Characterization and Genetic Manipulation of D-cysteine Desulfhydrase from *Solanum lycopersicum*

by

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AUTHOR'S DECLARATION

I hereby declare that I am the sole author of this thesis. This is a true copy of the thesis, including any required final revisions, as accepted by my examiners.

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Abstract

Progress in DNA sequencing of plant genomes has revealed that, in addition to microorganisms, a number of plants contain genes which share similarity to microbial 1-aminocyclopropane-1-carboxylate (ACC) deaminases. ACC deaminases break down ACC, the immediate precursor of ethylene in plants, into ammonia and α-ketobutyrate. We therefore sought to isolate putative ACC deaminase cDNAs from tomato plants with the objective of establishing whether the product of this gene is a functional ACC deaminase. It was demonstrated that the enzyme encoded by the putative ACC deaminase cDNA does not have the ability to break the cyclopropane ring of ACC, but rather that it utilizes D-cysteine as a substrate, and in fact encodes a D-cysteine desulfhydrase. Kinetic characterization of the enzyme has shown that it is similar to other previously characterized D-cysteine desulfhydrases. Using site-directed mutagenesis, it was shown that altering two amino acid residues within the predicted active site changed the enzyme from D-cysteine desulfhydrase to ACC deaminase. Concomitantly, it was shown that by altering two amino acids residues at the same position within the active site of ACC deaminase from *Pseudomonas putida* UW4 changed this enzyme into D-cysteine desulfhydrase.

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Chapter 1
Introduction

1.1 Ethylene

The gaseous phytohormone ethylene is one of the simplest known organic molecules with the ability to regulate plant growth and development. It was first recognized as a biologically active gas by the Russian plant physiologist Dimitri Neljubov in 1901, when he observed that etiolated pea seedlings grew upright in outside air but horizontally in the lab (Abeles, 1992). Neljubov demonstrated that the cause of this behavior was contamination by illuminating gas, where the active principle component was ethylene. Conclusive chemical proof that ethylene is a natural product of plants was provided by Gane in 1934, who collected and analyzed gases evolved from 60 pounds of ripening apples (Abeles, 1992).

Recognized as a biologically active gas for over a century now, effects of ethylene on plant development have been studied extensively; the phytohormone regulates many diverse metabolic and developmental processes, including germination, flower and leaf senescence, fruit ripening, leaf abscission, programmed cell death, root nodulation, and responsiveness to stress and pathogen attack (Abeles, 1992; Bleecker and Kende, 2000). Ethylene is vital to plant development and survival, however, large increase in ethylene levels due to various stresses, known as *stress ethylene*, can cause deleterious symptoms in plants, including the onset of senescence and an inhibition of plant root growth. Having multiple effects on plants, the biosynthesis of this hormone must be tightly regulated. This is achieved by a diverse array of factors including developmental cues and other phytohormones. Establishing how these factors act to modulate ethylene levels is fundamental to an understanding of ethylene functioning in plants.

1.2 Ethylene Biosynthesis, Perception and Signaling

The pathway of ethylene biosynthesis is well characterized in higher plants (for a review see Bleecker and Kende, 2000; Figure 1-1). Ethylene synthesis begins with the conversion of the amino acid methionine to S-adenosyl-methionine (AdoMet), which is then converted to the immediate ethylene precursor, 1-amioncyclopropane-1-carboxylic acid (ACC). ACC is formed from AdoMet through the action of ACC synthase (ACS) and the conversion of ACC to ethylene occurs through the enzyme ACC oxidase (ACO) (Kende, 1993; Figure 1-1). In addition to ACC, the reaction of ACC synthase also produces 5'-methylthioadenosise (MTA), which is utilized for the synthesis of a new methionine via a methionine salvage pathway. This pathway preserves the methylthio group through every revolution of the cycle at the cost of one ATP molecule. ACS and ACO are thought to be the major regulatory enzymes of ethylene biosynthesis, however, other enzymes such as those in the methionine cycle or those involved in methionine synthesis have also been implicated in regulating ethylene synthesis (Katz et al., 2006; Sauter et al., 2005). ACC may also be converted to 1-(malonyl-amino)cyclopropane-1-carboxylic acid (MACC) by ACC *N*-malonyltransferase. This reaction irreversibly decreases available levels of ACC in plant tissues.

Unlike the biosynthetic pathway, the ethylene signaling cascade is more complicated and therefore has been more difficult to study; many questions still remain unanswered. However, one attribute scientists have used extensively to study the signaling pathway is the so-called *triple response*. When treated with exogenous ethylene or when manipulated to produce high ethylene levels, the plants respond with the triple response. This response is characterized by the inhibition of hypocotyl and root cell elongation, swelling of the

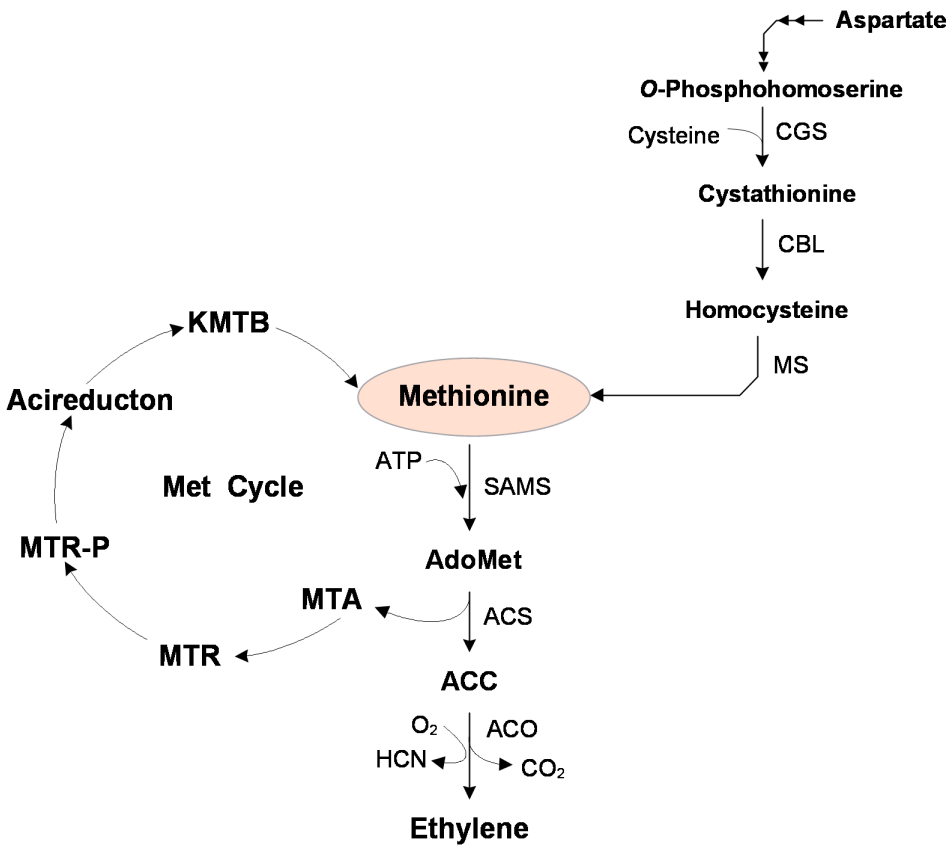


Figure 1-1. Ethylene biosynthesis and methionine metabolism. Abbreviations: ACC, 1-aminocyclopropane-1-carboxylate; ACS, 1-aminocyclopropane-1-carboxylate synthase; ACO, 1-aminocyclopropane-1-carboxylate oxidase; AdoMet, S-adenosyl-methionine; SAMS, S-adenosyl-methionine synthase; MS, methionine synthase; CBL, cystathionine-β-lyase; CGS, cystathionine-γ-synthase; MTA, 5'-methylthioadenosine; MTR, methylthioribose; MTR-P, methylthioribose phosphate; KMBT, 2-keto-4-methylthiobutyrate.

hypocotyls and exaggerated curvature of the apical hook. It is a highly specific ethylene response and it has been exploited to screen for a variety of mutants defective in ethylene signaling. More than a dozen ethylene perception mutants have been identified, and subsequently utilized to elaborate their role in ethylene signaling (Guo and Ecker, 2004). Generally, the mutants can be divided into three categories: constitutive triple-response mutants, ethylene-insensitive mutants and tissue-specific ethylene-insensitive mutants. The combination of molecular and genetic studies has defined the mostly linear ethylene response pathway, starting from hormone perception at the endoplasmic reticulum membrane to transcriptional regulation in the nucleus (Figure 1-2).

Ethylene is perceived by a family of membrane associated ethylene-binding receptors first identified in *Arabidopsis* and including ETHYLENE RESPONSE 1 (ETR1)/ETR2, ETHYLENE RESPONSE SENSOR 1 (ERS1)/ERS2 and ETHYLENE INSENSITIVE 4 (EIN4) (Bleecker and Kende, 2000; Johnson and Ecker, 1998; Stepanova and Ecker, 2000). All ethylene receptors have a sensor domain, a histidine kinase domain and a response domain, where they bind ethylene with the help of a copper cofactor. Upon ethylene binding, these receptors are inactivated and thus cannot activate the protein kinase CTR1 (constitutive triple response) anymore, also a negative regulator of the pathway. Consequently, the repression of the membrane protein EIN2 (ethylene-insensitive) by CTR1 is relieved. The activation of EIN2 in turn stabilizes the EIN3 transcription factor that regulates the expression of its immediate target genes such as ETHYLENE RESPONSE FACTOR1 (ERF1). ERF1 belongs to a large family of transcription factors that bind to a GCC-box present in the promoters of many ethylene-inducible or defense-related genes (Hao et al., 1998; Figure 1-2).

1.3 Regulation of Ethylene Biosynthesis

Nearly all plant tissues have the potential to produce ethylene, yet the amount of ethylene is relatively low in most cases. Ethylene levels increase drastically when a plant is under stresses such as wounding, flooding or drought, pathogen attack, and when exposed to harmful chemicals such as ozone and sulfur dioxide. During several growth and developmental process, including germination, leaf and flower senescence, and fruit ripening, ethylene production also increases. Ethylene can affect its own levels, either increasing (autocatalysis) or decreasing (autoinhibition) its rate of production, as seen in climacteric fruit ripening and during normal vegetative growth, respectively (Kende, 1993). Other hormones, such as auxin, abbscisic acid and cytokinins are also known to affect ethylene levels.

1.3.1 Developmental Factors Regulating Ethylene Levels

Studies to determine how developmental cues regulate ethylene synthesis have mainly focused on establishing how the expression patterns of ethylene synthesizing genes ACC synthase and ACC oxidase change through the process. The role of ACC synthase was first demonstrated in 1991 in transgenic tomato plants expressing an antisense *ACS* gene. The transgenic plants could not ripen unless exposed to exogenous ethylene (Rottmann et al., 1991; Theologis et al., 1993). Since the first gene was isolated by Van der Straten et al., (1990), at least eight *ACS* genes have been identified in tomato (*LEACS1A, LEACS1B* and *LEACS2-7*) (Rottmann et al., 1991; Shiu et al., 1998; Zarembinski and Theologis, 1994) whereas the *Arabidopsis* genome contains nine *ACS* genes, of which eight encode functional ACS proteins (ACS2, ASC4-9, ACS11) (Arabidopsis Genome Initiative, 2000; Yamagami et al., 2003). *ACS* genes have also been isolated from many other plants including melon,

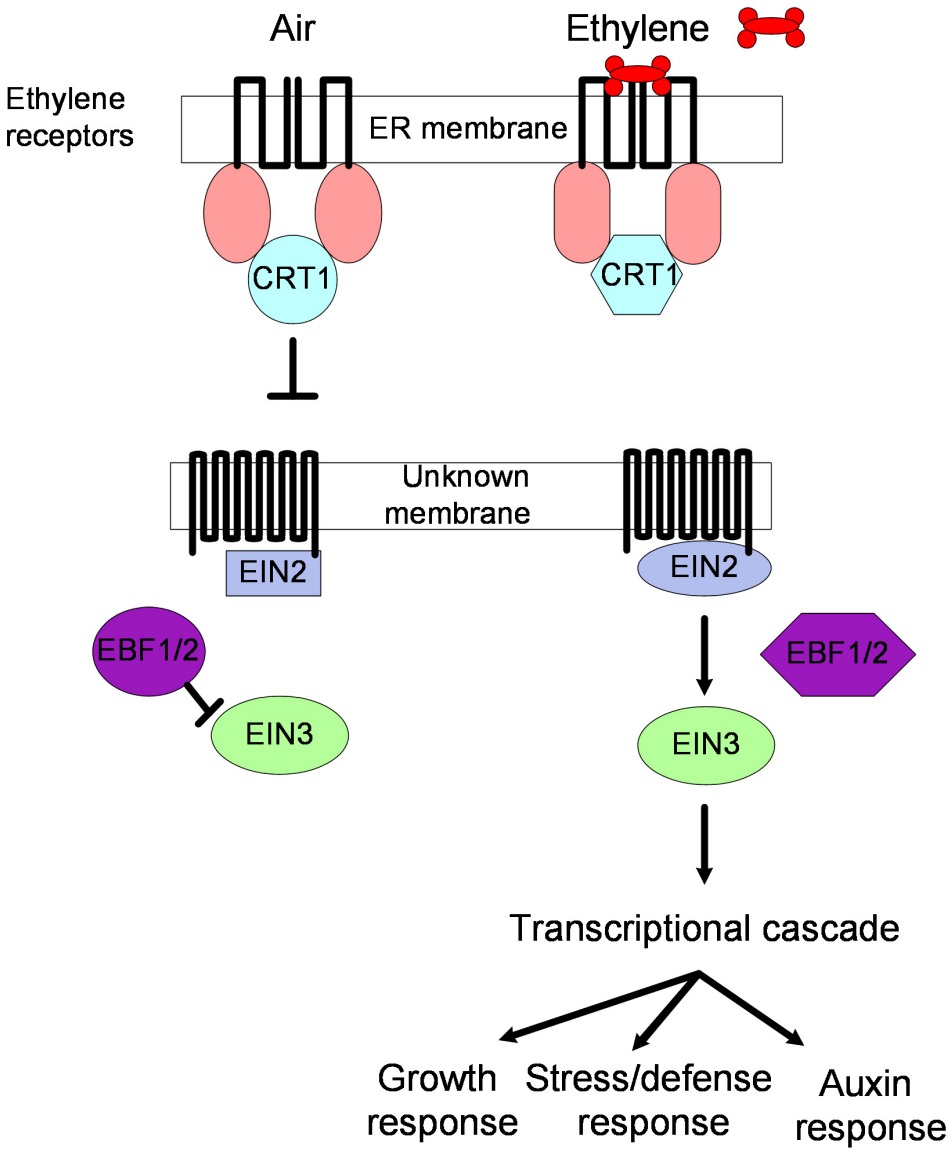


Figure 1-2. Ethylene perception and signal transduction. Ethylene is perceived by a family of ethylene-binding receptors located in the ER membrane. Upon ethylene binding, these receptors are inactivated and can not activate the protein kinase CTR1 (constitutive triple response). As a result, the repression of the membrane protein EIN2 (ethylene insensitive) by CTR1 is relieved. The resulting activation of EIN2 in the presence of ethylene stabilizes the EIN3 transcription factor, which brings ethylene signal transduction into the nucleus.

cucumber and citrus (Miki et al., 1995; Nakajima N., 1990; Wong et al., 1999).

