated in a 37°C water bath for 35 min. 0.9

ml DNPH was added and the mixture incubated at room temperature for 20 min. Finally, 1.7 ml 10% NaOH was added to stop the reaction. L-SD activity is expressed as micromoles pyruvate formed in 35 minutes by 100 Klett units of cells at 37°C.

5.2 *In vitro* L-serine deaminase assay

In vitro measurement of L-serine deaminase activity can be performed on either a crude protein extract or on purified protein. Both require activation by iron and dithiothreitol (DTT) (Newman *et al.* 1985a). The form of iron responsible is as yet unknown (Newman *et al.* 1990), but ferric salts ($FeCl_3$) were used in this study.

In this study, 4.5mM $FeCl_3$ and 225mM DTT (final concentration) were used to activate L-SD1 in crude extracts. Crude extracts, containing different amounts of protein, were added into reaction mixtures at different volumes and dilution ratios. 10 µl L-serine (100 mg/ml) was used as substrate and the final reaction volume was adjusted to 170 µl by the addition of 50 mM HEPES buffer. This reaction mixture was incubated in a 37°C water bath for 35 min, after which DNPH was added to stop the reaction and the level of keto acid was determined as previously described (Newman *et al.* 1980). Activity of purified proteins was measured in the same way but 1.8mM $FeCl_3$ was used.

The enzyme activity was related to amount of pyruvate. In both crude extract and purified protein assays, one unit of L-SD1 is defined as the amount of enzyme that produces 1 µmol of pyruvate per minute per mg protein at 37°C.

6. Protein concentration assay

Protein concentration was determined by using Bio-Rad protein assay reagent according to the manufacturer's protocol. Bovine serum albumin was used as standard.

7. Determination of plasmid maintenance

An appropriate dilution of cells was first plated on LB plates and the resulting colonies were replicated on LB plates containing 200μg/ml ampicillin.

8. Transformation

Electro-transformation was performed using Gene Pulser (Bio-Rad) according to directions provided by the manufacturer.

Preparations of competent *E. coli* cells using calcium chloride and chemical transformations were performed as described by Sambrook and Russell (2001).

9. DNA manipulation

9.1. DNA isolation and purification

Plasmid isolation, PCR product purification and gel extraction were performed using QIAGEN (Montreal, Canada) kits following their protocols.

9.2 Molecular biology methods

Recombinant DNA methods used in this study included standard procedures described by Sambrook and Russell (2001) and directions provided by suppliers of various reagents including restriction enzymes.

10. Gel electrophoresis

DNA agarose gel electrophoresis (usually 1% agarose) was carried out as described by Sambrook and Russell (2001).

11. Site-directed mutagenesis: construction of nine cysteine mutants

In vitro site-directed mutagenesis is one of the most commonly used methods in molecular biology. It is an invaluable technique for studying protein structure-function relationships and gene expression. The tools of site-directed mutagenesis are: a template with known sequence that one wants to change, a mutagenic primer (or primers), DNA polymerase and dNTPs. Quick-change site-directed mutagenesis utilizes a supercoiled double-stranded DNA (dsDNA) for the template and two synthetic oligonucleotide primers containing the desired mutation. The primers, each complementary to opposite strands of the template, are extended during temperature cycling by DNA polymerase. Incorporation of the oligonucleotide primers generates a mutated plasmid containing staggered nicks. Following temperature cycling, the product is treated with DpnI. The

DpnI endonuclease (target sequence: 5'-GM^6 ATC-3') is specific for methylated and hemimethylatd DNA and so digests the parental DNA template, leaving the mutation-containing synthetic DNA (Nelson *et al.,* 1992). The synthetic DNA is then transformed into JM109 competent cells.In this study, Dr. Jiang and I made site-directed mutations in the *sdaA* gene in order to change each of nine cysteine residues of L-SD1. Dr. Jiang made mutations in amino acids 290, 339, 366, 381 and 453. I changed amino acids 181, 219, 347 and 392.

First I used plasmid pMES22 (p*sdaA*) as the template. The 9 pairs of oligonucleotide primers (181f and 181r, 219f and 219r, 290f and 290r 339f and 339r, 347f and 347r, 366f and 366r, 381f and 381r, 392f and 392r, and 453f and 453r) were synthesized by Biocorp and are listed in Table 2. Each pair of primers introduces a new amino acid and a new specific enzyme cut site into the *sdaA* gene. PCR reactions were performed in a 50μl reaction mixture containing 8 ng template p*sdaA*, 0.08 μM of each of the two primers, 0. 2 mM each of the four deoxynucleoside triphosphates (dNTPs), 5 μl PCR buffer containing $MgSO_4$ and 2 units pfu DNA polymerase (MBI) were added. The reaction mixtures underwent the following PCR cycles in a DNA thermocycler (Interscience).

1) Denature DNA	94°C 40 sec
2) 18 cycles	94°C 40sec
	60°C 1 min

Table 2 Primers used for site-directed mutagenesis of the *sdaA* gene

Primer	Sequence (5' to 3')
181f	GAA CTG CTC GCG TAC GGT AAT GAA ACC GGC TAT
181r	ATA GCC GGT TTC ATT ACC GTA CGC GAG CAG TTC
219f	C TGG CAA ACC ATG CAG GCT AGC ATC GAT CAC GGG ATG AAC
219r	GTT CAT CCC GCG ATC GAT GCT AGC CTG CAT GGT TTG CCA G
290f	CG CCA ACC AAC GGT GCC GCG GGT ATC GTT CCG GCA GT
290r	AC TGC CGG AAC GAT ACC CGC GGC ACC GTT GGT TGG CG
339f	TCC GGT GCG GAA GTT GGT GCA CAG GGC GAA GTG GGT
339r	ACC CAC TTC GCC CTG TGC ACC AAC TTC CGC ACC GGA
347f	CAG GGC GAA GTG GGT GTT GCT AGC TCA ATG GCT GCT GCG GGT
347r	ACC CGC AGC AGC CAT TGA GCT AGC AAC ACC CAC TTC GCC CTG
366f	GGT AGC CCG GAA CAG GTT AGC GCT GCG GCG GAA ATT GGC ATG
366r	CAT GCC AAT TTC CGC CGC AGC GCT AAC CTG TTC CGG GCT ACC
381f	GAA CAC AAC CTT GGT TTA ACG GCC GAC CCG GTT GCA GGG CAG
381r	CTG CCC TGC AAC CGG GTC GGC CGT TAA ACC AAG GTT GTG TTC
392f	GGG CAG GTT CAG GTG CCG GCC ATT GAG CGT AAT GCC ATT G
392r	CAA TGG CAT TAC GCT CAA TGG CCG GCA CCT GAA CCT GCC C
453f	G GCA ATC AAA GTC CAG GGT GAC TAA TAC TTC TTA C
453r	G TAA GAA GTA TTA GTC ACC CTG GAC TTT GAT TGC C

The base pairs underlined are specific enzyme cut sites.

68°C 7min 30sec

2) cycle 72°C 6 min

PCR products were subsequently analyzed on a 1% agarose gel. Well-amplified samples were further digested with DnpI for 1hr at 37°C, transformed into JM109, and plated on LB-Amp plates for selection. Plasmids were isolated from ampicillin–resistant transformants, and tested for restricting enzymatic digestion. Each of the mutated plasmids was sent to University of Laval to be sequenced. All had the correct sequence. The plasmids were named p181, p219 etc where 181 or 219 corresponds to the 181 or 219 cysteine residues changed in the mutagenesis.

12. Construction of C-terminal his-tagged plasmid

12. 1. Construction of C-terminal his-tagged *sdaA* plasmid (pCH*sdaA*)

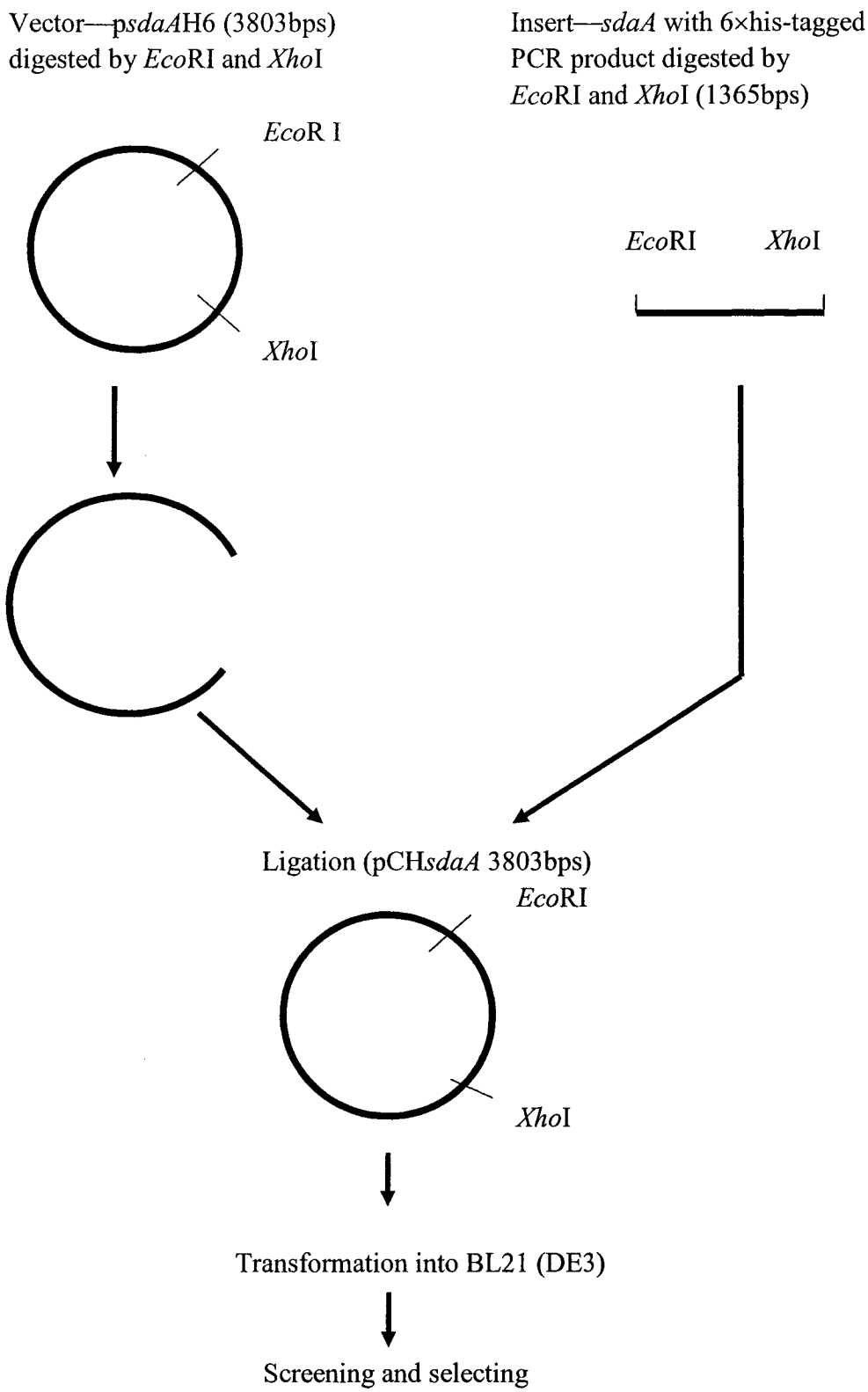
To construct a C-terminal his-tagged *sdaA,* I used p*sdaA* as template. Two primers C-his F and C-his R for PCR (Table3) were used to introduce *Eco*RI and *Xho*I cut sites into the *sdaA* fragment of p*sdaA*. In C-his R (reverse primer complementary to the C-terminal), the stop codon was removed from the native gene. A DNA fragment containing the *sdaA* gene and *Eco*RI-*Xho*I cut sites was amplified by PCR. This amplified DNA and the plasmid p*sdaA*H6 (a modified pT7-5 vector, carrying six histidines) were digested by *Eco*RI and *Xho*I, and then ligated to create the C-terminal his-tagged *sdaA* plasmid we named pCH*sdaA*. In this plasmid, a stop codon was

placed immediately following the His-tag for *sdaA,* and the two residues encoded by the *Xho*I site were inserted between *sadA* and the His-tag. The strategy for this construction is shown in Figure 5. PCR was performed in a final volume of 100 µl containing 0.4 ng template plasmid *psdaA*, 0.008 nM of each of the two primers, 200 µM each of dNTPs, pfu DNA polymerase buffer containing $MgSO_4$ 10 µl, and 1u pfu DNA polymerase (MBI). The reaction mixture underwent the following PCR cycles in a DNA thermocycler (Interscience):

1)	Denature DNA	94°C 40 sec
2)	18 cycles	94°C 40sec
		45°C 1 min
		68°C 4min
3)	1 cycle	72°C 6 min

PCR product(s) were analyzed on a 1% agarose gel. Well-amplified samples were further purified using a PCR purification kit (QIAGEN) designed to remove template and unincorporated primers. Purified PCR products were then digested with *Eco*RI and *Xho*I and the digestion products purified to obtain a DNA fragment containing *sdaA* insert. I also digested the plasmid p*sdaA*H6 with *Eco*RI and *Xho*I, ran an aliquot on a 1 % agarose gel and saw two bands, one at approximately 1.4 kb and the other at 2.4 kb. The 2.4 kb DNA fragment was extracted from the gel using a QIAGEN Gel extraction kit and ligated with the insert DNA by T4 DNA ligase. The ligated products were purified using a QIAGEN DNA purification kit. Finally, for overexpression of L-SD1, purified ligation

Figure 5 Construction of 6×His-tag *sdaA*—plasmid pCH*sdaA*



products were electro-transformed into BL21 (DE3) (Studier et al., 1991) and plated on LB-Amp plates for subsequent selection. Plasmid from one of the ampicillin-resistant colonies was isolated and confirmed to be the right clone by appropriate enzymatic digestion. This was the C-terminal his-tagged *sdaA* plasmid–pCH*sdaA*.