Although ACS activity has been recognized early on as the rate limiting step in ethylene biosynthesis, it has been demonstrated recently that ACO activity also increases in some plant tissues in response to ethylene (Kende, 1993). The fact that the rise in ACO activity precedes ACS activity in pre-climacteric fruit in response to ethylene was the first indication of importance of ACO activity in regulating ethylene levels (Lui et al., 1985). Further evidence came with the examination of *ACO* mRNA expression patterns in various tissues and during different developmental stages (Barry et al., 1996; Hamilton et al., 1990).

Similar to what has been observed for ACS, ACO is a member of a multi-gene family. However, unlike ACS this enzyme has proven difficult to study, due to the lack of an *in vitro* assay, and even more difficult to purify (Kende, 1993). The first *ACO* gene was identified through antisense expression of the ripening-related clone, pTOM13, at the time a gene of unknown function (Holdsworth et al., 1987a). Down-regulation of this gene in transgenic tomato produces plants which synthesize reduced levels of ethylene; the role of the enzyme was confirmed by expression of the pTOM13 clone in yeast and *Xenopus* oocytes, where it was directly shown to convert ACC into ethylene (Hamilton et al., 1991; Spanu et al., 1991). Since then, another three *ACO* genes have been identified in tomato (Barry et al., 1996; Blume and Grierson, 1997; Holdsworth et al., 1987b; Llop-Tous et al., 2000; Nakatsuka et al., 1998) and in numerous other plants including mung bean, peach, banana, apples and avocado (Callahan et al., 1992; Clendennen and May, 1997; Dong et al., 1992; Kim and Yang, 1994; McGarvey et al., 1990).

Fruit ripening is one of the developmental processes that has been studied extensively in terms of regulation of ethylene synthesis. It has been important commercially to

understand the ripening mechanisms of climacteric fruit such as melons, bananas and tomatoes; regulation of ethylene synthesis during fruit ripening is described below as an example of developmental cues that regulate ethylene.

A number of physical changes occur during fruit ripening such as changes in colour, texture and aroma. Fruit with different ripening mechanisms can be divided into two categories. *Climacteric*, in which a massive production of ethylene commences at the onset of the respiratory period and where exogenously applied ethylene induces ripening and endogenous production of ethylene. *Non-climacteric* in which respiration shows no dramatic change and ethylene production remains at constantly low levels. Tomato fruit begins as green, enters a mature green stage (pre-breaker stage), and acquires the first spot of visible colour (breaker stage) after which it becomes softer and the colour changes to a deep red. Throughout the process the characteristic increase in ethylene synthesis and perception are essential for the full completion of the ripening process (Oeller et al., 1991; Picton et al., 1993a).

Expression of both *ACS* and *ACO* has been investigated in a number of different climacteric fruit including apple, tomato, melon, pear, banana and fig (Barry et al., 1996; Dong et al., 1992; Lelievre et al., 1997; Liu et al., 1999; Miki et al., 1995; Nakatsuka et al., 1998; Olson et al., 1991; Ross et al., 1992; Rottmann et al., 1991). These plants are proposed to have two systems of ethylene regulation. System 1 functions during normal vegetative growth and produces basal ethylene levels that can be detected in all tissues including the non-climacteric fruit. System 2 functions during the ripening process of climacteric fruit and during senescence, where ethylene production is typically at a much higher level than in system 1.

A model to explain the transitions from system 1 to system 2 and the overall complexity of ethylene regulation in fruit is emerging based on studies carried out with tomato plants. Expression of two *ACS* genes, *LEACS2* and *LEACS4*, has been well documented in ripening fruit (Lincoln et al., 1993; Olson et al., 1991; Rottmann et al., 1991; Yip et al., 1992). Inhibition of *LEACS2* by antisense expression of the gene in transgenic plants caused down-regulation of both *LEACS2* and *LEACS4* and reduced ethylene synthesis to 0.1% of that usually produced by fruit during ripening (Oeller et al., 1991). Additionally, expression of *LEACS1A, LEACS3* and *LEACS6* has been demonstrated somewhat more recently in tomato fruit (Nakatsuka et al., 1998).

The use of tomato mutants has proven a useful tool in deciphering which *ACS* genes are ethylene regulated. For instance, the Never ripe (*Nr*) mutant cannot perceive ethylene due to a mutation in the NR ethylene receptor; additionally, fruit from the ripening inhibitor (*rin*) mutant do not show autocatalytic ethylene production and do not transmit the ethylene message downstream to ripening-related genes due to a mutation in the RIN transcription factor (Vrebalov et al., 2002; Wilkinson et al., 1995). Based on the studies utilizing these two mutants, it has been proposed that *LEACS1A* and *LEACS6* are involved in maintaining the system 1 ethylene production in green fruit. System 1 continues to function until the fruit is ready to proceed through the ripening pathway, whereupon a transition occurs, such that *LEACS1A* expression increases and *LEACS4* is induced. System 2 ethylene synthesis is initiated and sustained by ethylene dependent induction of *LEACS2* (Barry et al., 2000).

Furthermore, the importance of ACO in regulating ethylene synthesis during ripening process was also demonstrated using antisense technology (Hamilton et al., 1990; Picton et al., 1993b). Down-regulation of *LEACO1* expression in transgenic plants caused reduced

ethylene levels and impaired ripening. *LEACO1* has been shown to be the major *ACO* gene expressed in tomato fruit, but ripening related expression of *LEACO3* and *LEACO4* has also been reported (Barry et al., 1996; Nakatsuka et al., 1998). *LEACO1* and, at a lower level *LEACO3*, are expressed at the onset of ripening. *LEACO1* levels peak three days post-breaker and then fall back to levels observed at the breaker stage; *LEACO3* transcripts are only briefly expressed at the breaker stage before disappearing completely.

1.3.2 Post-transcriptional Regulation of ACS

Numerous studies have demonstrated the differential expression patterns of the *ACS* gene family to various environmental or developmental stimuli, but it has only recently become evident that ACS levels are also controlled by post-transcriptional mechanisms. The first evidence of possible post-transcriptional regulation was the observation that various ACS proteins have different half-lives. For instance, the half-life of wound induced ACS activity from pericarp tissue of green tomato fruit was found to be much shorter than that of ripening fruit (Kende and Boller, 1981). Many studies in *Arabidopsis* have led to the conclusion that post-transcriptional regulation of ACS must relay on the control of protein stability. These studies have utilized, among others, two classes of mutants which affect ethylene biosynthesis: *eto* (*ethylene-overproducer*) mutants which display constitutive ethylene response due to increased ethylene biosynthesis (Chae et al., 2003; Vogel et al., 1998a; Vogel et al., 1998b; Woeste et al., 1999); and *cin* (*cytokinin-insensitive*) mutants which synthesize reduced ethylene levels in response to cytokinin (Vogel et al., 1998a).

Analysis of etiolated Eto$^-$ mutant seedlings in *Arabidopsis* showed they have increased ACS activity but steady-state levels of *ACS* encoding transcripts. *eto2* and *eto3* mutations were shown to be the result of single base pair insertions and a single amino acid

change at the C-terminus of ACS5 and ACS9, respectively (Chae et al., 2003; Vogel et al., 1998b). Furthermore, *in vitro* analysis of tomato LEACS2 enzyme activity has illustrated that the deletion of 52 C-terminal amino acids increased enzymatic activity (Li et al., 1996). These findings have shown that the C-terminal domains of at least ACS5 and ACS9 in *Arabidopsis* and LEASC2 in tomato may have a regulatory role in protein stability.

To elaborate how the stability of the protein is regulated, studies have focused on yet another *ethylene-overproducer* mutant, *eto1*. The ETO1 protein is a BTB (Broad-complex, Tramtrack, Bric-à-brac) domain containing protein, a class of proteins that have been shown to link their substrate proteins to degradation pathways. The breakthrough came when ACS5 was shown to directly interact with ETO1, suggesting that it targets ACS5, and possibly other ACS isoforms for rapid degradation.

An important question arises regarding what mechanisms control and delay ACS protein degradation. While this question has not yet been answered for all ACS isoforms, data for some has shown that modification of ACS' C-terminus by phosphorylation is the key. It was reported that a C-terminal serine residue of LEACS2 is phosphorylated in wounded tomato fruit by a calcium-dependent protein kinase (CDPK); recombinant LEACS2 and LEACS3 were also shown to be phosphorylated *in vitro* (Tatsuki and Mori, 2001). Studies of a mitogen-activated-protein kinase (MAPK) pathway have revealed that a subset of ACS proteins are also substrates of MAPK phosphorylation (Liu and Zhang, 2004). Activation of the MAPK pathway in *Arabidopsis* resulted in stabilization of ACS6 protein *in vivo*, but MPK6 was also found to phosphorylate three serine residues from both ACS6 and ACS2 *in vitro*. Altering the target serine residues such that phosphorylation is blocked results in a protein that cannot be stabilized *in vivo* (Liu and Zhang, 2004). This data implies that

phosphorylation of ACS protects the protein from degradation, which in turn causes ACS to accumulate and ACS activity to increase as part of a pathway leading to increased ethylene production. Whether all ACS isoforms undergo this type of modification, and in turn stabilization, or whether it is specific to only certain ACS proteins remains to be determined.

1.3.3 Co-expression and Functional Heterodimerization of ACS

A recent in-depth study of the eight functional (*ACS2*, *ACS4*-9, and *ACS11*) and one non-functional *ACS1* genes from *Arabidopsis*, where transgenic *Arabidopsis* lines were constructed to express β-glucoronidase (GUS) and green fluorescent protein (GFP) reporter genes under the promoter of each of the gene family members, demonstrated their patterns of expression during plant development. All genes were expressed in 5-day-old etiolated or light-grown seedlings with a distinct expression pattern, except for *ACS9* which was expressed later in development (Tsuchisaka and Theologis, 2004b). Expression in various organs of adult plants and expression changes due to indoleacetic acid (IAA) treatment, wounding, cold, heat, anaerobiosis and Li^+ ions was also examined. Unique and overlapping gene expression patterns were observed, with extensive co-expression among the various gene family members during plant development (Tsuchisaka and Theologis, 2004b). These findings not only help in elucidating the mechanisms which control ethylene biosynthesis, but at the same time bring up an interesting question: what is the biological significance of having a multi-gene *ACS* family that is so extensively co-expressed? It has been suggested earlier that the presence of ACS isozymes may implicate tissue specific expression that satisfies the biochemical environment of the tissue in which the isozyme is expressed (Rottmann et al., 1991). More specifically, if the tissue in question contains low concentration of the ACS substrate, AdoMet, then this tissue would express an isozyme with

high affinity for its substrate. This notion would require that each isozyme carry distinct enzymatic properties; biochemical characterization of all ACS enzymes from *Ababidopsis* supports this idea (Yamagami et al., 2003).

The evidence that ACS enzymes, which have been shown to occur as homodimers, can also form functional heterodimers in a complementation assay in *Escherichia coli* has lead to the hypothesis that if functional heterodimerization occurs *in vivo* it may further enhance the isozyme diversity of the *ACS* gene family and provide additional physiological versatility (Tsuchisaka and Theologis, 2004a). Whether plants form functional heterodimers still remains to be proven; however, if this hypothesis is confirmed, it would corroborate that the patterns of differential co-expression and not just patterns of expression of the *ACS* gene family members play a major role in regulating ethylene levels in plants.

1.3.4 Stress Ethylene

If plants are exposed to conditions that threaten their ability to survive, the same biosynthetic pathway that produces ethylene for development, functions to produce what is referred to as *stress ethylene* (Figure 1-1). An increase in ethylene levels is observed in plants exposed to various types of biotic and abiotic stresses. The precise role of ethylene in disease or stress symptoms is still unclear due to a network of cross-communicating signaling pathways with other hormones which are induced together with ethylene in response to stress.

Similar to other growth and developmental processes, the biosynthesis of ethylene during stress has most intensively been studied at the level of the regulation of *ACS* and *ACO*. For instance, *ACO* genes are differentially expressed during pathogen attack or abiotic stress stimuli such as wounding, flooding or ozone exposure (Cohn and Martin, 2005;

Moeder et al., 2002; Nakatsuka et al., 1998; Nie et al., 2002; Woltering et al., 2005). On a more interesting note, Cohn and Martin (2005) have shown the involvement of the virulence/avirulance factors AvrPto and AvrPtoB from the pathogen *Pseudomonas syringae* pv. *tomato* in ethylene synthesis in a susceptible tomato cultivar. These pathogen derived proteins up-regulated two tomato *ACO* genes, *LEACO1* and *LEACO2*, whereas the other two tomato *ACO* genes remained unaffected. Furthermore, in *Arabidopsis*, *ACO* genes demonstrate significant abiotic and biotic stress-mediated gene regulation. The data implies that transcriptional control of *ACO* genes contributes to the regulation of ethylene production under stress.

However, the control of ethylene production under stress still remains largely attributed to the *ACS* genes. Tsuchisaka and Theologies (2004b) examined spatio-temporal expression patterns in response to different abiotic stress stimuli, including cold, heat, anaborosis, Li^+ ions and wounding. Similar to the developmental regulation of *ACS*, they found specific and partially overlapping patterns of expression among the various *ACS* gene family members. For instance, wounding of hypocotyl tissue resulted in inhibition of constitutively expressed *ACS1* and *ACS5* and induction of *ACS2*, *4*, *6*, *7*, and *8*. A similar pattern was observed with other stimuli (cold, heat, anaerobiosis) where constitutively expressed *ACS* genes were inhibited or expressed at significantly lower levels post-treatment and other *ACS* genes were induced, depending upon the stimuli.

1.3.5 Interaction of Ethylene with Other Hormones

Ethylene biosynthesis may also be regulated by interaction with other hormones, such as auxin, brassinosteroids, abscisic acid, gibberillic acid or cytokinin. Auxin is one of the most well-known inducers of ethylene production (Bleecker and Kende, 2000; Yang and

N.E., 1984). Interactions between ethylene and auxin have been described for adventitious root formation, root hair growth, and root elongation among others (Hirayama et al., 1999; Hua et al., 1998; Huang et al., 2003). These two hormones together stimulate petiole elongation during submergence, hypocotyl elongation in the light and phototropism (Iavicoli et al., 2003; Iordachescu and Verlinden, 2005; Ishigaki et al., 2004). These facts imply tight interaction between ethylene and auxin, their synthesis, transport and signaling. Indeed, it is well known that ethylene biosynthesis is regulated by auxin, primarily by activation of the transcription of ACC synthase (Hua et al., 1995; Tsuchisaka and Theologis, 2004b). It is however, less known that ethylene affects auxin, for instance, auxin distribution in the *Arabidopsis* apical hook or the fact that ethylene may inhibit auxin transport and signal transduction (Glick et al., 2007; Prayitno et al., 2006). Ethylene inhibition of auxin synthesis or functioning limits the amount of ACC synthase, ACC, and therefore ethylene.

Until recently, it was known that the *Arabidopsis ACS4* gene is an auxin primary response gene and that there are auxin-responsive motifs present upstream of this gene (Abel et al., 1995), but little information was available on the response of other *ACS* genes to auxin. Tsuchisaka and Theologis (2004b) have shown that not only are other *ACS* genes also regulated by auxin, but also that auxin enhances the expression of the genes that are constitutively expressed and alters their patterns of expression. On the other hand, auxin has no effect on *ACS1* and *ACS9* in *Arabidopsis*.

Cytokinin is another phytohormone that is known to modulate ethylene levels. For instance, it was reported that cytokinin induces ethylene biosynthesis in seedlings from several plant species (Vogel et al., 1998a; Vogel et al., 1998b). *Arabidopsis* seedlings grown in presence of cytokinin demonstrate elevated ethylene levels and triple response

morphology (Vogel et al., 1998b). This characteristic has been exploited to identify mutants that fail to increase ethylene production in response to cytokinin.

One such mutant identified as a loss-of-function in ACS5 (*cin*) produces severely reduced amount of ethylene in response to cytokinin, suggesting that this ACS isoform is cytokinin regulated. However, Northern blot analysis has revealed that cytokinin mainly increased ACS5 function by a post-transcriptional mechanism, as cytokinin treatment has little effect on the steady-state level of the *ACS5* transcript. Furthermore, analysis of Eto^{-} plants suggests that cytokinin could induce ethylene by modifying the ACS5 C-terminal domain in a manner that blocks ACS5 from being targeted for degradation (Chae and Kieber, 2005). Based on an analysis of *eto2* and *eto3* mutants, it has been concluded that cytokinin acts partially by blocking the C-terminus of ACS5, but also in an ACS C-terminus independent manner, which has not yet been characterized.

1.3.6 Other Mechanisms that Control Ethylene Levels

The biosynthetic pathway of the essential amino acid, one of only two sulfur containing amino acids, methionine, is placed at a regulatory junction, where it is required for protein synthesis and as a precursor for S-adenosyl-methionine (AdoMet). AdoMet serves as a methyl donor and a substrate for the synthesis of a number of metabolites, including polyamines and of course ethylene. The level of methionine in plant tissues is therefore highly regulated; however, the significance of *de novo* methionine synthesis and methionine recycling for ethylene synthesis has not been investigated in detail. Nevertheless, recently published reports suggest that both, *de novo* synthesis and recycling may play an important regulatory role.

Studies in *Arabidopsis* plants imply that the level of methionine is regulated mainly by the first enzyme unique to methionine biosynthesis, cystathionine-γ-synthase (CGS) (Amir et al., 2002; Hesse and Hoefgen, 2003; Kim et al., 2002; Figure 1-1), but the regulatory role of CGS in other plant species is not clear yet. Katz et al. (2006) have demonstrated that ethylene induces CGS gene expression and more notably that the level of free methionine is rate-limiting for ethylene biosynthesis in tomato fruit. This study also suggests that the massive wave of ethylene evolution which occurs during tomato fruit ripening requires an increase in *de novo* methionine synthesis through up-regulation of CGS gene expression.

The first committed step in ethylene synthesis, catalyzed by ACS, results in formation of ACC from AdoMet and release of 5'-methylthioadenosine (MTA) as a side product. The sulfur retained in MTA is recycled such that a methionine molecule is produced using the sulfur group of MTA and a ribose moiety in a cyclic pathway known as the Met or Yang cycle (Yang and Hoffman, 1984). It was predicted early on that the recycling of methionine from MTA is required for sustained ethylene synthesis (Baur and Yang, 1972). Bürstenbinder et al. (2007) have used an ethylene-overproducing *eto3* mutant and an *mtk* mutant which has a disrupted Met cycle to study and demonstrate the significance of Met cycle for ethylene synthesis. The study utilized double mutant *mtk/eto3* plants, which have high ACS activity and no capacity for regeneration of the ACS substrate AdoMet from MTA. They have shown that the Met cycle contributes to the maintenance of AdoMet homeostasis, especially when *de novo* AdoMet synthesis is limited. Moreover, Met cycle is required to sustain high rates of ethylene synthesis. It has been proposed, based on this data, that the regulation of Met cycle by ethylene may be restricted to plants that naturally produce high levels of ethylene for a

long period. Therefore, ethylene itself, methionine biosynthetic enzymes and methionine recycling enzymes may all be members of a regulatory loop that participate in ethylene production.

1.4 Lowering Ethylene Levels with Plant Growth Promoting Bacteria

Plant growth promoting bacteria have the ability to act as protective beneficial bacteria that can facilitate plant growth. However, these typically have little effect on plant growth when the plants experience an optimal and stress-free environment. A large number of these microorganisms have been isolated to date, each with one or more characteristic properties that under certain conditions may enhance plant growth. Some bacteria exert their beneficial effects *indirectly* by acting as biocontrols, where they may limit the growth of other microorganisms such as potential pathogens by synthesizing antibiotics, siderophores that sequester iron from the soil, enzymes such as protease or lipase that facilitate the lysis of fungal cells (Chet and Inbar, 1994; Haas et al., 1991). Others influence plant growth *directly*, e.g. by fixing atmospheric nitrogen, synthesizing phytohormones such as auxin or cytokinins or by synthesizing the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase, which can lower plant ethylene levels (Glick, 1995).

In the last few years, a number of the plant growth promoting bacteria that have been isolated and characterized synthesize ACC deaminase (Glick et al., 1995; Glick et al., 1998; Hontzeas et al., 2005; Jacobson, 1994; Ma et al., 2003b). In practice, these organisms have been used to protect plants against some deleterious effects caused by: flooding, organic toxicants, heavy metals, high salt and phytopathogens (Cheng et al., 2007; Farwell et al., 2007; Glick, 2003; Greenberg et al., 2006; Grichko and Glick, 2001; Mayak et al., 2004; Reed et al., 2005; Wang et al., 2000). The microorganisms that contain ACC deaminase are

able to promote plant growth by acting as a sink for the plant ethylene precursor ACC, thus protecting the plant from deleterious effects brought upon by high ethylene levels. Additionally, several transgenic plants, including tomato, canola and tobacco have been constructed to express the bacterial ACC deaminase. The plants have been reported to be more tolerant to pathogens, high salt and metals; they generally responded in a similar manner to the non-transformed plants that have been treated with ACC deaminase-containing plant growth promoting bacteria (Grichko and Glick, 2001; Robinson et al., 2001; Stearns et al., 2005).

A model has been developed to explain the effects of bacterial ACC deaminase in the promotion of plant growth (Glick et al., 1998; Figure 1-3). Briefly, the ACC deaminase-containing plant growth promoting bacteria bind to the surface of the seed or root; in response to plant synthesized compounds, including the amino acid tryptophan, the bacteria synthesize indoleacetic acid (IAA). Plant cells take up some of the bacterially synthesized IAA, which together with the endogenous IAA can stimulate plant cell elongation and proliferation as well as the synthesis of ACC synthase. Some of the ACC is exuded and taken up by the ACC deaminase-containing bacteria. To be utilized by the bacterium, ACC is cleaved via ACC deaminase into α-ketobutyrate and ammonia, where the products are used as a carbon and nitrogen source respectively. By taking up ACC, the immediate ethylene precursor, the bacteria lower the amount of ethylene that is formed within the plant tissues. When the plant is challenged with anyone of a variety of biotic and abiotic stresses, the direct consequence of this interaction is increased plant fitness, including increase in biomass.

1.5 ACC Deaminase

ACC deaminase was first isolated by Honma and Shimomura (1978) from *Pseudomonas* sp. ACP and since then has been characterized in a few other species, including the yeast *Hansenula saturnus*. The large portion of known biochemical properties of this enzyme can be attributed to the work done by Honma and co-workers (Honma, 1985; Honma et al., 1993; Jia et al., 1999; Minami et al., 1998; Ose et al., 2003; Walsh et al., 1981). However, others have also carried out a few biochemical studies of the enzyme (Hontzeas et al., 2004; Jacobson, 1994; Li et al., 1996; Liu et al., 1984; Zhao et al., 2003).

ACC deaminase is a multimeric enzyme, forming homodimers or homotrimers, depending of the source, with a subunit molecular mass of 35-42 kDa. The enzyme binds one pyridoxal phosphate (PLP) molecule at each subunit via the conserved lysine residue. PLP is not only an essential co-factor, but also involved in the catalysis of the cyclopropane ring.

The Km of various ACC deaminases for ACC as the substrate indicates that the enzyme does not bind the substrate with high affinity; the reported Km values range from 1.5 to 9.2 mM (Honma, 1978; Hontzeas et al., 2004; Minami et al., 1998; Walsh et al., 1981). The interest in the ACC deaminase catalytic reaction has not only been from a physiological perspective of lowering plant ethylene levels, but also due to its unique catalysis, which is still not completely understood.

To gain insight into the functioning of this PLP-dependent enzyme, the crystal structures of bacterial (*Pseudomonas* sp. ACP) and yeast (*Hansenula saturnus*) ACC deaminase as well as an ACC deaminase homologue without this activity from *Pyrococcus horikoshii* have been determined (Fujino et al., 2004; Karthikeyan et al., 2004b; Ose et al., 2003; Yao et al., 2000). The crystal structures, along with site-specific mutagenesis studies,

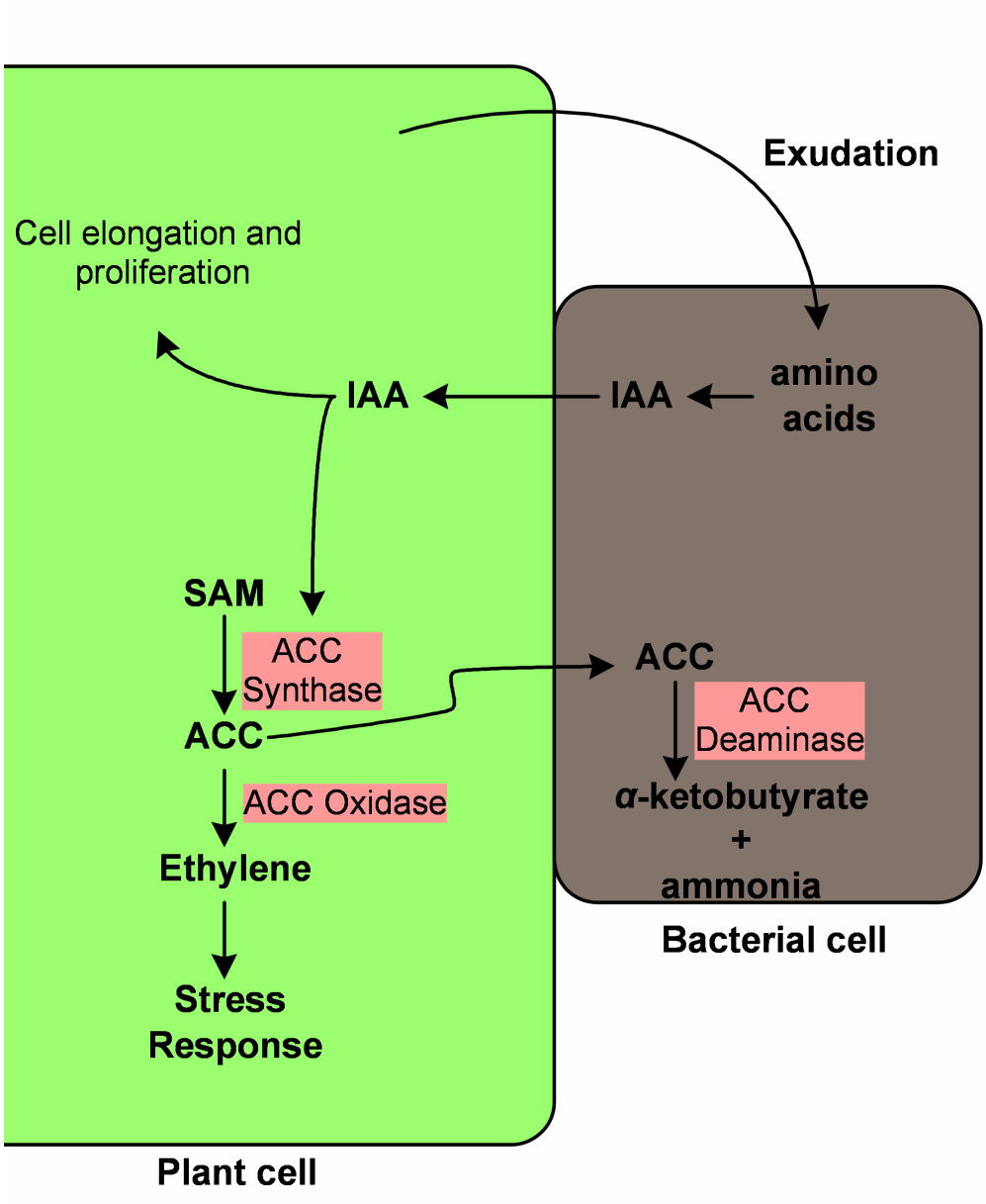


Figure 1-3. A model to explain how plant growth promoting bacteria with ACC deaminase promote plant growth under stress. Abbreviations: IAA, indoleacetic acid; SAM, S-adenosyl-methionine; ACC, 1-aminocyclopropane-1-carboxylate.