12. 2. Construction of plasmids pCH181, pCH219, pCH290, pCH339, pCH347, pCH366, pCH381, pCH392

Construction of these plasmids was performed in the same way as the construction of pCH*sdaA*, with the exception of the templates used. The templates for these plasmids were p181, p219, p290, p339, p347, p366, p381, and p392 respectively.

12. 3. Construction of plasmid pCH453

Construction of this plasmid was complicated by the proximity of the cysteine (residue 453) to the C-terminal of L-SD1 (454 amino acids). Therefore, p453 was used as the template and the reverse primer was designed to be complementary to the sequence of p453, not *sdaA* (Table 3). Procedures and reaction conditions were the same as used in the construction of pCH*sdaA*.

Table 3 Primers used in the construction of C-terminal his-tagged plasmids

Primers	Sequence (5' to 3')
C-his F	GAT GAA TTC AGG AGT ATT ATC G
C-his R	CGA GCT CGA GGT CAC ACT GGA CTT TG
C-his453 R	CGA GCT CGA GGT CGA CCT GGA CTT TG

The base pairs underlined are specific enzyme cut sites.

13. Crude extract of L-SD1

In order to prepare crude extracts containing L-SD1 or its mutated forms, I transformed p*sdaA* and its derivatives into CH22.

The *sdaA* gene is expressed in cells grown in either LB or glucose minimal medium, while the *sdaB* gene is only expressed in cells grown in LB and *tdcG* is expressed only under anaerobic conditions. Therefore, strains CH22 carrying wild type plasmid (p*sdaA*) and its derivatives were grown in glucose minimal ampicillin medium at 37°C under aerobic conditions. Cells were harvested in late exponential-phase growth and re-suspended in 50 mM HEPES buffer (1 g wet cells: 5 ml buffer). The cell suspension was sonicated, and debris removed by centrifugation at 10,000 rpm for 20 min. The supernatants were collected and stored at -86°C for assay.

14. Protein overexpression

Plasmid pCH*sdaA* and its derivatives were electro-transformed in to BL21 (DE3) for overexpression. Cells were grown in 5 ml LB medium plus 0.5% glucose and 200 μg /ml ampicillin at 37°C overnight. 1% (50 μl) was subcultured into 120 ml of the same medium. Expression was induced by addition of 0.4 mM Isopropylthiogalactoside (IPTG) when cell density reached an absorbance of 0.5 (OD_{600}). Cells were harvested about 3.5 hours after induction, and washed once with 1×binding buffer and re-suspended in the

same buffer (1g wet cell: 5 ml buffer). The cells were sonicated, centrifuged at 20000 rpm, 4°C for 20min, and the supernatant stored at -86°C for subsequent purification steps.

15. Purification of his- tagged protein by Ni-NTA column FPLC

To purify L-SD1 and its derivatives, an extract of *E. coli* BL21 (DE3) over-expressing the protein of interest was passed over a Ni-NTA (nickel-nitriloacetic acid) column. The his-tagged protein binds to the matrix through interaction of the histidine residues with the Ni ions and is then eluted with imidazole.

In this study, the purification was performed on a Ni-NTA superflow column by FPLC (2 Pharmcia P-500 pumps operated by a Pharmacia gradient programmer GP-250 plus). The procedure for purification was as follows: 8-10 ml of the supernatant of an extract of the overexpressed protein was loaded onto the Ni-column. The column was washed with 50 ml 100mM imidazole in 20 mM Tris-HCl (PH8.0) plus NaCl. Finally, 800 mM imidazole in the above buffer was used to elute L-SD1 or its derivatives from the column. The eluted fractions containing the purified protein were dialyzed and concentrated using Centrion concentrators (Amicon) in 50 mM HEPES buffer (pH 7.9) and stored at -86°C.

16. SDS-PAGE

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was

carried out with a Bio-Rad Mini Protean II dual slab cell according the instruction of the manufacturer.

Results

Since the 1960s, it has been recognized that iron-sulfur proteins exist in all life forms in the form of Fe_2S_2 and Fe_4S_4 clusters. Up to date, more than 100 different proteins containing Fe-S cluster(s) have been reported (Jeveson and Dennis, 2003). Bacterial L-SDs were also classified as Fe-S cluster enzymes (Grabowski *et al.,* 1993). Most Fe-S clusters are usually ligated to the protein by cysteine residues (Beinert *et al.,* 1997). L-SD1, a 454 amino acid protein from *E. coli* K-12, contains nine cysteine residues. The goal of this study was to investigate the role(s) played by the cysteines in L-SD1 activity in *E. coli* and to identify which cysteines are essential for the enzyme activity.

The experimental strategies employed in this study were the systematic replacement of each of the nine cysteine residues by site-directed mutagenesis; and the enzyme activity assays *in vivo* and *in vitro* especially with purified His-tagged protein from each mutant.

This section is divided into three parts. First, I report the results of site-directed mutagenesis studies of *E. coli sdaA* including the replacement of nine cysteines residues and the construction of C-terminal His-tagged *sdaA* and its derivatives. Second, the results of expression and overexpression studies of *sdaA* and its derivatives are given. Third, I describe the activity of L-SD1 coded by *sdaA* or its derivatives.

Part 1. Site-directed mutagenesis of *E. coli sdaA*

1.1. Replacement of nine cysteine residues by site-directed mutagenesis

To study the roles of the nine cysteines of L-SD1 in *E. coli* K-12, the nine cysteine residues were replaced by other amino acids (Table 4). Nine pairs of primers were designed to introduce alanine, serine or glycine as replacements for the cysteines. Each pair of primers also introduced a new specific enzyme cut site (see Table 2). All the mutated plasmids were digested by the appropriate enzymes. Sequencing of the mutated region of the plasmids confirmed the predicted sequence.

1.2. Construction of C-terminal his-tagged *sdaA* and derivatives

After creating the cysteine mutations, I wanted to obtain purified L-SD1 coded by wild type *sdaA* gene or its derivatives. Accordingly, the plasmid pCH*sdaA* and derivatives were constructed. Plasmid p*sdaA*H6 is a modified pT7-5 vector that carries a C-terminal 6×His. It contained the 1365bp *sdaA* gene from HB101 flanked by *Eco*RI and *Xho*I sites. I subsequently digested it with *Eco*RI and *Xho*I to obtain a 2.4 kb fragment for use as vector. In this construction, I utilized two primers, C-his F and C-his R, to amplify wild type *sda*A and its derivatives through PCR. The PCR products were approximately 1.4 kb DNA linear fragments containing wild type *sdaA* or mutant derivatives and *Eco*RI and *Xho*I enzyme cut sites. These 1.4 kb linear DNA fragments were digested by *Eco*RI and *Xho*I, then inserted into 2.4 kb p*sdaA*H6 vector resulting in a 3.8 kb recombinant DNA containing C-terminal his-tagged *sdaA* and derivatives. These can be digested by

Table 4 Replacements of nine cysteine residues by site-directed mutagenesis

Mutated Site (Amino acid #)	**Mutation**	**Changed base pairs** (Mutated base pair is underlined)	**Specific enzyme for digest**
181	Cys→Gly	GCGTACTGT→GCGTACGGT	*Bsi*WI
219	Cys→Ser	GCATGT→GCTAGC	*Nhe*I
290	Cys→Ala	GCCTGCGGT→GCCGCGGGT	*Sac*I
339	Cys→Ala	GGTTGCCAG→GGTGCACAG	*Apa*LI
347	Cys→Ser	GCCTGT→GCTAGC	*Nhe*I
366	Cys→Ser	TGCGTG→AGCGCT	*Afe*I
381	Cys→Ala	ACCTGCGAC→ACGGCCGAC	*Eag*I
392	Cys→Ala	GTGCCGTGC→GTGCCGGCC	*Ngo*M IV
453	Cys→Gly	GTCCAGTGT→GTCCAGGGC	*Bst*N I

*Eco*RI and *Xho*I, in addition to the specific enzymes for verification of the mutation.

Part 2. Expression of *sdaA* and derivatives and their enzyme activity assay *in vivo* or *in vitro*

2.1. Expression of *sdaA* and derivatives

Expression of *sdaA* and derivatives is needed for enzyme activity assay *in vivo* and *in vitro* with crude extracts. To express p*sdaA* and its derivatives, MEW28 (CH22) was selected as host cell, and plasmid p*sdaA* and derivatives were electro-transformed into it. CH22 is derived from wild type *E. coli* K-12 strain CU1008. The *sdaA* gene has been disrupted, thus CH22 has no L-SD1 activity. Because *sdaB* coding for L-SD2 can only be expressed in LB medium and *tdcG* coding for L-SD3 can only be expressed under anaerobic conditions (Su and Newman 1991, Shao and Newman 1993, Hesslinger *et al.* 1998), strains carrying p*sdaA* or derivatives were grown in glucose minimal medium under aerobic conditions to prevent the expression of L-SD2 and L-SD3. Therefore, all L-SD activity must be due to expression of L-SD1 from the plasmid copy of *sdaA*. I assayed L-SD1 activity in toluene-treated whole cells and in crude extracts. To ensure that differences in activity were not due to loss of the plasmids, I checked plasmid maintenance in each experiment. There was almost no loss of plasmids.

2.2. L-SD1 activity assay with CH22 carrying *sdaA* gene or derivatives *in vivo*

To know how the mutations affect L-SD1 activity, the enzyme activity assays were

performed *in vivo* in toluene-treated whole cells Results are shown in Table 5. The data indicated that CH22 alone had 2mu enzyme activity but with plasmid containing *sdaA* it had 77mu. Further, the mutations of *sdaA* had different affects on the enzyme activity. If L-SD1 activity of CH22p*sdaA* is taken as 100 percent, C181 had 118% L-SD1 activity. C339, C381, and C392 only had 0-1.3% enzyme activity (even less than CH22 itself (2.6%)). The other five mutations resulted in much lower activity. However, these were not all the same: C347 and C453 only had 6.5- 13% activity, C219, C290, and C366 had 22.1-30% enzyme activity. That suggests mutations of cysteine residues had different effects on L-SD activity *in vivo.* A change at 181 seems to have no great effect; a change at 219, 290, 347, 366, and 453 respectively decreased enzyme activity to different extents; and a change at 339, 381, and 392 caused the absolute loss of L-SD1 activity.

2.3. L-SD1 activity assay *in vitro* using a crude protein extract of CH22 containing *sdaA* or derivatives

We knew that mutations of nine cysteine residues had different effects on L-SD1 activity *in vivo.* To see if this was also true *in vitro,* I performed the L-SD1 activity assay *in vitro* using a crude extract of CH22 carrying p*sdaA* or derivatives. Crude extracts of L-SD1 are inactive *in vitro* and need to be activated by iron and DTT (Newman *et al.* 1985), so all extracts were treated with iron and DTT. Results are shown in Table.6. We found that the activity of C181 and p*sdaA* are almost the same; substitutions of C339,

Table 5 L-SD activity in CH22 and CH22 with p*sdaA* and its derivatives *in vivo*

Strain	Plasmid	L-SD activity (mu)	Percent p*sdaA* activity (%)
CH22	------	2 ± 0.2	2.6
CH22	p*sdaA*	77±1.9	100
CH22	C181	91± 2.3	118.2
CH22	C219	17±0.6	22.1
CH22	C290	18± 1.4	23.4
CH22	C339	1± 0.1	1.3
CH22	C347	5±0.5	6.5
CH22	C366	23± 1.2	29.9
CH22	C381	<0.8	<1
CH22	C392	1± 0.1	1.3
CH22	C453	10± 0.9	13

Cultures were grown in glucose minimal medium and assayed using standard protocols. Results represent the average of three trials. N.B. When the enzyme activity is lower than 0.8 mu, it is immeasurable.

Table 6 *In vitro* L-SD1 activity of crude protein extracts of CH22 carrying *sdaA* gene or derivatives.

Strain	Plasmid	L-SD activity (μmol of pyruvate/min/mg protein)	Percent p*sdaA* activity (%)
CH22	p*sdaA*	129 ± 7.4	100
CH22	p181	126 ± 11.4	97.7
CH22	p219	24 ± 1.4	18.7
CH22	p290	16± 1.0	12.4
CH22	p339	<2	<1.6
CH22	p347	6 ± 0.5	4.8
CH22	p366	11± 0.9	8.4
CH22	p381	<2	<1.6
CH22	p392	<2	<1.6
CH22	p453	29 ± 0.6	22.3

Cultures were grown in glucose minimal medium and assayed using standard protocols, except that iron was added at a concentration of 4.5mM. Results represent the average of three trials. N.B. When the enzyme activity is less than 2 μmol of pyruvate /min / mg protein, it is immeasurable.

C381 and C392 made the enzyme activity undetectable. Substitutions of C219, C290, C347, C366, and C453 decreased the enzyme activity that could still be detected and the degrees reduced were different either from each other or from they are in vivo. Even so, we can see the results obtained *in vitro* with crude extracts are really similar with that ones obtained *in vivo*.

Part 3. Overexpression of *sdaA* and derivatives and their enzyme activity assay

3.1. Overexpression of *sdaA* and derivatives

Though the assays *in vivo* and *in vitro* with crude extracts gave clear results, they do not show whether lowered activity is due to a changed enzyme or to changed stability. I therefore wanted to assay the activity of purified proteins, and that required the overexpression of *sdaA* and derivatives. In this study, BL21 (DE3) carrying a T7 RNA polymerase for high level specific expression was employed for the overexpression of the *sdaA* and its derivatives. Plasmids pCH*sda*A and C-terminal his-tagged cysteine mutants were electro-transformed into BL21 (DE3). The cells were grown in LB-Glu-Amp medium. After inducing by IPTG, cells were harvested, washed, sonicated and collected. Theses strains were also tested for the plasmid maintenance and again plasmid maintenance was high. Samples of crude extracts were run on SDS-PAGE and stained with Coomassie Brilliant Blue to check whether the target protein had been

overexpressed. Expression of L-SD1 protein would result in the presence of a strongly stained band at 51 kDa. Such bands were visible in extracts from transformed cells in all cases (Figure 6). This shows that *sdaA* and the derivatives have been successfully overexpressed.

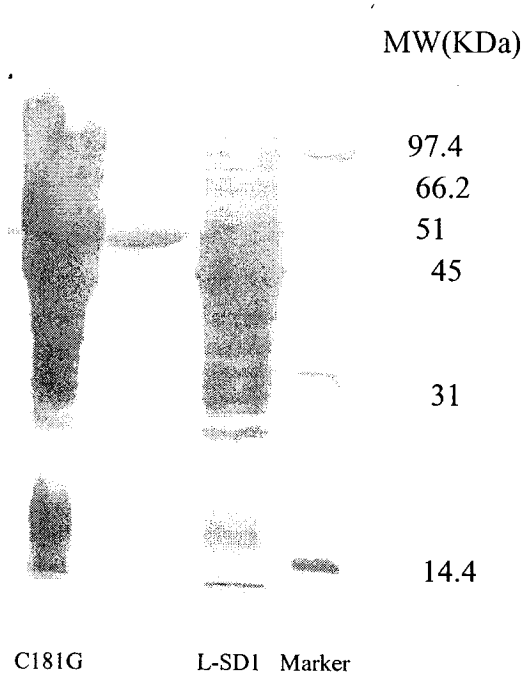
3.2. Purification of his-tagged proteins by Nickel Affinity FPLC

In this study, *sadA* gene was cloned and overexpressed in a 6×His tag system to get a His-Tagged fusion protein. This 6×His tag is small and normally does not interfere with the structure and function of the recombinant protein. In the present study, a tag removable by protease cleavage was not necessary.

The protein purification from crude extracts was performed through FPLC using a Ni-NTA column. Imidazole binds to nickel ions. Thus histidine, containing an imidazole ring, can also bind to nickel ions. The wild type and mutant L-SD1 contain a 6×His affinity tag that can bind to nickel ions without disturbing the structure and function of the recombinant protein.

Proteins in the crude extract that carry a His-tag bind to the column, and unbound protein is collected. When the flow-through was run on SDS-PAGE, there were no bands corresponding to the molecular weight of the His-tagged target protein L-SD1. The column was rinsed with washing buffer and this fraction collected and run on SDS-PAGE. There is less protein in this fraction (hence the more lightly stained

Figure 6 Overexpression of C-terminal his-tagged wild type L-SD1 and mutant derivatives



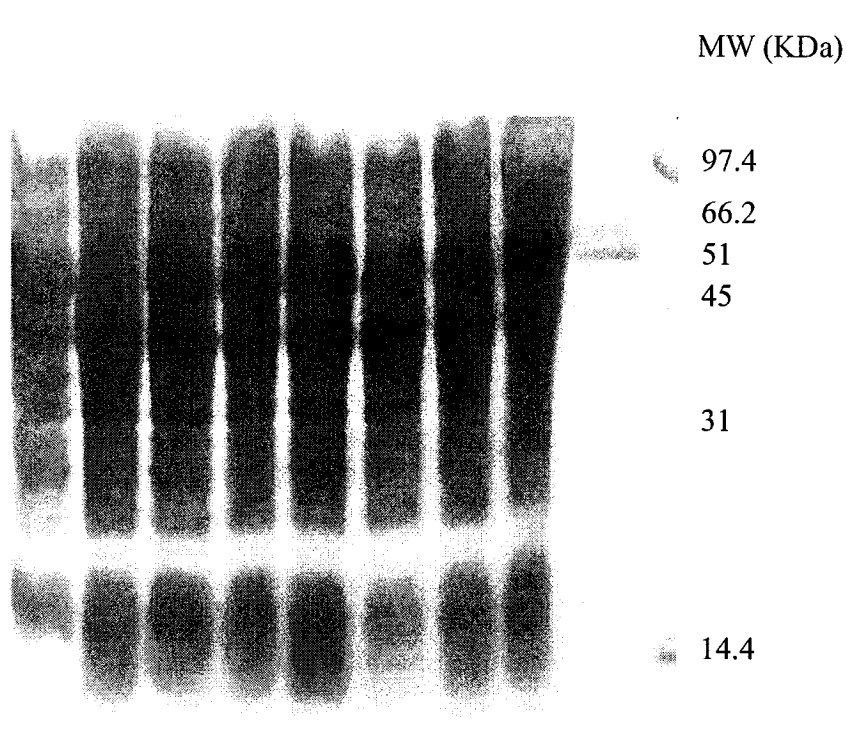
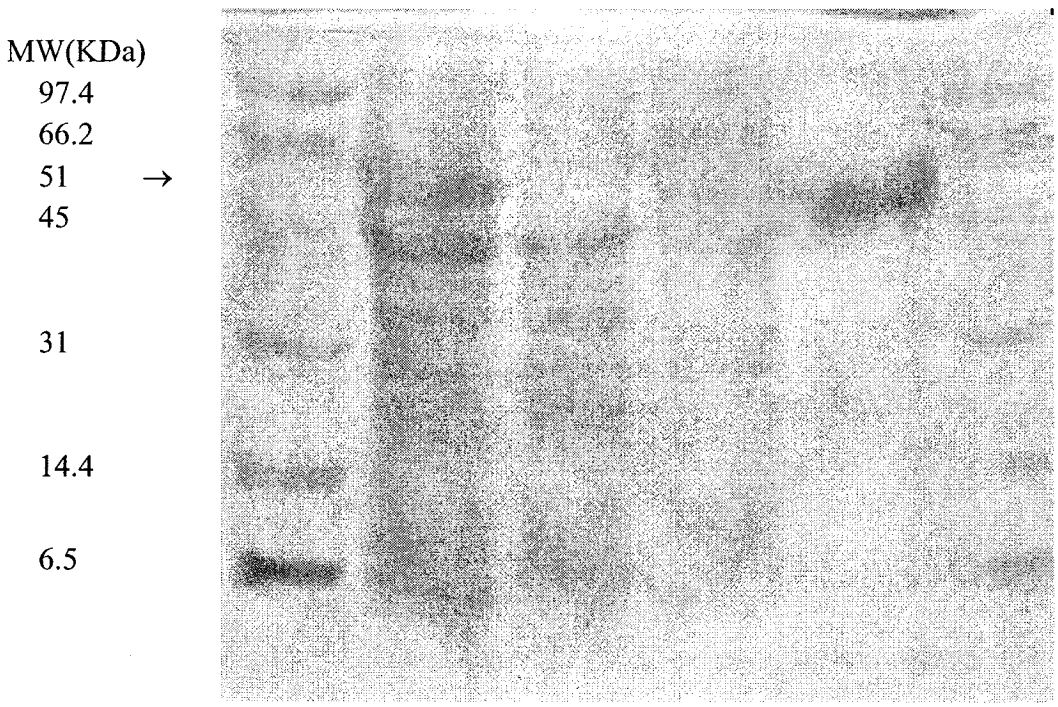


Figure 7 FPLC purification of 6Xhis-tagged protein L-SD1



bands), and again no bind at the molecular weight corresponding to L-SD1. When the concentration of imidazole in the buffer is increased, His-tagged proteins are eluted. This fraction contained protein corresponding in sign 51 KD (Figure 7). A SDS-PAGE of the ten purified C-terminal His-tagged L-SD1 proteins (wild type and its nine derivatives) is shown in Figure 8.

3.3. *In vitro* L-SD1 activity assay using purified proteins

L-SD1 activity assay using purified protein was performed as described for crude extract protein, except the concentration of iron was lowered to 1.8mM. The results are shown in Table 7. These results also revealed that a mutation at C181G had almost the same enzyme activity as wild type L-SD1. The mutations C219S, C290A, C347S, C366S and C453G had L-SD1 activity reduced to different degrees, but still detectable. No L-SD activity was detected in proteins of the mutations at C339A, C381A, and C392A (Table 7). That means the relative enzyme activity of wild type and mutant L-SD1s measured *in vivo* and *in* crude extracts or purified extracts are similar.