have allowed for identification of the essential amino acid residues for catalysis and substrate recognition. The studies have also indicated that ACC deaminase folds to form two domains, each of which has an open twisted α/β structure, similar to the β-subunit of the PLP-dependent enzyme tryptophan synthase. For most of the other PLP-dependent enzymes, two basic properties of PLP have been conserved: (i) PLP forms an external aldimine between its aldehyde group and the α-amino group of the substrate and (ii) PLP acts as an electron sink, withdrawing electrons from the substrate (John, 1995). The ACC deaminase ring opening reaction starts with conversion from an internal aldimine between the enzyme and PLP to an external aldimine between the substrate ACC and PLP. In most other PLP-dependent reaction the next step is the nucleophilic abstraction of either an α-proton or an α-carboxylate group. However, in this regard the ACC deaminase catalyzed reaction is considered as a special case, since the substrate does not contain an α-proton and the carboxylate group is retained in the product, ruling out the usual mechanisms. Walsh et al., (1981) proposed two possible routes for the ring fragmentation: (i) nucleophilic addition at the C_β methylene position followed by β-proton abstraction and (ii) direct β-proton abstraction leading to the cyclopropane ring cleavage. The exact mechanism is still unknown, with data to support both routes (i) and (ii) (Karthikeyan et al., 2004a; Ose et al., 2003).

1.6 Plant Encoded ACC Deaminase

With the progress in the sequencing of plant genomes, it has been suggested that there may exist a plant encoded ACC deaminase which could have a role in regulating plant ethylene levels. The sequences in tomato (Accession #BT013578), *Arabidopsis* (NM_103738), poplar (AI161555), birch (AY154652), rice (BAD16875), and corn (AY106365) share anywhere from 20 to 40% sequence identity at the amino acid level to

known bacterial ACC deaminase proteins. Two research groups have investigated the presence of a functional ACC deaminase in *Arabidopsis* with contradicting results (McDonnell et al., in press; Riemenschneider et al., 2005). Riemenschneider (2005) and co-workers have isolated the cDNA, expressed the protein in *E. coli* and characterized it after purification. They have shown that this enzyme has no activity towards ACC as the substrate, but rather that it breaks down D-cysteine into pyruvate, ammonia and hydrogen sulfide with a Km of 0.25 mM. They have also demonstrated the dependence of the enzyme on PLP, they have localized it to mitochondria and they have shown some data to suggest that the enzyme may be involved in regulating sulfur levels. However, the precise role of this enzyme, D-cysteine desulfhydrase, has not been reported yet, nor has it been shown that plants synthesize D- amino acids, including D-cysteine.

On the other hand, the data from McDonnell and co-workers (in press) suggests that this same enzyme does utilize ACC and breaks it down. They have argued that it utilizes ACC as the substrate on the basis of the ability of *E. coli* (that expresses a cloned cDNA) encoding this enzyme to grow on solid media with ACC as the sole nitrogen source. Additionally, they have also shown activity in plant tissues using plant protein extracts. The group has used the colorimetric assay in which 2,4-dinitrophnylehydrazine (2,4-DNP) is added to detect the product of the reaction, the α-keto acid, α-ketobutyrate. However, the 2,4-DNP is a compound that will react with any α-keto acid such that the accuracy of the assay when determining low activity in a setting as such (using plant protein extracts) is questionable.

However, results consistent with the hypothesis suggested by McDonnell and co-workers were observed when the cDNA encoding the enzyme was expressed in *Arabidopsis*

under the CaMV-35S promoter. They have used the triple response characteristic of plants to show that when grown in the presence of 1.6 μM ACC, the wild type seedlings exhibited a triple response, whereas the transgenic plants had significantly longer hypocotyls and no apparent triple response (i.e. the overexpressed enzyme utilized some of the added ACC, thereby protecting the plants from the ethylene production). Hence, the contradictory results from the two research groups still leave the question of whether plants encode a functional ACC deaminase unanswered.

In this work, we therefore examine whether tomato (*Solanum lycopersicum*) cDNA predicted as a putative ACC deaminase will encode for a protein with this activity. We examine both ACC deaminase and D-cysteine desulfhydrase activity of the recombinant enzyme. Using structural protein modeling and structural data available for bacterial and yeast ACC deaminase enzymes, we also explain why certain enzymes show ACC deaminase activity and others do not. Sequences sharing similarity to true ACC deaminase enzymes have been annotated as putative ACC deaminase for many organisms, yet only a fraction of these have been shown to have the ability to break the cyclopropane bond of ACC, and others have the ability to deaminate other substrates. We demonstrate that only two amino acid residues in the active site of ACC deaminase and D-cysteine desulfhydrase may have the control over which reaction is carried out.

Chapter 2
Experimental Procedures

2.1 Plant Growth and Tissue Collection

Seeds of tomato (*Solanum lycopersicum;* Bony Best variety, Ontario Seed Co #5750) were surface sterilized, by soaking in 70% (v/v) ethanol for one minute followed by soaking for ten minutes in 1% (v/v) bleach, then washed five times in sterilized Milli-Q water. Sterilized seeds were planted in Promix BX greenhouse mix (Premier Horticulture, Quakertown, PA, U.S.A) containing 75-85% (v/v) sphagnum peat moss, perlite, vermiculite, macronutrients (calcium, magnesium, nitrogen, phosphorus, potassium and sulfur), micronutrients (boron, copper, iron, manganese, molybdenum and zinc), dolomitic limestone, calcite limestone and a wetting agent. The plants were grown in the greenhouse during the months of June through September with a temperature of approximately 25°C during the day and 20°C overnight. The plants were under 16 h of light and 8 h of dark (supplemented with High Intensity Discharge (HID) lamps when necessary). They were watered as needed with tap water from above, and were fertilized every seven days, beginning on day fourteen, with a 10-52-10 fertilizer for the first three times, then with a 20-20-20 fertilizer containing 20% nitrogen, 20% phosphorous and 20% potassium (Plant Products, Brampton, ON). The plants were twice transplanted into successively larger pots; first when they reached the stage of having 3-4 true leaves, then when they grew to approximately 20 cm in height. At the flowering stage the plants were pollinated three times a week, always between 11:00 am and 1:00 pm.

The leaf tissue was collected at the mature green stage, prior to any noticeable yellowing and senescence; the fruit tissue, on the other hand, was collected at the breaker

stage, which is defined as the stage where first spot of pink/red colour appears at the blossom end. All tissue was flash-frozen in liquid nitrogen and was either used immediately or stored at -80°C until required.

2.2 Bacterial Strains, Plasmids and Culture Conditions

The bacterial strains, plasmids and constructs used in this study are described in Table 1. *Escherichia coli* DH5α (Invitrogen, Burlington, ON) or NovaBlue GigaSingles (Novagen, Mississauga, ON) were used as the host for initial construction and maintenance of recombinant plasmids. *Escherichia coli* BL21(DE3), which is a lambda DE3 lysogen, (Novagen, Mississauga, ON) was used as the host for recombinant protein expression of putative ACC deaminase from tomato, *Pseudomonas putida* UW4 ACC deaminase and all the constructed mutants. Unless otherwise specified, all *Escherichia coli* strains were cultivated aerobically at 37°C in Luria-Bertani (LB) broth (10 g/L tryptone, 5 g/L yeast extract, 5 g/L NaCl) or on solid media with 15 g/L agar added to the medium. When appropriate, antibiotics or other supplements were added at the following concentrations: 100 μg/ml ampicillin, 30 μg/ml kanamycin, 20 μg/ml tetracycline, 1 mM isopropyl β-D-1-thiogalactopyranoside (IPTG) or 40 μg/ml 5-bromo-4-chloro-3-indolyl- beta-D-galactopyranoside (X-Gal).

The plasmid pET30 Xa/LIC was purchased from Novagen (Mississauga, ON) and was used for recombinant protein expression of the putative ACC deaminase from tomato (Table 1). pBluescript SK (+) (Fermentas, Burlington, ON) was used for initial cloning procedures whereas pET30a (+) (Novagen, Mississauga, ON) was utilized for recombinant protein expression of *Pseudomonas putida* UW4 ACC deaminase.

2.3 RNA Extraction from Plant Tissues

RNA was extracted using the guanidine isothiocyanate (GIT) method (Davis et al., 1986), where all required solutions were made with RNase-free diethylpyrocarbonate (DEPC) treated water, and stored in RNase-free containers. Frozen tissue (<5 g) was added to a mortar and pestle (previously cooled with liquid nitrogen), and ground to a fine powder. The powder was added to 10 ml of denaturing solution (4 M guanidine isothiocyanate salt, 25 mM sodium acetate, pH 6.0, 0.8 M β-mercaptoethanol) in a 50 ml conical tube and vortexed. The mixture was filtered through two layers of MiraCloth (Calbiochem, Mississauga, ON) into 50 ml round-bottom SS-34 rotor centrifuge tubes, and centrifuged for 1 h at 4°C and 15,000 rpm (26,890 x g) in a Sorvall centrifuge. The supernatant was carefully layered onto 3.3 ml of 5.7 M cesium chloride, and RNA was pelleted by centrifuging for 23 h at 10°C and 115,915 x g. The aqueous phase was removed carefully and the pellet was washed once with 1 ml of 70% (v/v) ethanol, then air dried. Subsequently the pellet was re-suspended in 0.5 ml of DEPC water; two volumes of ice cold 100% ethanol and 100 µl of 3 M sodium acetate (pH 6.0) were added and RNA was precipitated overnight at -80°C. The samples were then centrifuged at 4°C and 14,000 rpm (20,798 x g) for 1 h after which 1 ml of 70% (v/v) ethanol was added to the pellet which was centrifuged at 4°C and 14,000 rpm (20,798 x g) for 30 min. The pellet was air dried and re-suspended in DEPC-treated water. Extracted RNA was aliquoted and stored at -80°C until necessary. The quality and quantity of total RNA sample was determined using spectrophotometric measurements at 230, 260, 280 and 320 nm. Purity of RNA was determined by the A_{260}/A_{280} and A_{260}/A_{230} ratios, which ideally should be ~2.0. Ratios below ~2.0 indicate possible protein or carbohydrate contamination, respectively. The integrity of total RNA was checked by denaturing formaldehyde agarose-gel electrophoresis

Table 2-1. Bacterial strains, plasmids and constructs used in this work.

Strains or plasmids	Description	Source/ reference
Strains		
Escherichia coli		
DH5α	*F80d/lacZ M15, recA1, endA1, gyr96, thi-1, hsdR17(rk-, mk+), sup E44, relA1, (lacZYA-argF)U169*	Invitrogen
NovaBlue	*endA1 hsdR17*(r_{K12}^{-} m_{K12}^{+}) *supE44 thi-1 recA1 gyrA96 relA1 lac* F'[*proA*$^{+}$ *B*$^{+}$ *lacI*^{q} *ZΔM15::Tn10* (Tc^{R})]	Novagen
BL21(DE3)	F^{-} *ompT hsdS*$_B$(r_B^{-} m_B^{-}) *gal dcm* (DE3)	Novagen
Plasmids		
pBluescript SK (+)	DNA cloning vector; Amp^{R}	Fermentas
pET30Xa/LIC	Bacterial expression vector designed for ligation independent cloning and high-level expression of target proteins fused with His-tag and S-Tag under T7 *lac* promoter; Kan^{R}	Novagen
pET30a (+)	Bacterial expression vector with the same features as pET30 Xa/LIC but not does not offer ligation independent cloning (LIC); Kan^{R}	Novagen
pBS-acd	pBluescript SK (+) carrying a putative *acdS* gene from tomato in the *Eco*RV site (Accession # EU639448)	This study
pRKLACC	Broad host range plasmid pRK415 with the *acdS* gene from *Pseudomonas putida* UW4, under the control of the *lac* promoter; Tc^{R}	(Holguin and Glick, 2001)
ET30-acd (*)	Plasmid pET30Xa/LIC with a putative tomato *acdS*; the recombinant protein contains an N-terminal 6xHis-tag; Kan^{R}	This study
ET30-Tm1 (▪)	Derivative of (*) containing the point mutation S358E	This study
ET30-Tm2	Derivative of (*) containing the point mutation T386L	This study
ET30-Tm3	Derivative of (▪) containing the point mutation T386L	This study
ET30-UW4 (**)	Plasmid pET30a (+) with the *Pseudomonas putida* UW4 *acdS* gene cloned directionally into *Eco*RV and *Hind*III sites; Kan^{R}	This study
ET30-Um1 (▪▪)	Derivative of (**) containing the point mutation E295S	This study
ET30-Um2	Derivative of (▪▪) containing the point mutation L322T	This study

acdS=ACC deaminase gene

as described below (Sambrook and Russell, 2001).