Figure.8. Purification of C-terminal his-tagged L-SD1 proteins and its nine cystein mutations on SDS-PAGE

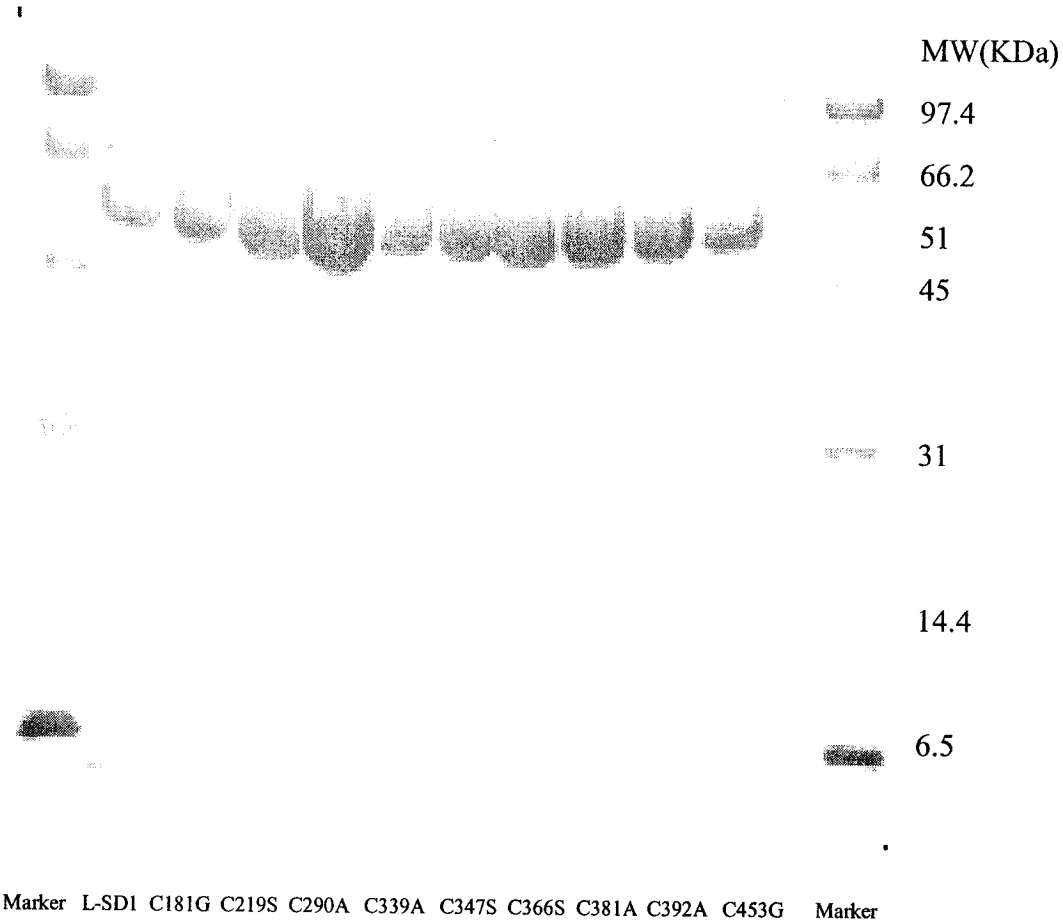


Table 7 *In vitro* L-SD1 activity assay in BL21pCH*sdaA* and derivatives with purified protein

Strain	Plasmid	L-SD1 activity (μmol of pyruvate /min/mg protein)	Percent p*sdaA* activity(%)
BL21	pCH*sdaA*	199± 5.6	100
BL21	pCH181	208 ±4.4	104.3
BL21	pCH219	118 ± 3.0	59.4
BL21	pCH290	106± 4.0	53.3
BL21	pCH339	<2	<1
BL21	pCH347	114± 6.5	57.5
BL21	pCH366	131 ± 6.0	65.6
BL21	pCH381	<2	<1
BL21	pCH392	<2	<1
BL21	pCH453	84± 4.5	42.2

The strains were grown in LB-Glu-Amp medium, induced by 0.4mM IPTG and assayed using standard protocol containing 1.8mM iron in the reaction. Results represent the average of three trials. N.B. When the enzyme activity is less than 2 μmol of pyruvate / min / mg protein, it is immeasurable.

Discussion

In this study, site-directed mutagenesis was employed to replace the nine cysteine residues of L-SD1 in *E. coli* K-12. I measured L-SD1 activity *in vivo* and *in vitro* for both the wild type L-SD1 and mutants. The important cysteine residues for the activity of L-SD1 were identified by comparing L-SD1 activity of the wild type and mutant strains. In this section, I will first present evidence to demonstrate that cysteine residues play an important role in L-SD1 activity of *E. coli* K-12. Three critical cysteine residues are widely conserved in L-SDs containing Fe-S cluster(s) and these are essential for the activity of L-SD1 from *E. coli* K-12. Then I will also give evidence to support the fact demonstrated by Cicchillo *et al* (2004) that L-SD1 is an enzyme containing Fe-S cluster and this cluster is essential for the enzyme activity. Finally, I will discuss some problems in this study and suggest future research to more fully explore the questions.

Part 1 Do the nine cysteine residues play an important role in L-SD1 activity in *E. coli* K-12?

Cysteine can be part of catalytic sites of proteins or serve as metal ion binding sites and play an important role in protein structure and activity. The *E. coli* enzyme, L-SD1, is composed of 454 amino acids including nine cysteines. If these cysteine residues are important for L-SD1 activity, substitution by other amino acids will impair enzyme

activity. Otherwise, L-SD1 activity will not be affected. If these cysteine residues are essential for L-SD1 activity, there won't be any activity. The substitution of ones that are not essential to the mechanism will give reductions in activity or no change. In this study, I have introduced substitutions of each of the nine cysteine residues respectively into the wild-type protein by site-directed mutagenesis, and then assayed the enzyme activity *in vivo* (whole cell assay) and *in vitro* with crude extracts and purified protein respectively. The results presented in Table 5, 6 and 7 indicate that the substitution of cysteine residues in L-SD1 causes three different effects depending on the location of the substituted cysteine: a complete loss of enzyme activity, a great reduction in activity, or almost no change in L-SD1 activity.

1.1. Cysteine at positions 339, 381 and 392 are essential for the enzyme activity of L-SD1 *in E. coli* K-12

Results obtained in this study indicate that C339A, C381A and C392A completely lost enzyme activity. In *in vivo* activity assays, the levels of L-SD1 activity were so low that they were actually similar to the baseline of CH22, a strain with a disrupted *sdaA* gene. It suggested that these three cysteines at positions 339, 381 and 392 are essential for the enzyme activity of L-SD1 in *E. coli* K-12.

1.2. Cysteine residues at position 219, 290, 347, 366, and 453 have function on L-SD1 activity in *E. coli K-12*

L-SD1 activity assays of mutants C219S, C290A, C347S, C366S and C453G *in vitro* using either crude extracts or purified proteins were consistent (Table 6, and 7). The purified proteins retain approximately 42.2-65.6% of activity. *In vivo*, mutants C219S, C290A, C347S, C366S and C453G had 6.5–29.9% of L-SD1 activity. Therefore, these cysteine residues in L-SD1 effect the conformation of the enzyme, though they are of lesser importance.

1.3. Cysteine at position 181 might be non-essential for L-SD1 activity

The L-SD1 activity of mutant C181G was very similar to the wild type enzyme either *in vivo* or *in vitro* assay. This suggests that cysteine at position 181 may not be important for the activity of L-SD1 from *E. coli* K-12. However, the function of cysteine at position 181 and the reason why it is not essential for enzyme activity remain unclear.

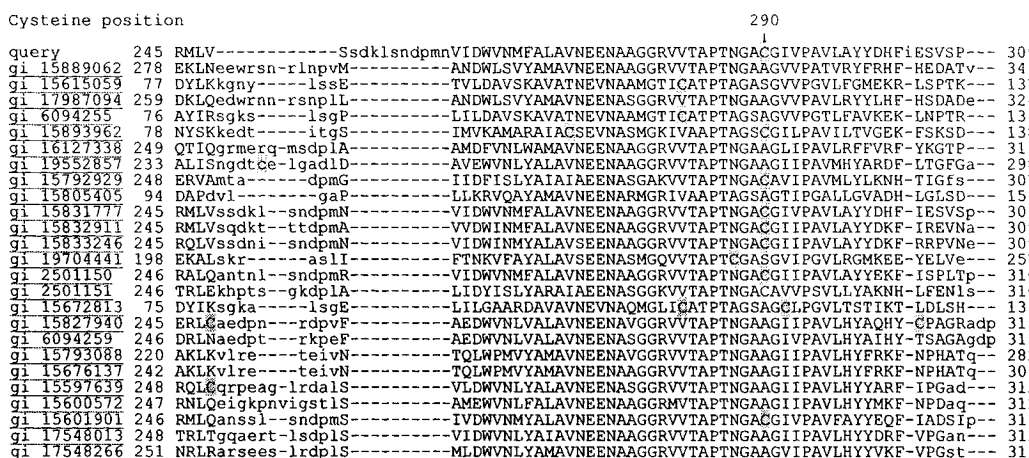
1.4. Cysteine at positions 339, 381 and 392 are high conserved in L-SDs from different organisms

In 1997, Hofmeister and coworkers compared the sequences of L-SD from *P. asaccharolyticus,* the sequences of L-SD1 and L-SD2 from *E. coli* K-12 and the sequences of a putative L-serine deaminase from *H. influenzae*. They found that the four

cysteine residues at position of 181, 339 381 and 392 in *E. coli* L-SD1 are conserved in these four L-SD molecules (Hofmeister *et al.* 1997).

To know how common each of the cysteine residues is in homologue enzymes, I performed a blast search with the amino acid sequence starting with the 168th residue of L-SD1 from *E. coli* K-12 against NCBI protein database as of June 2004. The sequence alignment is shown in Figure 9. A more detailed sequence comparison was illustrated in Table 8 and 9. Sequence alignment results reveal that: 1) the number of cysteine residue is different in different organisms. It may vary from 4 to 20. That means all of the 9 cysteine residues in *E. coli* L-SD1 may be highly conserved in some L-SDs. However, that may not be the case in other L-SDs; 2) the cysteine residues 339, 381 and 392 of L-SD1 from *E. coli* K-12 are high conserved in almost L-SDs. This is similar to aconitase which also has three conserved cysteine residues; and 3) even though these three cysteine residues are at slightly different positions in molecules from different organisms, the distance between them is constant, i.e. the distance between cysteine 339 and cysteine 381 is always 42 amino acids; and the distance between cysteine 381 and 392 is always 11 amino acids. We may say there is a motif GCQ-X_{40}-CDP-X_7-PC (where G, C, Q, D, P represents a glycine, cysteine, glutamine, aspartic acid, proline residue respectively and X represents any other amino acid) conserved in most of L-SDs. This structural character is also very interesting and may be very important for L-SD. The results presented in this study i.e. only cysteine residues in 339, 381 and 392 are essential for the enzyme activity of *E. coli* L-SD1, which supports our assumption.

Figure.9. Comparesion of L-SD1 from *E. coli* with other L-SDs



```
Cysteine position                          339     347                  366          381
                                             |       |                    |            |
query         310 -DIYTRYFMAAGAIG-ALYKMNASISGAE--VGCQGEVGVACSMAAAGLA--ELLGGSPEQVCVAAEIGMEHN--LGLTC 381
gi 15889062   344 -DDVRDFLLTAAAIG-GIIKHNASISGAE--VGCQGEVGSAAAMAAAGLA--AVMGGSPEQIENAAEIALEHH--LGMTC 415
gi 15615059   138 -EQKVRFLFTSGAFG-FVVANNASISGAA--GGCQAEVGSATGMAAAALV--ELAGGTPSQSAEAMAIALKNM--LGLVC 209
gi 17987094   325 -KGIRDFLLTSAAIG-GVIKHNASISGAE--VGCQGEVGSASAMAAAGLA--AVLGGSPEQIENAAEIALEHH--LGMTC 396
gi 6094255    137 -EQMIRFLFTAGAFG-FVVANNASISGAA--GGCQAEVGSASGMAAAAIV--EMAGGTPEQSAEAMAITLKNM--LGLVC 208
gi 15893962   139 -DELTKALFTASGIG-MLIAKNATLSGAE--GGCQAECGSAAAMGAGAVV--EMLGGTPEMALDAASIVIKNV--LGLVC 210
gi 16127338   314 -EQIRTFLLTAAAIG-ALYKRNASISGAE--VGCQGEVGVACSMAAAGLA--AALGGTNAQIENAAEIGMEHN--LGLTC 385
gi 19552857   299 -EQARTFLYTAGAVG-IIIKENASISGAE--VGCQGEVGSASAMAAAGLC--AVLGGSPQQVENAAEIALEHN--LGLTC 370
gi 15792929   308 dEKVIEFLLTAMLIG-SFYKKNASISGAE--AGCQAEIGSASSMAAAAMA--TVLGANAFKACNAAEMAMEHH--LGLTC 380
gi 15805405   152 -EQLVDPMILAAGVG-KAISKRMFISGAA--GGCQAEIGSSAAMAAAAVV--ELLGGTPRAAVHAASLALMNT--IGLVC 223
gi 15831777   310 -DIYTRYFMAAGAIG-ALYKMNASISGAE--VGCQGEVGVACSMAAAGLA--ELLGGSPEQVCVAAEIGMEHN--LGLTC 381
gi 15832911   310 -NSLARYLLVASAIG-SLYKMNASISGAE--VGCQGEVGVACSMAAAGLA--ELLGASPAQVCIAAEIAMEHN--LGLTC 381
gi 15833246   310 -RSIARYFLAAGAIG-ALYKMNASISGAE--VGCQGEIGVACSMAAAGLT--ELLGGSPAQVCNAAEIAMEHN--LGLTC 381
gi 19704441   258 -KHILRGLAIAGLIG-NLVKYNATISGAE--GGCQAEVGTACSMAAAMAT--YFMGGNTDQIEYAAESAMEHH--LGMTC 329
gi 2501150    311 -EIIERYLLAAGMIG-SLYKMNASISGAE--VGCQGEVGVACSMAAAGLA--EILGGNPLQVCIAAEIAMEHN--LGLTC 382
gi 2501151    311 qKAINDFLLTSAAIG-YLYKKNASLSGAE--AGCQAEIGVASSMAAGGLA--HLCQATTQQVLIASEIAMEHH--LGLTC 383
gi 15672813   136 -EQQVEFLFVAGAFG-LAIANNATISGAE--GGCQAEVGSASAMASAALV--LAAGGSAEQAGYAIAIVLQNM--LGLIC 207
gi 15827940   311 dDTSVRFLLTAGAIG-SLYKELASISGAE--VGCQGEVGSAASMAAAGLA--EILGGTPAQVENAAEIAMEHS--LGLTC 383
gi 6094259    312 dDVTVRFLLTAGAIG-SLFKERASISGAE--VGCQGEVGSAAAMAAAGLA--EILGGTPRQVENAAEIAMEHS--LGLTC 384
gi 15793088   283 -ERVENFLLTAGAIG-ILYKTNASISGAD--VGCQGEVGVACSMAAGAYA--EVIGGTPKQVENAAEMAMEHH--LGLTC 354
gi 15676137   305 -ERVENFLLTAGAIG-ILYKTNASISGAD--VGCQGEVGVACSMAAGAYA--EVIGGTPKQVENAAEMAMEHH--LGLTC 376
gi 15597639   313 dDGVVRFLLTAAAIG-ILYKENASISGAE--VGCQGEVGVACSMAAGALC--EVLGGSVQQVENAAEIGMEHN--LGLTC 385
gi 15600572   313 dRDVVDYLLAAAAVG-ILCKKNASISGAE--VGCQGEVGSACAMAAAGLA--EVLGATPAQVENAAEIGLEHN--LGLTC 385
gi 15601901   311 -DIIERYLLTCSFIG-SLYKMNASISGAE--VGCQGEVGVACSMAAAGLT--EILGGSPEQVCIAAEIAMEHN--LGLTC 382
gi 17548013   313 dNGVVDFLLTAGAIG-MLYKMNASISGAE--VGCQGEVGVACSMAAGALA--AVLGGTPAQAENAAEIGMEHN--LGLTC 385
gi 17548266   316 dNGIVEFLLTAAAIG-IIYKETASISGAE--VGCQGEVGVACSMAAAALA--AVMGGTPDQVENAAEIGMEHN--LGMTC 388
gi 15928109   139 -EQMIDFLFTSALFG-RVVANNASVAGAT--GGCQAEVGSASAMAAAAAV--AIFGGSPEASGHAMALAISNL--LGLVC 210
gi 15965161   322 -EGIRDYLLTAAAIG-GIIKHNASISGAE--VGCQGEVGSASAMAAAGLA--AVMGGTPEQIENAAEIALEHH--LGMTC 393
gi 15900048   138 -EQQLDFLFAAGAFG-LVIANNASISGAE--GGCQAEVGSASAMSAAALT--LAAGGTPYQASQAIAFVIKNM--LGLIC 209
gi 15675924   138 -KEQLEFLFTAGAFG-LVIGNNASISGAE--GGCQAEVGSAAAMSAAALV--KAAGGTSHQASQAIAFVIKNL--LGLVC 209
gi 16765167   310 -DIYTRYFLAAGAIG-ALYKMNASISGAE--VGCQGEVGVACSMAAAGLA--ELLGASPEQVCVAAEIGMEHN--LGLTC 381
gi 16765526   308 pGALTRFFLTAAAIA-MLFKQNASILGSE--VGCQGEIGVACSMAAAGLA--ELMGASVEQTLSAAEIAMEHH--LGLTC 380
gi 16766276   310 -NSLARYMLVASAIG-SLYKMNASISGAE--VGCQGEVGVACSMAAAGLA--ELLGGSPAQVCIAAEIAMEHN--LGLTC 381
gi 16766539   310 -NSIARYLLSAGAIG-MLYKMNASISGAE--VGCQGEVGVACSMAAAGLT--ELLGGSPAQVCIAAEIAMEHN--LGLTC 381
gi 15641313   310 -KDYIRYFAASGAIG-GLYKRNASISGAE--VGCQGEVGVACSMAAAGLA--ELLGGSPEQVCMAAEIAMEHN--LGLTC 381
gi 15601424   329 -KQLKDFLAVSGAIG-ILYKTNASISGAE--VGCQGEVGVSSSMAAAGLT--ALRGGSNEQICMAAEIAMEHS--LGMTC 400
gi 16122025   310 -EIFIRYFLASGAIG-ILYKMNASISGAE--VGCQGEVGVACSMAAAGLA--ELLGASPIQVCIAAEIGMEHN--LGLTC 381
gi 15803651   312 -RSIARYFLAAGAIG-ALYKMNASISGAE--VGCQGEIGVACSMAAAGLT--ELLGGSPAQVCNAAEIAMEHN--LGLTC 383
gi 15802227   310 -DIYTRYFMAAGAIG-ALYKMNASISGAE--VGCQGEVGVACSMAAAGLA--ELLGGSPEQVCVAAEIGMEHN--LGLTC 381
gi 15803319   310 -NSLARYLLVASAIG-SLYKMNASISGAE--VGCQGEVGVACSMAAAGLA--ELLGASPAQVCIAAEIAMEHN--LGLTC 381
gi 12230012   311 qKSINDFLLTSAAIG-YLYKKNASLSGAE--AGCQAEIGVASSMAAGGLA--HLCQATTQQVLIASEIAMEHH--LGLTC 383
gi 16800992   137 -EDMVNFLFTAGAFG-YVVANNAFISGAA--GGCQAEIGSASAMASAAIV--AAAGGTPEQSAHAMAMTMKNM--LGLVC 208
gi 13470860   323 -PSIRDFLLTAAAVG-GIIKTNASISGAE--VGCQGEVGSASAMAAAGLC--AVMGGTPEQVENAAEIALEHH--LGMTC 394
gi 266996     310 -DIYTRYFMAAGAIG-ALYKMNASISGAE--VGCQGEVGVACSMAAAGLA--ELLGGSPEQVCVAAEIGMEHN--LGLTC 381
gi 2507444    310 -NSLARYLLVASAIG-SLYKMNASISGAE--VGCQGEVGVACSMAAAGLA--ELLGASPAQVCIAAEIAMEHN--LGLTC 381
gi 16131004   131 -RSIARYFLAAGAIG-ALYKMNASISGAE--VGCQGEIGVACSMAAAGLT--ELLGGSPAQVCNAAEIAMEHN--LGLTC 202
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Cysteine position      392                                                            453
                          |                                                              |
query         382 DPVAGQVQVPC-IERNAIASVKAINAARMALRRTSAPRv-SLDKVIETMYETGKDMNAKYRETSRG----GLAIKVQCD 454
gi 15889062   416 DPIAGLVQVPC-IERNALGAVKAVTAASLALKGDGQHFv-PLDACIETMRQTGNDMSEKYKETSTG----GLAVNVVEC 488
gi 15615059   210 DPVAGLVEVPC-VKRNAIGASIAITAADMALAGIQSRI--PTDEVIDAMYKIGQAMPVAYKETAQG----GLAATPKGR 281
gi 17987094   397 DPIAGLVQVPC-IERNALGAVKAVTAASLAVKGDGKHFv-PLDACIETMRQTGHDMSERYKETSQG----GLAVNVVAC 469
gi 6094255    209 DPVAGLVEVPC-VKRNAMGASNAMIAADMALAGITSRI--PCDEVIDAMYKIGQTMPTALRETGQG----GLAATPTGR 280
gi 15893962   211 DPVAGLVEVPC-SKRNVSGAVNAITTADMVMAGVGSII--PFDDSVSAMYRVGKQLPCELRETALG----GLAVTKKGQ 282
gi 16127338   386 DPIGGLVQIPC-IERNAMGAIKAIDAARLALLGDGQHSv-SLDKVIATMKRTGEDMNEIYKETSMGglavGLSVNRVEC 462
gi 19552857   371 DPVGGLVQIPC-IERNAIAAMKSINAARLARIGDGNNRv-SLDDVVVTMAATGRDMLTKYKETSLG----GLATTLGFP 443
gi 15792929   381 DPVAGLVQIPC-IERNAFGAIKAISAARMAMTRKSTPMv-SLDEVIETMYETGKDMNYKYKETSLG----GLATNLKTV 454
gi 15805405   224 DPVGGYVEVPC-VSRNAFFAVHAVSAAQLALAQLESFI--PPDEVLGAMASVGRLMPAALRETAEG----GLAQTPTGL 295
gi 15831777   382 DPVAGQVQVPC-IERNAIASVKAINAARMALRRTSAPRv-SLDKVIETMYETGKDMNAKYRETSRG----GLAIKVQCD 454
gi 15832911   382 DPVAGQVQVPC-IERNAIAAVKAVNAARMALRRTSEPRv-CLDKVIETMYETGKDMNAKYRETSRG----GLAMKIVAC 454
gi 15833246   382 EPVAGQVQIPC-IERNAINAVKAVNAARMAMRRTSAPRv-SLDKVIETMYETGKDMNDKYRETSRG----GLAIKVVCG 454
gi 19704441   330 DPVGGYVIIPC-IERNAICAVRAINTATYCMSTDGKHTi-SFDEVIKTMKETGKDMCSAYKETSDG----GLAKYYDRI 402
gi 2501150    383 DPVGGQVQVPC-IERNAIASVKAINASRMALRRTTNPRv-TLDKVIETMYETGKDMNAKYRETSQG----GLAVKIVCN 455
gi 2501151    384 DPVGGLVQIPC-IERNVLGAIKAISASKLALEDEYKPKv-SLDEVIATMYATGKDMNEKYKETSLG----GLAKTLKC- 455
gi 15672813   208 DPVAGLVEIPC-VHRNAMGASQAMISADMALAGVKTVI--PVDEVVNTMYNVGRSLPAAFRETAEG----GLAQTPTGR 279
gi 15827940   384 DPIAGLVQIPC-IERNAISAGKAINAARMAMRGDGTHRv-SLDEVIATMRATGRDMNFKYKETAAG----GLALAVNIV 456
gi 6094259    385 DPIAGLVQIPC-IERNAISAGKAINAARMALRGDGIHRv-TLDQVIDTMRATGADMHTKYKETSAG----GLAINVAVN 457
gi 15793088   355 DPVGGLVQIPC-IERNGIAAEKALKLGTLALLEDGTDKkvSLDEVIRTMLQTGRDMKSTYKETSLA----GLAATLRKK 428
gi 15676137   377 DPVGGLVQIPC-IERNGIAAEKALKLGTLALLEDGTDKkvSLDEVIRTMLQTGRDMKATYKETSLA----GLAATRVVE 450
gi 15597639   386 DPVGGLVQVPC-IERNAMASVKAINAARMALRGDGQHFi-SLDKVIRTMRQTGADMKSKYKETARG----GLAVNSVRV 458
gi 15600572   386 DPVGGLVQVPC-IERNAIAAVKAINAAQMALRGDGQHFi-SLDKVIRTMRDTGADMHDKYKETSRG----GLAVNAIEC 458
gi 15601901   383 DPVGGQVQVPC-IERNAIAAVKAINASRMALRRTTSPRv-TLDKVIETMYETGKDMNAKYRETSQG----GLAIKVVCS 455
gi 17548013   386 DPVGGLVQIPC-IERNAMASVKAVNAARMALRGDGTHYv-SLDSVIKTMRETGADMKTKYKETARG----GLAVNIVEC 458
gi 17548266   389 DPVGGLVQIPC-IERNAMGAIKALNASRMALKGNGQHYv-SLDSVIKTMRETGADMKTKYKETSRG----GLAVNVIEC 461
gi 15928109   211 DPVAGLVEIPC-VMRNAIGSGNALISADLALAGIESRI--PVDEVIEAMDKVGRNLPASLRETGLG----GLAGTPTGE 282
gi 15965161   394 DPVAGLVQVPC-IERNALGAVKAVTAASLALKGDGKHFv-PLDACIETMRQTGADMNEKYKETSTG----GLAVNVVEC 466
gi 15900048   210 DPVAGLVEVPC-VKRNAMGASFAFIAADMALAGIESKI--PVDEVIDAMYQVGASMPTAFRETAEG----GLATTPTGR 281
gi 15675924   210 DPVAGLVEVPC-VKRNALGASFALVAADMALADIDSQI--PVDEVIDAMYQVGSAMPTAFRETAEG----GLAATPTGR 281
gi 16765167   382 DPVAGQVQVPC-IERNAIASVKAINAARMAMRRTSAPRv-SLDKVIETMYETGKDMNAKYRETSRG----GLAIKVQCD 454
gi 16765526   381 DPLGGQVQIPC-IERNAISAVKAINAATMAMSRVSEPCi-SLDEIIAAMYETGKDMSAKYRETYHG----SLGKIQPRK 453
gi 16766276   382 DPVAGQVQVPC-IERNAIASVKAVNAARMAVRRTSEPRv-CLDKVIETMYETGKDMNAKYRETSRG----GLAMKIVTC 454