2.3.1 Formaldehyde Gel Electrophoresis

To determine RNA integrity and to show that no degradation of the sample had occurred prior to downstream applications and use, the method of denaturing formaldehyde gel electrophoresis was utilized. Prior to the preparation of the gel, the electrophoresis apparatus including the combs and trays were soaked in 10% (w/v) SDS solution (prepared in DEPC water), then rinsed with DEPC water to ensure RNase free conditions. All necessary solutions were prepared using only DEPC treated water; any necessary equipment, such as glassware was baked at 250°C overnight.

To prepare a 1% (w/v) mini gel, 0.5 g of agarose was added to 5 ml of 10X MOPS buffer (0.2 M 3-[N-morpholino]propanesulfonic acid (MOPS), 50 mM sodium acetate, 10 mM EDTA, pH 7.0) and 36 ml of water; it was melted in a microwave oven and then cooled to 60°C. In the fume hood, 9 ml of 37% formaldehyde solution was added, mixed and the gel was poured; it was left in the fume hood to polymerase for at least 30 min. When preparing the RNA samples to be analyzed, the following was mixed in an Eppendorf tube: 7 µl of RNA (as little as 2 µg of RNA can be visualized), 3 µl of 10X MOPS, 5 µl of 37% formaldehyde, 15 µl of deionized formamide and 0.5 µl of 0.5 µg/µl ethidium bromide. The samples were heated at 65°C for 5 minutes and cooled on ice immediately. Three µl of loading buffer (50% glycerol (v/v), 1 mM EDTA, 0.25% bromophenol blue (w/v), 0.25% (w/v) xylene cyanol) was added and the samples loaded. The gel was run in 1X MOPS at a constant voltage of 100V. Once the dye front had traveled at least 3 cm, the gel was examined under UV light. RNA integrity was assessed by the appearance of major rRNA bands. For un-degraded RNA these bands exhibit little tailing and the upper band appears at

least as bright as the lower one. RNA from leaf tissue contains several smaller rRNAs in addition to the two major bands.

2.4 Primers and Oligonucleotides

All oligonucleotides used for RT-PCR, PCR, DNA sequencing or site-directed mutagenesis were purchased from Sigma-Aldrich, Canada. The sequences of the oligonucleotides that were used in this study are given in Table 2. Restriction sites in the corresponding primers are highlighted in light grey, start and stop codons are given in bold; mutated codons in primers used for mutagenesis are underlined; fwd = forward primer; rev = reverse; sequence in italics indicates pET30 Xa/LIC complementary sequence necessary for ligation independent cloning. Where indicated as "5' P, PAGE" primers where phosphorylated at 5' end and PAGE purified.

2.5 Cloning of Putative ACC deaminase from *S. lycopersicum* (tomato)

2.5.1 Primers

To obtain the putative ACC deaminase cDNA from tomato, the primers F(BT013578) and B(BT013578)-1 were used (Table 2). Both the forward and reverse primer sequences were based on the DNA sequence from GenBank with the accession number BT013578. This sequence has not yet been annotated, but it shares sequence similarity to other putative ACC deaminase genes from plants and has some sequence similarity to bacterial ACC deaminase genes. BT013578 is a 1492 bp cDNA sequence initially isolated from tomato fruit, with a predicted open reading frame (ORF) of 1254 bp. The above primers were designed based on the sequence outside the predicted ORF. Since there was limited sequence information based

Table 2-2. Primers and oligonucleotides used in this work.

Name	Sequence	Description
F(BT013578)	5'-GAAAGGGGAAAGGTTAAAAATGTCG-3'	BT013578 specific; outside ORF, fwd
B(BT013578)-1	5'-CTCAACTAGTCTTGGATGTAGGC-3'	BT013578 specific; outside ORF, rev
XaSense2	5'-*GGTATTGAGGGTCGC***ATG**TCGAGTTGCCAATGGAGTAG-3'	pET30Xa/LIC cloning, fwd
XaAntisense2	5'-*AGAGGAGAGTTAGAGCC***TCA**GAACATTTTGCCGATGCC-3'	pET30Xa/LIC cloning, rev
Primer1	5'-GCAATTAGGGAATTGGAGC-3'	BT013578 expression, fwd
Primer3	5'-GAGCTAACTCCAGCAGTGATT-3'	BT013578 expression, rev
Primer4	5'-ATTGTTGTAGCTTGTGGCAGTTTC-3'	BT013578 expression, fwd
Primer5	5'-GGATCGTCGCAGACACAAAATGC-3'	BT013578 expression, rev
UW4-F-EcoRV	5'-ATGATATC**ATG**AACCTGAATCGTTTTGAACG-3'	UW4 ACCD, fwd, *Eco*RV
UW4-R-HindIII	5'-ATAAGCTT**TCA**GCCGTTGCGAAACAGGAAGC-3'	UW4 ACCD, rev, *Hind*III
Tomato-M1-F	5'-TGACCCTGTCTACGAAGGTAAAGCAGCTT-3'	ET30-Tm1 S358E, fwd; 5' P, PAGE
Tomato-M1-R	5'-AGAATAACACCTGTGGTTTCAGCAACTTG-3'	ET30-Tm1, S358E, rev; 5' P, PAGE
Tomato-M2-F	5'-TCTGTTCATACACCTGGGTGGGCTACTAG-3'	ET30-Tm2 T386L, fwd; 5' P, PAGE
Tomato-M2-R	5'-ATCTTTCTTCCCTCCCACTTTGTTGGATT-3'	ET30-Tm2, T386L, rev; 5' P, PAGE
UW4-M1-F	5'-CGATCCGGTCTACAGCGGCAAATCCATGC-3'	ET30-Um1, E293S, fwd; 5' P, PAGE
UW4-M1-R	5'-GTCAACACCCCCTCAAGACTGCCGCACAG-3'	ET30-Um1 E293S, rev; 5' P, PAGE
UW4-M2-F	5'-TCTTTATGCCCACACCGGCGGCGCACCTG-3'	ET30-Um2, L322T, fwd; 5' P, PAGE
UW4-M2-R	5'-ACTTTGGAGCCGTCAGGGAATTCCCCGCG-3'	ET30-Um2, L322T, rev; 5' P, PAGE
F20	5'-CCTTGACTGCTATCTCATCTTACGC-3'	Tomato gene sequencing, fwd
F23	5'-TTGACCGTTTAGTTGGAGCACAC-3'	Tomato gene sequencing, fwd
F29	5'-AACCACAGGTGTTATTCTTGAC-3'	Tomato gene sequencing, fwd
F31	5'-TTGACGGAATCACTGCTGGAG-3'	Tomato gene sequencing, fwd
B29	5'-CACTTTGTTGGATTCTCGCCC-3'	Tomato gene sequencing, rev
UW4 Seq 1	5'-TATGGAGGGCTCGGGTTTGTCGGCTTC-3'	UW4 ACCD sequencing, fwd
UW4 Seq 2	5'-CGAAATCACTGAAGAGGATGTGGTGC-3'	UW4 ACCD sequencing, fwd

on which of the primers could be designed, and as these regions of the gene sequences typically tend to have a large number of repeats, especially in the 3' end where the poly-adenylation signal is located, optimization of primers and their position was limited. Instead, the polymerase chain reaction (PCR) was optimized to isolate the cDNA of interest, as described below.

2.5.2 Reverse-transcriptase PCR (RT-PCR)

The reverse transcription reaction was carried out using the Invitrogen SuperScript III reverse transcriptase and buffers (Burlington, ON). The following components were combined on ice: 10 µl 2X RT reaction mix (containing oligo$(dT)_{20}$, random hexamers, $MgCl_2$, and dNTPs in a buffer formulation that has been optimized by the manufacturer), 2 µl RT enzyme mix (200 U/µl reverse transcriptase and RNaseOUT™ Recombinant Ribonuclease Inhibitor), and 1 µg of RNA from leaf tissue, with a final volume, using DEPC treated water of 20 µl. The reaction was gently mixed and the tube contents were incubated at 25°C for 10 min. Following that, the reaction was incubated at 42°C for 50 min and then terminated at 85°C for 5 min and chilled on ice for 5 min. One µl (2 U) of *E. coli* RNase H was added to the mixture and incubated at 37°C for 20 min. A negative control reaction without the Invitrogen SuperScript III reverse transcriptase was done to determine if there was DNA contamination in the extracted RNA sample. The samples were used immediately for PCR.

Hot-start PCR was performed with the KOD Hot Start DNA Polymerase (Novagen, Mississauga, ON). The 50 µl reaction was set up on ice and included: 5 µl of 10X PCR buffer (1X final), 5 µl dNTP (final 0.2 mM), 2 µl of $MgSO_4$ (final 1 mM), 1 nmol of each forward and reverse primers, 2 µl of reverse transcription reaction as template, 1 µl (1U)

KOD Polymerase and 30 µl of sterile water. PCR was performed in the PTC-100™ Programmable Thermal Controller (MJ Research Inc., Watertown, MA, U.S.A.) using the following amplification conditions:

94°C for 5 min
94°C for 30 sec
55°C for 30 sec } 35 cycles
72°C for 40 sec

2.5.3 Cloning into pBluescript SK (+) and Gene Sequence Analysis

The PCR products from the above reactions were separated by gel electrophoresis on a 0.7% (w/v) agarose gel. The band of interest was excised from the gel and purified using a QIAgen-QIAquick Gel Extraction Kit (Mississauga, ON) as described in the "Other general protocols" section. Next, the fragment was cloned into the pBluescript SK(+) plasmid; since the KOD Pol produces blunt ends, the plasmid DNA was digested with a blunt ended cutter, *Eco*RV (Fermentas, Burlington, ON) by incubating the reaction at 37°C for 2 h in the appropriate buffer, then terminating by incubation at 65°C for 20 min. To promote efficient cloning, a vector to DNA insert ratio of 1:3 was used. The amount of vector DNA after digestion and the amount of insert post extraction from the gel was estimated by running another DNA gel and comparing the band intensities to the DNA ladder. The ligation reaction was set up using a Rapid DNA Ligation Kit (Fermentas, Burlington, ON) in the following manner: 145 ng of insert, 68 ng of digested plasmid DNA, 4 µl of 5X rapid ligation buffer, 1µl of T4 DNA ligase (5U) and water for a total reaction volume of 20 µl. The reaction was incubated at 22°C for 5 min, then used to transform *E.coli* DH5α (Invitrogen, Burlington, ON) as described in the "Other general protocols" section.

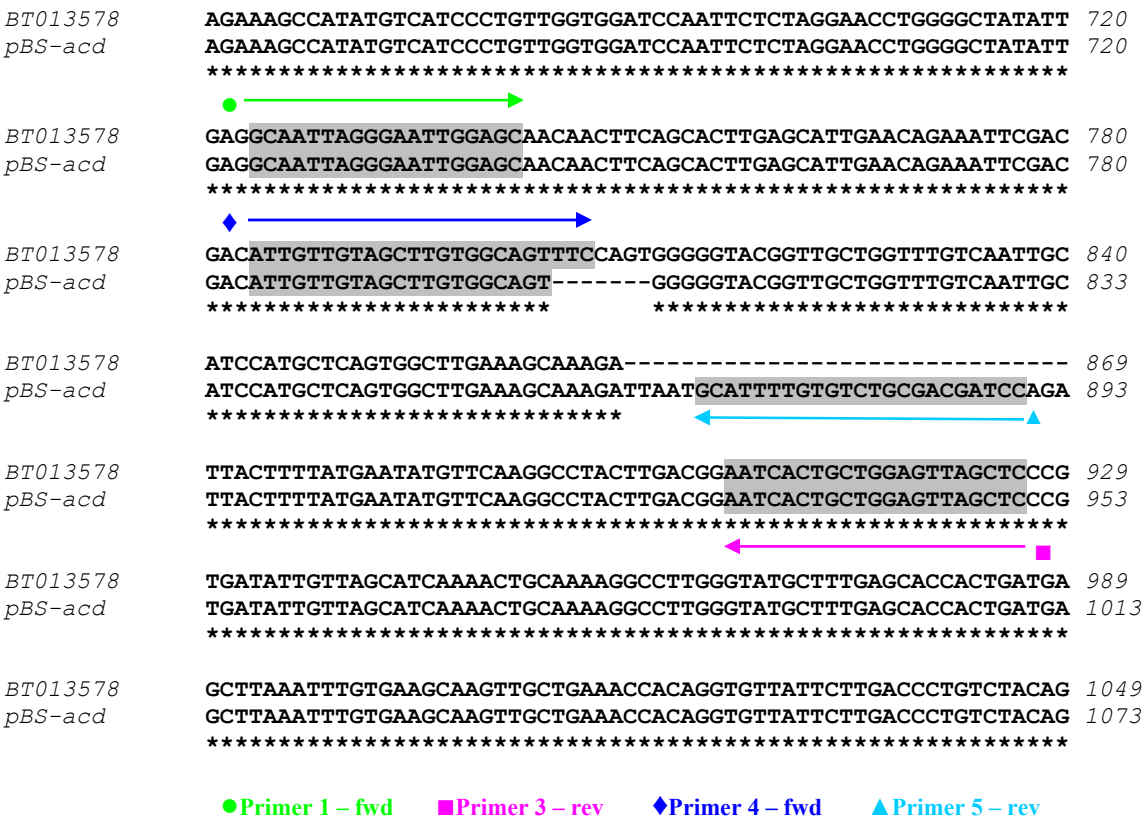
Post-transformation, random white colonies were picked from the plate and screened for presence of the insert and tested to determine the insert orientation. Plasmid DNA was extracted from these colonies (refer to "Other general protocols" section), and digested with *Bam*HI (Fermentas, Burlington, ON), then subject to gel electrophoresis. When the insert is not present, only one band is visible on the gel since pBluescript SK (+) has one *Bam*HI recognition sequence in its multiple cloning site (MCS). However, when the insert is present, two bands are visible. The insert was sequenced at the York University Core Molecular Biology and DNA Sequencing Facility (York University, Toronto, ON). This construct is referred to as pBS-acd; the isolated cDNA sequence has been deposited to GenBank with the Accession # EU639448.

2.6 Determining Whether BT013578 is Expressed

Since the putative ACC deaminase sequence that was isolated from tomato differs from the published sequence in GenBank (BT013578) in the region between ~780 bp and 890 bp by having a 7 bp gap and an "extra" 31 bp of coding region (Figure 2-1), a PCR strategy was devised to test whether BT013578 version is expressed in Bony Best variety of *Solanum lycopersicum*.

2.6.1 Rationale for Primer Design

Differences between the pBS-acd insert sequence (EU639448) and the BT013578 sequence were exploited to determine what is expressed. Two primers, Primer 1 and Primer 3, were designed to be used as positive controls, which would bind both pBS-acd (EU639448) and BT013578 cDNA. Primer 4 was designed to specifically bind BT013578 in



1(●)+3(■) – positive control; amplifies both (if pBS-acd then 227 bp product; if BT013578 then 203 bp)
4(♦)+3(■) – if amplified, then BT013578 is expressed; product of 143 bp
1(●)+5(▲) – if amplified, then pBS-acd is expressed; product of 167 bp

Figure 2-1. A portion of the alignment between the pBS-acd cDNA (EU639448) and the BT013578 cDNA where the sequence difference between the two occurs. Numbers to the right of the alignment represent base pair positions. Gray shading and arrows indicate primer positions. Description below the alignment explains the three different RT-PCR reactions that were set up for each of the templates (leaf, breaker fruit, pBS-acd plasmid DNA) when testing for the expression of BT013578 in Bony Best variety of tomato.

the region where pBS-acd contains the 7 bp gap. In order for DNA polymerase to perform the reaction, a primer at its 3' hydroxyl end must be attached to the template. If BT013578 is expressed, when Primer 4 and Primer 3 are used in the PCR reaction, a product of 143 bp would be observed; on the other hand, pBS-acd (EU639448) would not be amplified at all in this reaction since the Primer 4 hydroxyl end would not be attached. Furthermore, to confirm the expression of the isolated cDNA (pBS-acd (EU639448)), the second difference between the two sequences was utilized. Primer 5 was designed as the reverse primer that binds in the region which only occurs in the pBS-acd cDNA (the "extra" 31 bp region). This third reaction would be set up with Primer 1 and Primer 5; when the pBS-acd cDNA is selectively amplified using these two primers a product of 167 bp is observed (Figure 2-1).

2.6.2 RT-PCR

Two reverse transcription reactions were carried out as previously described (Section 2.5.2) using RNA extracted from leaf tissue as well as from breaker fruit. The PCR reactions were performed using the GoTaq® Green Master Mix (Promega, Madison, WI, U.S.A.); each 25 µl reaction was set up with 12.5 µl of Master Mix, 1 µl of each of forward and reverse primers (from 20 µM stock), 1 µl of reverse transcription reaction or 1 ng of pBS-acd plasmid DNA as the template and sterile water for a total of 25 µl. A total of nine reactions were set up, three for each template (leaf, breaker fruit and the pBS-acd plasmid DNA) and each with the above described primers (Figure 2-1). PCR was carried out in a PTC-100^{TM} Programmable Thermal Controller (MJ Research Inc., Watertown, MA, U.S.A.) using the following amplification conditions:

95°C for 2 min
95°C for 30 sec
55°C for 30 sec } 35 cycles
72°C for 30 sec

Three µl of each amplification reaction was examined on a 3% (w/v) agarose gel. The product sizes were determined using AlphaEaseFC (Alpha Innotech Corporation, San Leandro, CA, U.S.A.) software and its built-in feature for determining molecular weight based on the gel mobility of the fragments in the DNA ladder.

2.7 Protein Expression

2.7.1 Sub-cloning into the Expression Vector pET30 Xa/LIC

For the purposes of protein expression, the construct called pBS-acd was used as the template to subclone the cDNA into the pET30 Xa/LIC (Novagen, Mississauga, ON) expression vector. pET30 Xa/LIC is provided by the manufacturer as a linear double stranded molecule with sticky ends designed for ligation independent cloning. Briefly, when amplifying the gene of interest for cloning into pET30 Xa/LIC, the primers must be designed such that their 5' end contains the complementary sequence to that of the sticky end of the vector. This allows for fast, directional and efficient cloning of the insert into the vector without the need for restriction enzyme digestion or ligation.

Forward primer XaSense2 and reverse primer XaAntisense2 (Table 2) were used in the PCR reaction to prepare the insert for cloning into pET30 Xa/LIC. The reaction was set up with KOD Hot Start DNA Polymerase (Novagen, Mississauga, ON): 5µl of 10X PCR buffer (1X final), 5 µl dNTP (final 0.2 mM), 2 µl of $MgSO_4$ (final 1 mM), 2.5 µl of each forward and reverse primers (1 nmol of each), 1 ng of pBS-acd plasmid DNA as the template, 1 µl (1U) KOD Polymerase and 30 µl of sterile water. PCR was completed in the

PTC-100™ Programmable Thermal Controller (MJ Research Inc., Watertown, MA, U.S.A.). using the following amplification conditions:

94°C for 5 min
94°C for 30 sec
51°C for 30 sec } 35 cycles
72°C for 40 sec

Following PCR, the entire reaction was loaded on a 0.7% agarose gel and the band was excised from the gel and purified using the QIAgen-QIAquick Gel Extraction Kit (refer to "Other general protocols" section). The concentration of the PCR product after purification was determined by running another DNA gel and comparing the band intensity to the intensities of the bands that are part of the DNA ladder.

To prepare the PCR product for cloning into pET30 Xa/LIC, the product was treated with T4 DNA Polymerase. Two reactions were assembled in the following manner: 0.2 pmol of purified PCR product (or β-galactosidase gene; positive control provided by the manufacturer), 2 µl of 10X T4 DNA Pol buffer, 2 µl of 25 mM dGTP, 1 µl of 100 mM DTT, 0.4 µl T4 DNA Pol (from 2.5 U/µl stock) and water up to 20 µl in total. The reaction was started with the addition of the enzyme; it was gently stirred with a pipette tip and incubated at 22°C for 30 min. Then, T4 DNA polymerase was inactivated by incubation for 20 min at 75°C. The prepared insert was used immediately in subsequent steps, but it could be stored at -20°C for up to several months.

The vector to insert annealing reaction was performed by combining 1 µl of pET30 Xa/LIC vector (Xa/LIC Cloning Kits, Novagen, Mississauga, ON) with 2 µl of T4 DNA polymerase treated insert (0.02 pmol) then incubating the mixture at 22°C for 5 min. 1 µl of 25 mM EDTA was added for a total of 4 µl reaction volume. The mixture was further incubated at 22°C for 5 min; 1 µl of the annealing reaction was used directly to transform *E.*

coli NovaBlue GigaSingles as described in the "Other general protocols" section. The colonies were screened by extracting plasmid DNA and determining the plasmid size. Prior to protein expression, the insert was also sequenced (York University, Toronto) to determine if any polymerase induced changes had occurred. Moreover, plasmid DNA which had been confirmed as having no polymerase induced changes was used to transform the protein expression host, BL21(DE3) following the standard transformation protocol. This strain is referred to as ET30-adc.

2.7.2 Optimization of Target Protein Expression

To determine whether the recombinant protein is expressed and what the optimum IPTG concentration is, a small scale induction protocol was utilized along with sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) analysis. Three ml of LB containing kanamycin at 30 µg/ml was inoculated with ET30-acd and incubated overnight at 37°C with shaking. The following day, 50 ml of fresh LB with kanamycin was inoculated with the entire 3 ml of the overnight culture and incubated at 37°C with shaking until the cells reached mid-log phase (OD_{600} of ~0.5). One ml of culture was sampled as an un-induced control. The cells were collected by centrifugation for 1 min at 14,000 rpm (20,798 x g) and the pellet was re-suspended in 100 µl of 1X SDS-PAGE gel loading buffer (100 mM Tris-Cl, pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 200 mM DTT). The sample was stored at -20°C until used for SDS-PAGE analysis. The remainder of the culture was induced with 0.1, 0.4 or 1.0 mM IPTG. The induction was continued at 30°C with sufficient shaking to provide aeration. One ml sample of the culture was collected at various time points (following 1, 3, 4, 6 h of induction) and processed/suspended as described above. Once all the samples were collected, they were thawed on ice, warmed to

room temperature and 0.15OD_{600} units of the original culture was examined on a 10% (w/v) SDS-PAGE gel (refer to "Other general protocols").

2.7.3 Determining the Solubility of Expressed Protein

To determine if the recombinant protein is expressed in soluble form or if it forms aggregates and inclusion bodies, the following procedure was used. Five ml from a 10 ml overnight culture was used to inoculate 50 ml of fresh LB with kanamycin (30 µg/ml). The culture was grown to mid-log phase (OD_{600} ~0.5) at 37°C at which point a 1 ml sample was collected and the cell pellet was re-suspended in 50 µl of 1X SDS-PAGE sample buffer (un-induced sample). The sample was stored at -20°C, and the remaining culture induced with 0.1 mM IPTG for 5 h at 30°C. After the induction period, another 1 ml sample was collected, and the remaining culture was harvested by centrifugation at 4,000 x g for 20 min at 4°C. The collected cell pellet was re-suspended in 5 ml of lysis buffer for native purification (50 mM NaH_2PO_4, 300 mM NaCl, 10 mM imidazole); the cells were lysed by adding 1 mg/ml lysozyme and incubating on ice for 30 min. Afterwards, the suspension was sonicated 6X for 10 sec with 10 sec pauses at 200-300 W. The lysate was kept on ice at all times; it was centrifuged at 10,000 x g for 30 min and 4°C. The supernatant was saved on ice, representing the soluble fraction, whereas the pellet was re-suspended in another 5 ml of lysis buffer, and represents insoluble protein and cellular debris. The un-induced, induced, soluble and insoluble fractions were examined by SDS-PAGE.

2.8 Protein Purification

2.8.1 Culture Preparation

Fifty ml of the overnight culture grown at 37°C was used to inoculate 700 ml of fresh LB with kanamycin (30 μg/ml). The freshly inoculated culture was grown at 30°C with vigorous shaking for 1 h, at which point the OD_{600} of the culture was ~0.4-0.5. The culture was induced with 0.1 mM IPTG for 6 h at 30°C; to harvest the cells, the culture was centrifuged at 8,000 rmp (9,715 x g) for 15 min at 4°C using a Sorvall centrifuge and a SLA-1500 rotor. The collected pellet was stored at -20°C overnight, or if frozen for longer periods of time, then at -80°C.

2.8.2 Purification

The recombinant protein, expressed with an N-terminal 6X His-tag was purified under native/non-denaturing conditions using Ni-NTA Superflow resin (QIAgen Inc., Mississauga, ON). Briefly, the pellet was suspended in 4 ml of lysis buffer (Table 3), after being thawed on ice for ~15 min (or quickly thawed in a 35°C waterbath for 3-4 min). To lyse the cells, lysozyme was added at a concentration of 1 mg/ml; the suspension was incubated on ice for 30 min, and then sonicated 6X for 10 sec with 10 sec breaks at 200-300 W. To obtain the soluble fraction that would be applied to the column, the sonicated suspension was centrifuged at 10,000 x g for 30 min at 4°C. The supernatant was applied to 0.5 ml of prepared Ni-NTA resin (1 ml of 50% slurry was carefully poured into disposable 11 ml columns, and the resin was allowed to settle; it was then washed with water to remove any ethanol, then 10 column volumes of lysis buffer) and was allowed to pass through the column using gravity flow. After the supernatant passed through the column, the column was

washed with wash buffers of gradually increasing imidazole concentrations. Wash 1 and wash 2: 4 ml of lysis buffer; wash 3 and wash 4: 4 ml of 20 mM imidazole wash buffer; wash 5: 4 ml of 40 mM imidazole wash buffer; wash 6: 4 ml of 50 mM imidazole wash buffer. The recombinant protein was eluted off the column with 4X 0.5 ml elution buffer.

Table 2-3. Composition of buffers used for purification of the recombinant protein(s) on Ni-NTA column.

	Lysis buffer	20 mM wash buffer	40 mM wash buffer	50 mM wash buffer	Elution buffer
NaH_2PO_4	50 mM	50 mM	50 mM	50 mM	50 mM
NaCl	500 mM	1 M	1 M	1M	300 mM
Imidazole	10 mM	20 mM	40 mM	50 mM	250 mM
β-mercaptoethanol	20 mM	--	--	--	--
PMSF	1 mM	--	--	--	--
pH 8.0	Yes	Yes	Yes	Yes	Yes

2.8.3 Gel Filtration

After purification using affinity chromatography, fraction(s) containing the protein of interest were used in gel filtration. This method was utilized for the purposes of buffer exchange. In order to store the purified protein and test for activity, the elution buffer was unsuitable; hence, gel filtration was used to obtain the recombinant protein in the final storage buffer (20 mM potassium phosphate, pH 7.0, 15% glycerol, 10 μM PLP, 0.1 mM DTT, 0.1 mM EDTA).