gi 16766539   382 DPVAGQVQIPC-IERNAINAVKAVNAARMALRRTSEPRv-SLDKVIETMYETGKDMNDKYRETSRG----GLAIKVVCG 454
gi 15641313   382 DPVAGQVQVPC-IERNGIAAVKAINSTRMALRRSSAPRv-SLDKVIETMLETGKDMNAKYRETSQG----GLAIKVIC- 453
gi 15601424   401 DPIGGLVQVPC-IERNAMGAVKAINASRMALKRTSKCLi-SLDKVIETMYQTGKDMNKKYRETSLG----GLALIHLAP 473
gi 16122025   382 DPVAGQVQVPC-IERNAIASVKAINSARMAMRRTSEPRv-SLDKVIETMFETGKDMNAKYRETSRG----GLAIKVQCT 453
gi 15803651   384 EPVAGQVQIPC-IERNAINAVKAVNAARMAMRRTSAPRv-SLDKVIETMYETGKDMNDKYRETSRG----GLAIKVVCG 456
gi 15802227   382 DPVAGQVQVPC-IERNAIASVKAINAARMALRRTSAPRv-SLDKVIETMYETGKDMNAKYRETSRG----GLAIKVQCD 454
gi 15803319   382 DPVAGQVQVPC-IERNAIAAVKAVNAARMALRRTSEPRv-CLDKVIETMYETGKDMNAKYRETSRG----GLAMKIVAC 454
gi 12230012   384 DPVGGLVQIPC-IERNVLGAIKAISASKLALEDEYKPKv-SLDEVIATMYATGKDMNEKYKETSLG----GLAKTLKC- 455
gi 16800992   209 DPVAGLVEVPC-VKRNALGSSQAIISADMALAGIKSRI--PCDEVIEAMHRVGLQMPSSLRETAEG----GLAATPTGR 280
gi 13470860   395 DPVGGLVQVPC-IERNALGAVKAVTAASLAIKGDGIHFv-PLDAAIETMRQTGLDMNEKYKETSLG----GLAVNVVEC 467
gi 266996     382 DPVAGQVQVPC-IERNAIASVKAINAARMALRRTSAPRv-SLDKVIETMYETGKDMNAKYRETSRG----GLAIKVQCD 454
gi 2507444    382 DPVAGQVQVPC-IERNAIAAVKAVNAARMALRRTSEPRv-CLDKVIETMYETGKDMNAKYRETSRG----GLAMKIVAC 454
gi 16131004   203 DPVAGQVQIPC-IERNAINAVKAVNAARMAMRRTSAPRv-SLDKVIETMYETGKDMNDKYRETSRG----GLAIKVVCG 275
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This figure is multiple sequence alignment .The query is the amino acid sequence that the first amino acid is the 168th residue of L-SD1 from *E. coil* K-12. The other amino sequences of homologue L-SDs come from NCBI protein database. The cysteine residue is marked by light grey. Cysteine position 181, 219, 290, 339, 347, 366, 381, 392, and 453 represent the site of cysteine residues of L-SD1 from *E. coli* K-12.

Table 8 Comparison of sequence of L-SD1 from *E. coli* K-12 with the sequence of L-SD from other organism

Organism	Cysteine Position	Number of cyteine	Conserved Sequence
Agrobacterium tumefaciens str. C58	17,252,373,415,426,458,488	7	VGC^{373}QG.....MTC^{415}DPI.....QVPC^{426}IE
Bacillus halodurans C-125	111,167,209,220,	4	GGC^{167}QA.....LVC^{209}DPA.....EVPC^{220}VA
Brucella melitensis 16M	78,212,213,233,354, 396,407,439,469	9	VGC^{354}QG.....MTC^{396}DPI..... QVPC^{407}IE
Bacillus subtilis	110,166,208,219	4	GGC^{166}QA.....LVC^{208}DPV.....EVPC^{219}VK
Clostridium acetobutylicum ATCC 824	13,101,120,168,172,210,221	7	GGC^{168}QA..... LVC^{210}DPV.... .EVPC^{221}SK
Caulobacter crescentus CB15	91,223,343,351,385,396,462	7	VGC^{343}QG.....LTC^{385}DPI..... QIPC^{396}IE
Corynebacterium glutamicum ATCC 13032	55,160,207,241,328,344,370,381,450	9	VGC^{328}QG..... LTC^{370}DPV... .. QIPC^{381}IE
Campylobacter jejuni subsp. jejuni NCTC 11168	29,96,130,183,208,211,289,338, 365, 380, 391, 454	11	AGC^{338}QA.. .... LTC^{380}DPV..... QIPC^{391}IE
Deinococcus radiodurans R1	45,181,223,234,	4	GGC^{181}QA..... LVC^{223}DPV... EVPC^{234}VS
Escherichia coli O157:H7	181,219,290,339,347,366,381,392,453	9	VGC^{339}QG..... LTC^{381}DPV... QVPC^{392}IE
Escherichia coli O157:H7	112,181,217,290,339,347,366,381, 392,420,454	11	VGC^{339}QG..... LTC^{381}DPV.....QVPC^{392}IE
Escherichia coli O157:H7	181,290,339,347,366,381, 392,420,453	8	VGC^{339}QG..... LTC^{381}EPV..... QIPC^{392}IE
Fusobacterium nucleatum subsp. nucleatum ATCC	25586 139,153,236,287,295,329,340, 347,358,384	10	GGC^{287}QA.....MTC^{329}DPV..... IIPC^{340}IE
Haemophilus influenzae	182,210,291,340,348,367,382,393,454	9	VGC^{340}QG.....LTC^{382}DPV.....QVPC^{393}IE
Helicobacter pylori	42,183,219,291,340,360,383,394,455	9	AGC^{340}QA.....LTC^{383}DPV.....QIPC^{394}IE
Lactococcus lactis subsp. lactis II1	403 109,119,165,207,218	5	GGC^{165}QA.....LIC^{207}DPV..... EIPC^{218}VH
Mycobacterium leprae TN	44,90,98,189,248,303,341,383,394,458	9	VGC^{341}QG..... LTC^{383}DPI..... QIPC^{394}IE
Mycobacterium tuberculosis	74,166,182,199,200,220,342,384,395,461	10	VGC^{342}QG..... LTC^{384}DPI..... QIPC^{395}IE
Neisseria meningitidis Z2491	147,154,175,184,312,320,354,365,439	9	VGC^{312}QG.....LTC^{354}DPV.....QIPC^{365}IE
Neisseria meningitidis MC58	169,176,197,216,334,342,376,387,461	9	VGC^{334}QG.....LTC^{376}DPV.....QIPC^{387}IE
Pseudomonas aeruginosa PA01	184,222,227,251,343,351,359,385,396,458	10	VGC^{343}QG.....LTC^{385}DPV.....QVPC^{396}IE
Pseudomonas aeruginosa PA01	183,221,230,343,351,385,396,458	8	VGC^{343}QG.....LTC^{385}DPV.....QVPC^{396}IE
Pasteurella multocida subsp. Multocida str.Pm70	182,220,291,320,340,348,367,382,393,454	10	VGC^{340}QG.....LTC^{382}DPV.....QVPC^{393}IE
Ralstonia solanacearum GMI1000	184,222,227,343,351,385,396,458	8	VGC^{343}QG.....LTC^{385}DPV..... QIPC^{396}IE
Ralstonia solanacearum GMI1000	28,187,203, 215,220, 346,354,388,399,461	10	VGC^{346}QG.....LTC^{388}DPV.....QIPC^{399}IE
Staphylococcus aureus subsp. Aureus N315	112, 168,210,221	4	GGC^{168}QA.....LVC^{210}DPV..... EVPC^{221}VK
Sinorhizobium meliloti 1021	209,230,351,393,404,436,466	7	VGC^{351}QG.....MTC^{393}DPV... QVPC^{404}IE
Streptococcus pneumoniae TIGR4	111,121,167,209,220,	5	GGC^{167}QA..... LIC^{209}DPV.....EVPC^{220}VK
Streptococcus pyogenes M1 GAS	111,121,167,209,220	5	GGC^{167}QA..... LVC^{209}DPV..... EVPC^{220}VK
Salmonella typhimurium LT2	181,219,290,339,347,366,381,392,453,	9	VGC^{339}QG..... LTC^{381}DPV.....QVPC^{392}IE
Salmonella typhimurium LT2	4,27,59,73,181,197,241,289,297, 338,346,380,391,417	14	VGC^{338}QG..... LTC^{380}DPV..... QIPC^{391}IE
Salmonella typhimurium LT2	112,181,290,339,347,366,381,392,420,454,	10	VGC^{339}QG..... LTC^{381}DPV.....QVPC^{392}IE
Salmonella typhimurium LT2	28,181,290,339,347,366,381,392,453	9	VGC^{339}QG.....LTC^{381}DPV..... QIPC^{392}IE
Vibrio cholerae O1 biovar eltor str. N16961	102,181,219,290,339,347,366,381,392,	9	VGC^{339}QG..... LTC^{381}DPV.....QVPC^{392}IE

Organism	Cysteine Position	Number of cyteine	Conserved Sequence
Vibrio cholerae O1 biovar eltor str. N16961	238,358, 385,400 ,411,436,475	7	VGC[358]QG.....MTC[400]DPI.....QVPC[411]IE
Yersinia pestis	181,219,290,339,347,366,381,392,453	9	VGC[339]QG..... LTC[381]DPV... QVPC[392]IE
Escherichia coli O157:H7 EDL933	112,181,217,290,339,347,366,381,392,420,454,	11	VGC[339]QG..... LTC[381]DPV.....QVPC[392]IE
Escherichia coli O157:H7 EDL933	181,219,290,339,347,366,381,392, 453	9	VGC[339]QG..... LTC[381]DPV.....QVPC[392]IE
Escherichia coli O157:H7 EDL933	112, 181,217,290,339,347,366,381,392, 420,454	11	VGC[339]QG..... LTC[381]DPV.....QVPC[392]IE
Helicobacter pylori J99	32,183,219,291,341,360,383,394,455	9	VGC[341]QA..... LTC[383]DPV..... QIPC[394]IE
Listeria innocua Clip11262	110,166,208,219,247	5	GGC[166]QA..... LVC[208]DPV.....EVPC[219]VK
Mesorhizobium loti MAFF303099	231,352,368,394,405,467	6	VGC[352]QG..... MTC[394]DPV... QVPC[405]IE
Escherichia coli	181,219,290,339,347,366,381,392, 453	9	VGC[339]QG..... LTC[381]DPV.....QVPC[392]IE
Escherichia coli	112,181,290,339,347,366,381,392,420,454	10	VGC[339]QG..... LTC[381]DPV.....QVPC[392]IE
Escherichia coli	2,111,160,168,186,202,213,274	8	VGC[160]QG..... LTC[202]DPV..... QIPC[213]IE

Table 9 Comparison of cysteines corresponding to *E. coli* L-SD1 with homologous L-SDs

Organism	Cysteine corresponding to *E. coli* L-SD1								
	181	219	290	339	347	366	381	392	453
Agrobacterium tumefaciens str. C58	-	+	-	+	-	-	+	+	-
Bacillus halodurans C-125	-	-	-	+	-	-	+	+	-
Brucella melitensis 16M	-	+	-	+	-	-	+	+	-
Bacillus subtilis	-	-	-	+	-	-	+	+	-
Clostridium acetobutylicum ATCC 824	+	-	+	+	-	-	+	+	-
Caulobacter crescentus CB15	-	+	-	+	+	-	+	+	-
Corynebacterium glutamicum ATCC 13032	-	+	-	+	-	-	+	+	-
Campylobacter jejuni subsp. jejuni NCTC 11168	+	-	+	+	-	+	+	+	-
Deinococcus radiodurans R1	-	-	-	+	-	-	+	+	-
Escherichia coli O157:H7	+	+	+	+	+	+	+	+	+
Escherichia coli O157:H7	+	-	+	+	+	+	+	+	-
Escherichia coli O157:H7	+	-	+	+	+	+	+	+	+
Fusobacterium nucleatum subsp. nucleatum ATCC 25586	+	-	-	+	+	-	+	+	-
Haemophilus influenzae	+	+	+	+	+	+	+	+	+
Helicobacter pylori	+	+	+	+	-	-	+	+	+
Lactococcus lactis subsp. lactis Il1403	-	-	-	+	-	-	+	+	-
Mycobacterium leprae TN	-	-	-	+	-	-	+	+	-
Mycobacterium tubrculosis	+	+	-	+	-	-	+	+	-
Neisseria meningitidis Z2491	+	+	-	+	+	-	+	+	-
Neisseria meningitidis MC58	+	+	-	+	+	-	+	+	-
Pseudomonas aeruginosa PAO1	+	+	-	+	+	-	+	+	-
Pseudomonas aeruginosa PAO1	+	+	-	+	+	-	+	+	-
Pasteurella multocida subsp. multocida str. Pm70	+	+	+	+	+	+	+	+	+

Organism	Cysteine corresponding to *E. coli* L-SD1								
	181	219	290	339	347	366	381	392	453
Ralstonia solanacearum GMI1000	+	+	-	+	+	-	+	+	-
Ralstonia solanacearum GMI1000	+	+	-	+	+	-	+	+	-