The gel filtration column was packed, tested and calibrated as follows: 10 ml column with Sephadex G-25, fine grade beads (formerly Pharmacia; GE Healthcare Bio-Sciences Inc., Baie d'Urfé, QC) were prepared by swelling 2.5 g of Sephadex G-25 powder in 13.5 ml of 0.1 M Tris-Cl, pH 8.0. The mixture was incubated for 1 h at 90°C, and then cooled to room temperature. It was poured carefully in one motion, avoiding air bubbles, into an empty

column of 1 cm in diameter. The column was then calibrated using 100 µl of dextran blue solution (0.025 g dissolved in 2 ml of 50% (v/v) ethanol solution; filtered), which determined the void volume of the column. The packed column was further calibrated with a 2 mg/ml BSA solution, prepared in elution buffer. The point at which the protein elutes, as well as the fraction where salt from elution buffer elutes from the column was monitored by testing each fraction for protein content and conductivity. When not in use, the column was thoroughly washed then stored in 20% (v/v) ethanol.

Prior to applying the sample and exchanging the buffer, the gel filtration column (10 ml of resin in a 1 x 12.75 cm column) was equilibrated with at least 20 ml of the final storage buffer. Either 0.5 or 1 ml of sample (depending upon where the recombinant protein eluted from the Ni-NTA column) was slowly and carefully applied to the column; once the sample entered the column (i.e. no liquid above the column was left), 10 ml of storage buffer was applied. When ~2 cm of the buffer was left above the column, another 10 ml of buffer was applied. The samples were collected in 1 ml aliquots, up to 16 ml, to ensure that no protein would be lost in case it was sticking or interacting with the column. The fractions were tested for protein concentration, and those containing the recombinant protein were aliquoted and stored at -20°C. The recombinant protein was frozen and thawed only once prior to all downstream analysis (activity, pH and temperature optima curves).

2.8.4 Mass Spectrometry

To determine which proteins had co-purified with the protein of interest, mass spectrometry was utilized. After running the samples on an SDS-PAGE gel, the spots of interests were excised from the gel; the gel pieces were washed with water and destained with 50 mM NH_4HCO_3/50% (v/v) acetonitrile (ACN). Proteins were reduced by incubation

with 10 mM DTT in 100 mM NH_4HCO_3 at 50°C for 30 min, and then alkylated by incubating with 55 mM iodoacetamide in 100 mM NH_4HCO_3 for 30 min in the dark. After being dehydrated with 100% ACN and air-dried, the gel pieces were rehydrated for ten minutes in a trypsin solution (Promega, Madison, WI, U.S.A.) in a ratio of approximately 1:10 (w/w) of trypsin:protein. Fifty µl of 50 mM NH_4HCO_3 (pH 8.0) was added to the gel pieces and the proteins were digested at 37°C for 18 hours. The peptides were extracted by vortexing and then concentrated to 10 µl in a Savant SpeedVac. The samples were cleaned using the C-18 ZipTip system (Millipore, Billerica, MA, U.S.A) and eluted with 5 µL of 50% ACN. One µL of 1% formic acid was added to the eluate to protonate the peptides.

Mass spectrometry was performed with a Waters Micromass Q-TOF Ultima using nano-spray injection as the sample delivery method. Proteins were identified either by the PEAKS software 3.1 (Ma et al., 2003a) (Bioinformatics Solutions Inc., Waterloo, ON), which combines auto *de novo* sequencing and database searching, or by the MASCOT peptide-fingerprinting algorithm (Perkins et al., 1999).

2.8.5 Densitometry

Subsequent to SDS-PAGE analysis of gel filtration samples, densitometry was performed to determine the purity of recombinant protein. TotalLab TL100 Control Center (Nonlinear Dynamics, Durham, NC, U.S.A.) software and its 1D gel analysis tool were used. The background was subtracted by placing a manual baseline; Band% value from the Management Window was used as the value for percent purity of the recombinant protein.

2.9 Enzymatic Characterization

2.9.1 Activity

The recombinant protein was tested for two activities: ACC deaminase activity and D-cysteine desulfhydrase. Both assays were performed in the same manner, using a lactate dehydrogenase (LDH) linked protocol (Figure 2-2). For the initial activity test, the reactions were set up using 0.1, 1 and 10 mM D-cysteine or ACC as substrates. The 250 µl reactions were set up in a 96-well plate as follows: 50 mM potassium phosphate, pH 8.0, 0.13 mM NADH, 5 U of LDH from rabbit muscle and 5 µl of purified protein; to initiate the reaction each substrate was added. The mixture was pipetted quickly to mix the contents, placed at 37°C and the change in absorbance at 340 nm was monitored immediately. For calculation purposes, the NADH molar absorption coefficient of 6.22 x $l0^3$ M^{-1} cm^{-1} at 340 nm was used. When shown, the error bars represent standard error.

2.9.2 Km Determination

To determine the Km value for the recombinant protein, D-cysteine was used as the substrate at concentrations of 0.02, 0.03, 0.05, 0.1, 0.2, 0.5, and 1 mM. At each concentration, the reactions were set up as previously described, each containing five replicates. Therefore, the Km of the enzyme was determined at 37°C and pH 8.0.

2.9.3 pH Optima

The reactions were carried out in the following buffers: phosphate at pH 6.2 – 8.0, pyrophosphate at pH 7.6 – 9.0, and glycine at pH 9.0 – 10.4. Five µl of purified recombinant protein, 1 µl of D-cysteine (from 50 mM stock; 0.8 mM final) and 54 µl of each

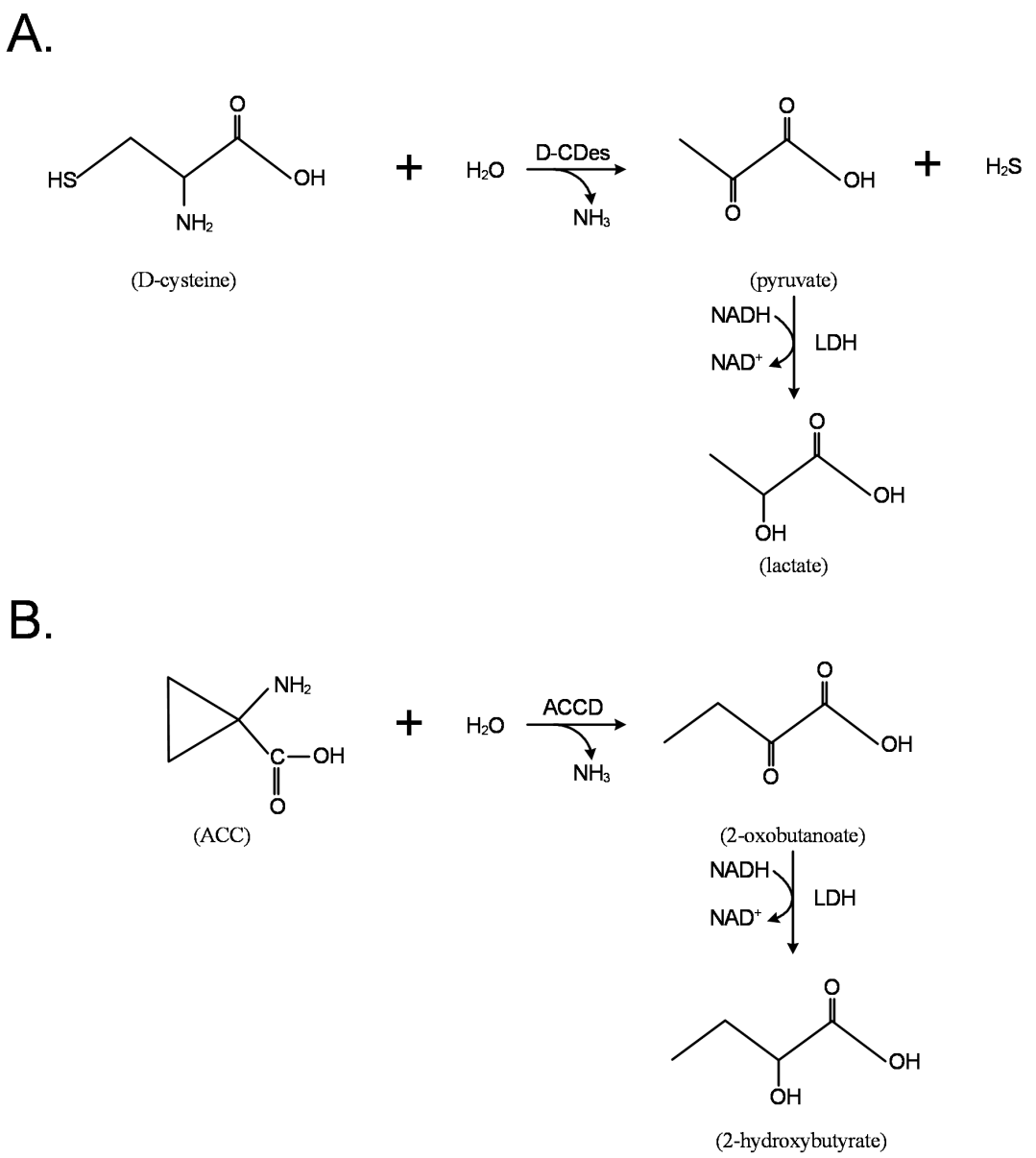


Figure 2-2. D-cysteine desulfhydrase and ACC deaminase reactions linked to LDH. *A*: D-cysteine desulfhydrase (D-CDes) utilizes D-cysteine as the substrate, breaks it down to pyruvate, ammonia and hydrogen sulfide. In presence of lactate dehydrogenase (LDH), D-CDes activity is monitored by a decrease in the absorbance at 340 nm as LDH converts pyruvate into lactate. In the process of the LDH reaction, the co-enzyme NADH is also converted to NAD; NADH, but not the NAD has an absorption peak at 340 nm. *B*: The ACC deaminase (ACCD) reaction converts ACC into 2-oxobutanoate (or α-ketobutyrate), which in presence of LDH, is converted into 2-hydroxybutyrate. As the LDH reaction proceeds, a decrease in the absorbance at 340 nm is observed due to the consumption of NADH.

representative buffer was incubated at 37°C for 15 min. Subsequently, the pyruvate concentration was determined by adding 900 µl of 0.56 N HCl and 150 µl of 0.2% (w/v) 2,4-dinitrophenylhydrazine (DNP; in 2 N HCl). The mixture was then incubated at 30°C for 30 min; 1 ml of 2 N NaOH was added and the absorbance determined at 540 nm. Reactions for each pH point were set up in four replicates. For a representative pyruvate standard curve, please refer to the Appendix.

2.9.4 Temperature Optima

All reactions were set up as described for the pH optima; however, these measurements were carried out at the determined pH optimum of 7.6. The temperature dependence of the reaction was tested from 10 -70°C. Only the first 15 min incubation was carried out at the desired temperature; when determining the pyruvate formed the incubation was carried out at 30°C as previously described.

2.9.5 Determining PLP Dependence

The reaction progress was monitored in presence of aminooxyacetic acid (AOA), a known inhibitor of PLP dependent enzymes. The reactions were set up at pH 7.6 in phosphate buffer; the buffer, the enzyme and AOA in the concentration range of 5 to 10 000 µM were incubated for 15 min at 30°C. After the incubation other components of the reaction, as described above, were added and the reaction progress and the absorbance at 340 nm was monitored. Each reaction point was tested in triplicates.

2.10 Three-dimensional Structure Prediction

The nucleotide sequence was translated into protein using the ExPASy Translate tool (http://ca.expasy.org/tools/dna.html), and the reading frame corresponding with the highest

sequence similarity to bacterial ACC deaminase was chosen. Structure prediction of putative ACC deaminase from tomato, shown to lack this activity, has been based on the availability of the three-dimensional models of the yeast *Hansenula saturnus* ACC deaminase (Yao et. al., 2000), PDB code 1F2D, *Pseudomonas* sp. ACP ACC deaminase (Karthikeyan et. al., 2004b), PDB code 1TYZ, as well as the *Pyrococcus horikoshii* ACC deaminase homologue (PH0054) (Fujino et. al., 2004), PDB code 1J0A. These potential templates were chosen with a BLAST search within the non-redundant SWISS-PROT Protein Sequence database and PDB database. However, the *P. horikoshii* protein was selected as the template for prediction of the 3D structure, since it carries the highest sequence similarity to the sequence for tomato enzyme. The multiple sequence alignments were performed using the MUSCLE program (Edgar, 2004), with default parameters, and was edited using BioEdit multiple sequence alignment editor (Hall, 1999). For the modeling procedure, only the sequence region for which the 3D structure of the template is available was considered. As a consequence, the model does not include the first 74 amino acids of the isolated protein, as well as the last 21 amino acids. The secondary structure was predicted with the SOPMA method (Geourjon and Deleage, 1994) which determines the consensus of different prediction methods and does not perform a multiple sequence alignment. PredictProtein server (http://www.predictprotein.org) was used for further secondary structure predictions with PHD (Rost, 1996), as well as to search for motifs with PROSITE (Hofmann et al., 1999). The secondary structure of 3D models has been assigned with the program DSSP through the ENDscript server (Gouet et al., 2003; Kabsch and Sander, 1983). The SWISS-MODEL server (http://swissmodel.expasy.org//SWISS-MODEL.html), along with the program Swiss-pdbViewer (http://www.expasy.org/spdbv/) were used to build the three-dimensional models

according to the comparative protein modeling method and to generate images of all represented proteins. Swiss-pdbViewer was also used for any refinements; energy computations were done with the GROMOS96 implementation in Swiss-pdbViewer. The stereochemical quality of the model was verified with the program PROCHECK (Laskowski et al., 1993). The search for structural classification was performed on SCOP (Murzin et al., 1995) and CATH (Orengo et al., 1997).