Staphylococcus aureus subsp. aureus N315	-	-	-	+	-	-	+	+	-
Sinorhizobium meliloti 1021	-	+	-	+	-	-	+	+	-
Streptococcus pneumoniae TIGR4	-	-	-	+	-	-	+	+	-
Streptococcus pyogenes M1 GAS	-	-	-	+	-	-	+	+	-
Salmonella typhimurium LT2	+	+	+	+	+	+	+	+	+
Salmonella typhimurium LT2	+	-	+	+	+	-	+	+	-
Salmonella typhimurium LT2	+	-	+	+	+	+	+	+	-
Salmonella typhimurium LT2	+	-	+	+	+	-	+	+	+
Vibrio cholerae O1 biovar eltor str. N16961	+	+	+	+	+	+	+	+	+
Vibrio cholerae O1 biovar eltor str. N16961	-	+	-	+	-	+	+	+	-
Yersinia pestis CO92	+	+	+	+	+	+	+	+	+
Escherichia coli O157:H7 EDL933	+	-	+	+	+	+	+	+	+
Escherichia coli O157:H7 EDL933	+	+	+	+	+	+	+	+	+
Escherichia coli O157:H7 EDL933	+	-	+	+	+	+	+	+	-
Helicobacter pylori J99	+	+	+	+	-	-	+	+	+
Listeria innocua Clip11262	-	-	-	+	-	-	+	+	-
Mesorhizobium loti MAFF303099	-	+	-	+	-	-	+	+	-
Escherichia coli	+	+	+	+	+	+	+	+	-
Escherichia coli	+	-	+	+	+	+	+	+	-
Escherichia coli	-	-	+	+	+	+	+	+	+
Percent of 45 sequences with cysteine at the homologous position (%)	62	53	47	100	56	38	100	100	29

Part 2 L-SD1 from *E. coli* K-12 belongs to an iron-sulfur cluster containing enzyme family.

2.1. L-SD1 from *E. coli* K-12 contains an iron-sulfur cluster.

L-SDs from many bacteria such as *P. asaccharolyticus* (Grabowski *et al.* 1991), *C. sticklandii* (Zinecker *et al.* 1998), *C. propionicum* (Hofmeister *et al.*1994) and *C. jejuni* (Velaydhan *et al.* 2004), have been proved to be Fe-S cluster enzymes. L-SD1 from *E. coli* K-12 has been demonstrated that it is an enzyme containing Fe-S cluster (Cicchillo *et al.,* 2004). Here I give some evidences to support this. First of all, the L-SD1 shows significant sequence homology with L-SD from *P. asaccharolyticus*, in particular three conserved cysteine residues. Secondly, like L-SD from *P. asaccharolyticus*, L-SD1 is a PLP-independent deaminase. Thirdly, previous studies from our lab have shown that L-SD1 from *E. coli* K-12 is inactive both *in vivo* and *in vitro*. It can be activated by incubation with Fe and DTT in the presence of L-serine (Newman *et al.* 1985a). Studies also revealed that purified L-SD1 from *E. coli* K-12 is active, but is quickly inactivated on exposure to air. Activity is restored upon incubation with iron, sulfide, and DTT under anaerobic conditions (Newman and Sun, unpublished results). These well-known characteristics exist among the other enzymes with Fe-S cluster. The results from this study also support it. L-SD1 activities with both crude extracts and purified proteins were only observed while the samples were incubated with Fe and DTT for at least 10 minutes. After that, L-SD1 activity increased gradually during incubation period with Fe and DTT in the presence of L-serine under aerobic conditions. This also implies that L-SD1 from

E. coli K-12, present in either crude extracts or as purified protein, is inactive, but can be reactivated by incubation with Fe and DTT in the presence of L-serine. Taken together, the evidence supports that L-serine deaminase 1 from *E. coli* K-12 contains an Fe-S cluster. Then, if it does contain Fe-S cluster, which kind of Fe-S cluster is it?

In the year 1985, it was found that L-SD1 is very unstable and L-serine could stabilize and protect it (Newman *et al.* 1985a). This also occurs in L-SDs from other bacteria (Zinecker *et al.* 1998, Velaydhan *et al.* 2004). The mechanism for this was investigated in L-SD from *P. asaccharolyticus* by electron paramagnetic resonance (Hofmeister *et al.* 1994). It was demonstrated that the substrate L-serine binds to L-SD very near the $[3Fe\text{-}4S]^{+}$ cluster and protects the $[3Fe\text{-}4S]^{+}$ cluster. For the $[4Fe\text{-}4S]^{2+}$ cluster of the active enzyme, L-serine may prevent it from oxidation and loss of an iron atom. Because L-serine has the same effect on L-SD1 from *E. coli* K-12 and L-SD from *P. asaccharolyticus*, one would expect that they might share the same mechanism. If this is true, it suggests L-SD1 from *E. coli* K-12 also contains an 4Fe-4S cluster in its active form, and L-serine protects the enzyme by binding to the Fe-S cluster. It is now reported by that the L-SD1 purified anaerobically contains a 4Fe-4S cluster which is required for the enzyme catalysis activity. And only 3Fe-4S cluster was found in reconstituted L-SD1 without dithionite (Cicchillo *et al.,* 2004). All of these support that L-SD1 is an enzyme containing 4Fe-4S cluster.

2.2. Cysteine residues 339, 381 and 392 are essential for the 4Fe-4S cluster of L-SD1 from *E. coli* K-12

In aconitase, three cysteine residues: cysteine 358, cysteine 421, and cysteine 424 are bound to three iron molecules of 4Fe-4S/3Fe-4S cluster respectively. These bindings cause cysteine residues ligation to and interaction with the Fe-S cluster (Beinert *et al.* 1989, 1996). It is known that L-SDs from *P. asaccharolyticus, C. sticklandii, C. propionicum* and *C. jejuni* also have four conserved cysteine residues, which are thought to coordinate a [4Fe4S] cluster (Velayudhan *et al.* 2004).

L-SD1 from *E. coli* K-12 contains nine cysteine residues. As mentioned previously, the substitution of cysteine residues at positions 339, 381, and 392 leads to complete loss of enzyme activity (Table. 5, 6 and 7. These results indicate that cysteine residues 339, 381, and 392 are not only essential for enzyme activity, but also most likely for the iron-sulfur cluster that exists. Replacement of these three cysteine residues would disrupt the iron-sulfur cluster and render it unable to interact with the substrate L-serine and deaminate it. Thus, it is understandable that the replacements of cysteine residues at either of three positions make L-SD1 lost its activity.

If cysteine residues 339, 381 and 392 are essential for the iron-sulfur cluster of L-SD1, what is their function? As described above, the proposed iron-sulfur cluster of L-SD1 contains four iron atoms. If one of the iron atoms is labile, then the other three iron atoms are involved in the link to the cysteine residues. If cysteine residues 339, 381, and 392 are essential for the iron-sulfur cluster, each of them should interact with one of the

three iron atoms. This is similar to the mechanism demonstrated for aconitase (Beinert *et al.* 1989).

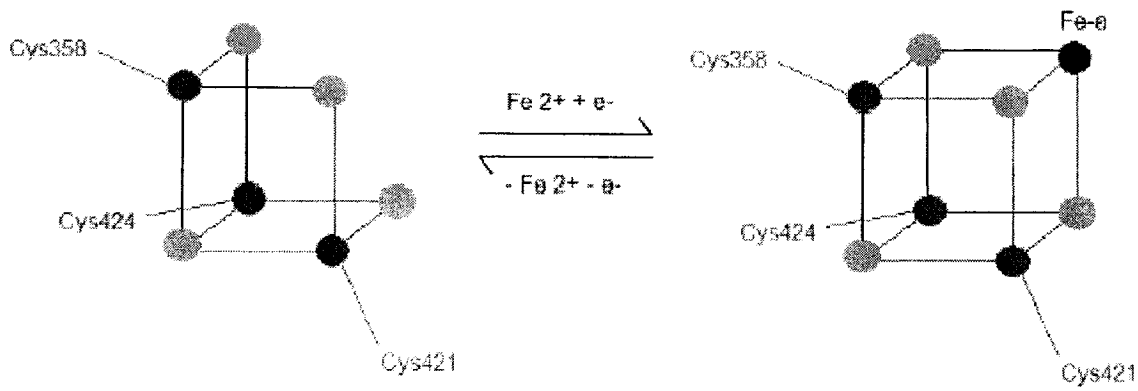
2.3. Aconitase model and the mechanism of catalysis of L-SD1 in *E. coli* K-12

2.3.1 Aconitase model: the Fe-S cluster of aconitase reacts directly with an enzyme substrate

Aconitase is one of the enzymes containing an iron-sulfur cluster(s). Beinert and his colleagues studied its mechanism very well (1989, 1996, 2003). When the enzyme is inactive, the Fe-S cluster is [3Fe-4S] cluster form. Each of the three cysteine residues (residues 358, 421, and 424) are bound to one of the three iron molecules of [3Fe-4S] cluster respectively. These three iron molecules also link to four water molecules. Addition of $Fe2^{+}$ under reducing conditions converts the [3Fe-4S] cluster to a [4Fe-4S] cluster, which activates the enzyme. When the active enzyme is exposed to air, the $[4Fe\text{-}4S]^{2+}$ cluster is oxidized to a $[4Fe\text{-}4S]^{3+}$ cluster. Being unstable, this cluster can easily lose one Fe^{2+} and be inactivated $[3Fe\text{-}4S]^{+}$ cluster (Figure10). Since the fourth iron is always inserted in the same site in the cluster and is essential for the enzyme activity, it is named active iron-Fe_a. This labile Fe_a has no cysteine ligand and is ligated to three sulfides and three oxygens: one from water and two from the substrate (citrate or isocitrate) hydroxyl and one carboxyl, respectively. When one water molecule is

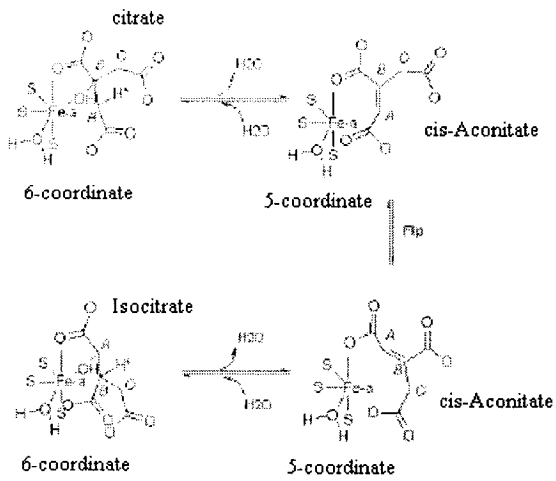
eliminated from the substrate, Fe_a is bound to the same three sulfides and only one carboxyl oxygen of the substrate by a covalent bond (Figure 11) (Beinert *et al.*, 1996).

Figure 10 Interconversion between [3Fe-4S] and [4Fe-4S] cluster of Aconitase.



This figure is adapted from Beinert *et al.*, 1996.

Figure 11 Binding of substrate to Fe_a.



This figure is adapted from Beinert *et al.*, 1996

2.3.2. The mechanism of catalysis of L-SD1 in *E. coli* K-12

It is known that L-SD1 contains 4Fe-4S at its active form and this cluster is required for the enzyme activity (Cicchillo *et al* 2004). This enzyme uses the similar mechanism which is demonstrated in aconitase (Hofmeister *et al.* 1997, Cicchillo *et al*

2004). Thus, the inactive and activation of L-SD1 from *E. coli* K-12 may be explained as following.

Like in aconitase, all the three cysteine residues 339, 381 and 392 are respectively held to one of three iron molecules of [3Fe-4S]/[4Fe-4S] cluster. The [4Fe-4S] cluster is formed by inserting a fourth iron into the [3Fe-4S] cluster. When L-SD1 is exposed to air, the $[4Fe\text{-}4S]^{2+}$ cluster within the enzyme is oxidized to an unstable $[4Fe\text{-}4S]^{3+}$ cluster that easily loses a Fe^{2+} ion to produce the inactive form, $[3Fe\text{-}4S]^{+}$ cluster. Then it can explain why L-SD1 purified in aerobic condition is inactive and can be reactivated by Fe and DTT. The mechanism of catalysis of L-SD1 in *E. coli* K-12 may be hypothesized as following. Active 4Fe-4S cluster of L-SD1 directly interacts with L-serine. The fourth Fe within the [4Fe-4S] cluster is an active iron (Fe_a) and not stable. It coordinates three sulfides and L-serine (Figure 12). How does L-serine bind to the fourth iron? There are two different hypotheses for the coordination of L-serine to 4Fe-4S cluster.

Hofmeister *et al* (1994) proposed that the fourth iron molecule binds to two oxygen molecules of L-serine. One is the β-hydroxyl group and the other is the carboxyl group of L-serine that results in a free enamine. Pyruvate and ammonia are produced due to the tautomerism and hydrolyzation of the enamine (Figure 13).

Figure 12 L-serine binding to Fe-S cluster of L-SD1 of *E. coli* K-12

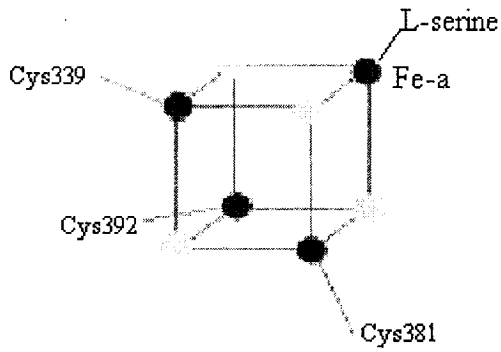
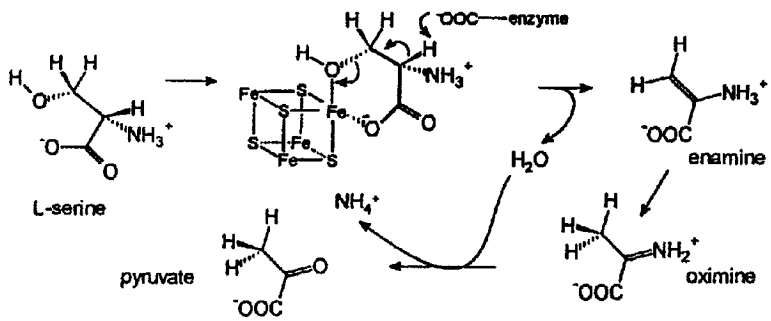


Figure 13 Proposed mechanism for Fe-S cluster-containing L-SD1 of *E. coli* K-12

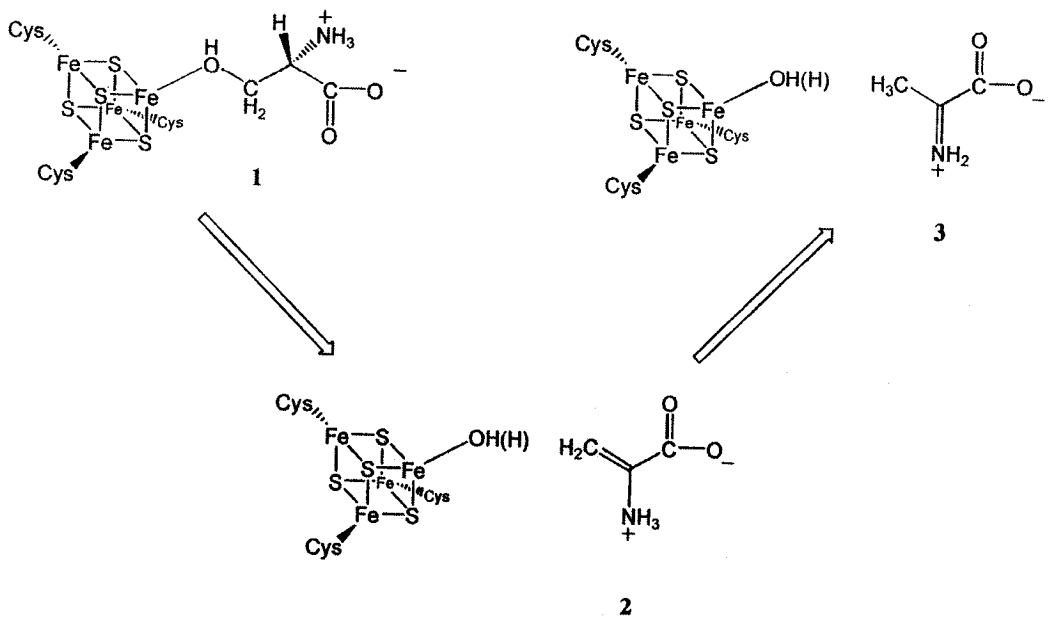


This figure is adapted from Hofmeister *et al.*, 1994

Because the hydroxyl group and one of the carboxylate oxygens of L-serine were

not in the same plane, Cicchillo *et al* (2004) thought that the molecule would be in a conformation that wouldn't allow proper overlap for the elimination reaction if both of them were coordinated to the Fe_a. Cicchillo *et al* (2004) proposed that the fourth iron molecule only binds to one oxygen molecule, the oxygen of β-hydroxyl group of L-serine. Dehydratation of L-serine leads to formation of 2-amino-2-propenoic acid which is tautomerized to 2-imino-propionic acid. The later hydrolyzes to produce pyruvate and ammonia (Figure.14) (Cicchillo *et al.*, 2004).

Figure 14 Proposed mechanism for Fe-S cluster-containing L-SD1 of *E. coli* K-12



This figure is adapted from Cicchillo *et al.*, 2004.

Part 3 Some considerations and further research suggestions

In this study, I demonstrated that cysteine residues 339, 381, and 392 were essential for the activity of L-SD1 from *E. coli* K-12. Cysteine 181 seems not essential for the activity of L-SD1. This is unexpected and rather surprising, since it is one of four conserved cysteine residues in L-SD1 from *E. coli* and L-SD from *P. asaccharolyticus*. In this study, cysteine residues 319, 381 and 392 were substituted with alanine, and cysteine 181 was substituted with glycine. The difference between alanine and glycine is the side chain (R-group) that is a functional group. This side chain in glycine is a hydrogen atom. Substitution with glycine could also introduce conformational flexibility into the protein backbone. This may result in no effect on function of protein. Thus mutation C181G may have no signification change on enzyme activity. The side chain of alanine is a methyl and lacks unusual backbone dihedral angle preferences. Substitution with alanine removes the function of side chain of the substituted amino acid. To confirm whether cysteine residue 181 is not important for the enzyme activity, we can substitute it with alanine or other amino acids in future study.

L-SD1 is an enzyme containing 4Fe-4S which interacts directly with L-serine as active site (Cicchillo *et al.,* 2004). This study indicated cysteine residues 339, 381, and 392 act as a link for three irons within this 4Fe-4S cluster. Further experiments, with X-ray crystallographic and spectroscopic methods such as electron paramagnetic resonance (EPR) and Mössbauer Spectroscopy, will be very helpful to confirm the mechanism model.

Lastly, I would like to mention again about the stability of L-SD1. It is reported that the activity of L-SD1is difficult to assay, because it is not stable (Newman *et al.,* 1989, 1993). In this study, we also had this problem for both of wild type L-SD1 and mutant L-SD1. The reason is remains unknown, however, one possible explanation is that the assays were done in aerobic condition, and it is a known fact that this enzyme is very sensitive to oxygen. In future, if the enzyme can be purified and measured under anaerobic condition, this problem may be avoided.

Summary

L-SDs are widespread in nature and have been purified and characterized from different sources such as bacteria, yeast, and plants. Enzymes from different sources may have different mechanisms of catalysis. It is known that bacterial L-SDs are highly specific for L-serine and PLP-independent and can convert L-serine to pyruvate, ammonia and water. The L-SDs from *P. asaccharolyticus* and *C. propionicum* contain a Fe-S cluster instead of PLP at the active site. In *E. coli* K-12 there are three kinds of L-SD, namely L-SD1, L-SD2, and L-SD3 that are respectively coded by *sdaA, sdaB,* and *tdcG.* L-SD1 coded by *sdaA* gene consists of 454 amino acids that include 9 cysteine residues located at positions 181, 219, 290, 339, 347, 366, 381, 392 and 453. Cicchilo and his colleagues have demonstrated that this enzyme contains 4Fe-4S cluster and this cluster play a key role in the enzyme activity. The cysteine residues are of interest since cysteine is one of two sulfur-containing amino acids. It can bind to the iron atom in Fe-S cluster and catalysis enzyme reaction.The goal of this study was to investigate the role(s) played by the cysteines in L-SD1 activity in *E. coli* K-12 and to identify which cysteines are essential for activity.

For these objects, the first step of the experiments in this study was the systematic replacement of each of the nine cysteine residues by site-directed mutagenesis via PCR. Then I transformed these plasmids into CH22 for expression. For overexpression and purification of *sdaA* and derivatives, the his-tagged *sdaA* and derivatives were

constructed. I cloned 1365bp of the wild type *sdaA* gene and its derivatives into a modified pT7-5 vector that carries a C-terminal 6×His-p*sdaA*H6 which was predigested with *Eco*RI and *Xho*I to obtain a 2.4 kb DNA fragment. After that, the his-tagged *sda*A and derivatives were transformed into BL21 (DE3)..

To know how the mutations of cysteine residues affect L-SD1 activity, L-SD1 activity assays were performed *in vivo* and *in vitro* with crude extracts and purified proteins. Comparing L-SD1 activity in the wild type and mutant strains identified the cysteine residues important for the activity of L-SD1. The results indicated that mutations of different cysteine residues had different effect on L-SD1 activity. A change at 181 seems to have no great effect; a change at 219, 290, 347, 366, and 453 respective decreased enzyme activity to varying extents; and a change at 339, 381, and 392 caused the absolute loss of L-SD activity.

Blast results show that three cysteines at positions 339, 381 and 392 as well as the motif GCQ-X_{40}-CDP-X_7-PC (where G, C, Q, D, P represents a glycine, cysteine, glutamine, aspartic acid, proline residue respectively and X represents any other amino acid) are highly conserved in most L-SDs depending Fe-S cluster among all species. The other six cysteine residues are also conserved among some bacteria L-SDs. Thus from the results in this study, I conclude that: 1) Eight of nine cysteine residues play an important role in L-SD1 activity in *E. coli* K-12 except cysteine 181. 2) Cysteine at positions 339, 381 and 392 are essential for L-SD1 activity; 3) Cysteine at position 181 might be non-essential for L-SD1 activity. Additionally, the results reported here supported that L-SD1

from *E. coli* K-12 contain a Fe-S cluster as well as L-SD from *P. asaccharolyticus* which is thought to use a similar mechanism as that elucidated for aconitase.Since L-SD1 is a Fe-S cluster containing enzyme, cysteine residues 339, 381 and 392 are probably essential for this Fe-S cluster. These three cysteine residues bind to the three iron molecules of the Fe-S cluster respectively. This Fe-S cluster as the active site of L-SD1 directly takes part in the deamination of L-serine.

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