2.11 Mutagenesis Studies

2.11.1 Sub-cloning of the *P. putida* UW4 ACC Deaminase into pET30

For consistency purposes and to purify all recombinant proteins with the same approach using the N-terminal 6X His-tag, the gene for *P. putida* UW4 ACC deaminase was sub-cloned into the pET30 vector. Using PCR, the construct pRKLACC (Holguin and Glick, 2001) as the template and primers UW4-F-EcoRV and UW4-R-HindIII (Table 2), the gene was amplified to contain *Eco*Rv and *Hind*III restriction enzyme sites. The PCR reaction was carried out with the KOD Hot Start DNA Polymerase (Novagen, Mississauga, ON). The 50 µl reaction was set up on ice: 5µl of 10X PCR buffer (1X final), 5 µl dNTP (final 0.2 mM), 2 µl of $MgSO_4$ (final 1 mM), 1 nmol of each forward and reverse primers, 1 ng of template plasmid DNA, 1 µl (1U) KOD Polymerase and 30 µl of sterile water. PCR was performed in the PTC-100^{TM} Programmable Thermal Controller (MJ Research Inc., Watertown, MA, U.S.A.) using the following amplification conditions:

94°C for 5 min
94°C for 30 sec
55°C for 30 sec } 35 cycles
72°C for 40 sec

Following PCR, the product was cleaned with the QIAqick PCR Purification Kit (please see "Other general protocols section"), digested with the appropriate restriction enzymes and ligated into already digested pET30 vector, in a similar fashion as described previously. The DNA sequence of the insert was confirmed by DNA sequencing analysis.

2.11.2 Site-directed Mutagenesis

ET30-Tm1, ET30-Tm2, ET30-Tm3, ET30-Um1 and ET30-Um2 mutants were created by specifically mutating one or two amino acid residues, as described in Table 1 using a Phusion Site Directed Mutagenesis Kit (New England Biolabs, Mississauga, ON), and the primers shown in Table 2 (Figure 2-3); note that the primers were phosphorylated and purified using PAGE prior to use. ET30-Tm3 and ET30-Um2, which contain two point mutations, were obtained using ET30-Tm1 and ET30-Um1 as templates, respectively. The other two tomato mutants were constructed using ET30-acd as the template, and ET30-Um1 was obtained when ET30-UW4 was used as the template.

Each PCR reaction was set up and performed by modifying the instructions provided by the manufacturer. The reactions were set up using a higher amount of template (100-250 pg) and a longer extension time than recommended. Briefly, the PCR reactions were set up as follows: 10 µl of 5X Phusion HF Buffer, 1 µl of 10 mM dNTPs, 1.25 µl of each primer (from 20 µM stock), 0.5 µl of template (100-250 pg), 0.5 µl of Phusion Hot Start DNA polymerase (2U/µl) and water for a total of 50 µl. The reactions were cycled as follows:

98°C for 30 sec
98°C for 15 sec
65°C for 20 sec } 25 cycles
72°C for 7 min
72°C for 5 min

Five µl of the PCR reaction was examined using a 0.7% (w/v) agarose DNA gel; the remaining reaction products were purified using the QIAquick PCR Purification Kit, as described in the "Other general protocols" section. After purification, the concentration of DNA was determined using ND-100 (NanoDrop Technologies, Inc., Wilmington, DE, U.S.A.), and 25 ng was ligated as follows: a 10 µl ligation reaction was set up by adding the DNA, 5 µl of 2X Quick Ligation Buffer and 0.5 µl of Quick T4 DNA Ligase (buffers and enzymes provided in the Phusion Site Directed Mutagenesis Kit). The mixture was incubated at room temperature for 15 min and used immediately for transformation. Plasmid DNA was recovered from the potential mutants and sequenced; initially the sequencing was restricted to the region where the mutation occurs, as a screening method. Generally, the mutation efficiency was high, such that on average no more than three potential mutants had to be screen to obtain the desired mutation. The complete sequence for each mutant was obtained thereafter. Multiple sequence alignment of the constructed mutants, for both the tomato and *P. putida* UW4 enzymes is provided in the Appendix.

2.11.3 Protein Purification

The cultures were induced with IPTG and the altered proteins were purified for all mutants as well as for the native *P. putida* UW4 ACC deaminase using the same procedure described previously. For a representative SDS-PAGE gel picture of purified samples for *P. putida* UW4 ACC deaminase, please refer to the Appendix. Contrary to the purification of the tomato enzyme and its mutants, the purification of the UW4 ACC deaminase was achieved to almost 100% purity. Densitometry, but not mass spectrometry was carried out for all samples. The deviation from the previously described protocol included the omission of the gel filtration step, but utilization of a diafiltration protocol to exchange the buffer.

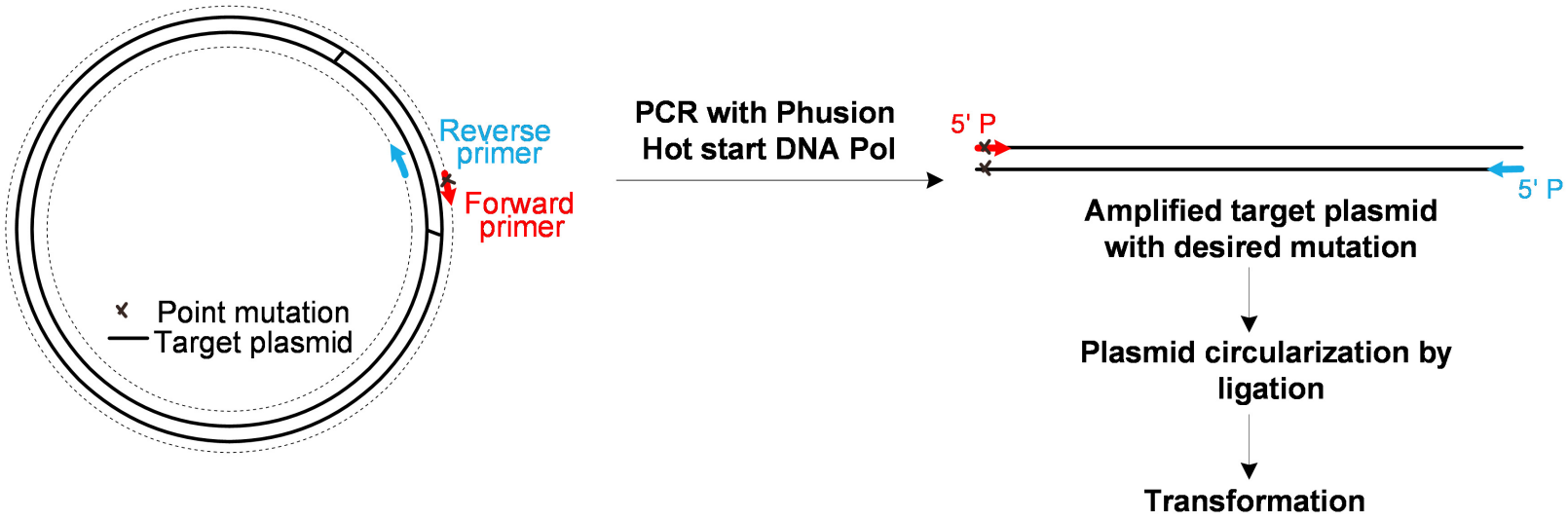


Figure 2-3. Summary of the site-directed mutagenesis protocol. Note that the mutation is introduced in the forward primer. Forward and reverse primers are designed back-to-back and they must be phosphorylated at the 5' end.

Vivaspin 6 (Sartorius Stedim Biotech, Mississauga, ON) columns were used, where 0.5 ml of protein was diluted with 5.5 ml of storage buffer immediately after elution from the Ni-NTA column. Centrifugation was carried out at 4,500 x g and 4°C until 0.5 ml of solution remained in the column. Following that, the protein was diluted again using 5.5 ml of storage buffer and this centrifugation and washing step was repeated twice. The procedure resulted in most of the salt from elution buffer being removed (0.17 mM salt is estimated to be remaining as opposed to 300 mM that is found in elution buffer); the final 0.5 ml of purified protein was aliquoted and stored in storage buffer at -20°C.

2.11.4 Analysis of the Mutants

All mutants were tested for activity towards both ACC and D-cysteine at substrate concentrations that bracketed the known Km values for similar enzymes. The activity assays were carried out as previously described, using the lactate dehydrogenase linked assay. The reported values for ACC deaminase activity are based on the reactions carried out at 10 mM ACC; the values for D-cysteine desulfhydrase activity are based on reactions set up at 0.8 mM D-cysteine. Km determination for the double mutant of UW4 ACC deaminase (ET30-Um2) are based only on the values at 1 mM D-cysteine and below. All reactions were set up in triplicates and also included a negative control without substrate.

2.12 Other General Protocols

The protocols described in this section were used routinely for cloning purposes and protein analysis.

2.12.1 DNA Purification

After certain DNA manipulations such as PCR amplification or restriction endonuclease digestion, QIAquick PCR Purification Kit (Qiagen Inc., Mississauga, ON) was used to remove unwanted primers and enzymes from the DNA samples. The DNA was purified according to the manufacturer's instruction; it was eluted in sterile water instead of Buffer EB, in volumes ranging from 30 to 50 µl. If not otherwise specified, the post-purification DNA concentration as well as the purity was determined with the ND-100 (NanoDrop Technologies, Inc., Wilmington, DE, U.S.A.).

2.12.2 Recovery of DNA from Gels

DNA fragments were recovered from agarose gels using the QIAquick Gel Extraction Kit (Qiagen Inc., Mississauga, ON). The DNA fragment was excised from the agarose gel with a clean, sharp scalpel and the gel slice was weighed in a microtube. Following gel excision, the fragment was purified according to the manufacturer's instructions, where the fragment was eluted from the column with 30 to 50 µl of water. The purity was determined with the ND-100 (NanoDrop Technologies, Inc., Wilmington, DE, U.S.A.).

2.12.3 Transformation

All transformation reactions were carried out using heat shock and $CaCl_2$ competent cells. For instance, up to 10 µl of a ligation mixture (or appropriate amount of plasmid DNA) was added to competent cells that had been previously thawed on ice. The cells were gently mixed, then incubated on ice for 20 min. The cells were then heat shocked at 42°C for 40 sec, and placed on ice immediately for 2 min. While still on ice, LB medium was

added to a total volume of 1 ml. The culture was recovered for 1 h by shaking at 37°C. Depending on the transformation efficiency of the competent cells, up to 200 µl aliquots were plated onto LB solid media with appropriate antibiotics. The plates were incubated at 37°C overnight.

2.12.4 Isolation of Plasmid DNA

Plasmid DNA was extracted from bacterial cells using the Wizard *Plus* SV Minipreps DNA Purification System (Promega, Madison, WI, U.S.A). 5-10 ml of overnight bacterial culture was centrifuged to collect the pellet; isolation was carried out according to the manufacturer's instructions. Plasmid DNA was eluted in 50-100 µl of water.

2.12.5 Determination of Protein Concentration

Protein concentrations were measured according to the instructions from Bio-Rad Laboratories; the assay is based on the method of Bradford (1976). Briefly, the protein BSA standards as well as the samples were assayed in triplicates. The assay was performed in 96 well plates, where 10 µl of either the standard or the appropriately diluted sample was pipetted and 200 µl of diluted Bio-Rad reagent added (diluted with water in the ratio of 1:4 Bio-Rad reagent to water, then filtered). The samples were mixed, and then incubated for 10 min at room temperature; the absorbance at 595 nm was measured. A typical standard curve is shown in the Appendix.

2.12.6 Sodium Dodecyl Sulfate Polyacrylamide Gel Electrophoresis (SDS-PAGE)

Proteins were routinely analyzed on 10 or 12% polyacrylamide gels. A separating gel was prepared by mixing together, in order: 2.0 ml of water, 1.25 ml of 1.5 M Tris-base, pH 8.8, an appropriate volume of 40% acrylamide/bis solution, 50 µl 10% SDS, 50 µl 10% ammonium persulfate and 5 µl of TEMED. The mixture was immediately poured between two glass plates to approximately 1 cm bellow the comb teeth. A thin layer of isopropanol was added to the top of the gel to keep the surface flat and to speed the polymerization process by preventing the gel solution from coming into contact with air and oxygen. After the separating gel had polymerized, the layer of isopropanol was removed and a stacking gel was prepared (1.7 ml of water, 0.3 ml of 1 M Tris-base pH 6.8, acrylamide/bis solution (final 5%), 25 µl of 10% SDS, 15 µl of 10% ammounium persulfate and 2.5 µl of TEMED). The stacking gel was poured on top of the separating gel, and then the comb was inserted immediately. Samples were prepared by mixing appropriate amount of protein, water and SDS-PAGE loading buffer (1X buffer contains 100 mM Tris-Cl, pH 6.8, 4% (w/v) SDS, 0.2% (w/v) bromophenol blue, 20% (v/v) glycerol, 200 mM DTT); these were then incubated in a boiling water bath for 5 min, cooled to room temperature and centrifuged for 30 sec. After polymerization and sample preparation, the samples were loaded and the gel was run in electrophoresis buffer (1 L of 5X buffer consists of 15 g of Tris-base, 72 g of glycine and 5 g of SDS). The gel was run at 100 volts through the stacking gel, then at 140 volts through the separating gel.

Chapter 3
Results

3.1 Cloning of Putative ACC Deaminase

With the progress in the sequencing of plant genomes, including tomato, gene sequences bearing similarity to bacterial ACC deaminases have been identified in plants (*Arabidopsis*, accession # NM_103738; poplar, accession # AI161555; birch, accession # AY154652; rice, accession # BAD16875; corn, accession # AY106365). The tomato sequence (accession number BT013578) shares ~30% identity with bacterial ACC deaminases at the protein level. The plant encoded sequences, however, are more similar among each other, sharing an identity of ~70% or higher. To isolate and clone the putative ACC deaminase from *Solanum lycopersicum*, Bony Best variety, primers F(BT013578) and B(BT013578)-1 (Table 2) were designed such that they would bind outside the predicted open reading frame of the BT013578 sequence; the amplified product would be 1408 bp in length. The cloning procedure was carried out by first reverse transcribing the RNA from leaf tissue, then using the first strand cDNA as the template in a PCR reaction with the above primers. The overall isolation and cloning procedure is illustrated in the Figure 3-1. The amplified fragment was cloned into the *Eco*RV site of pBluescript SK(+), then sequenced from both directions; the insert sequence of the pBS-acd construct is provided in the Appendix.

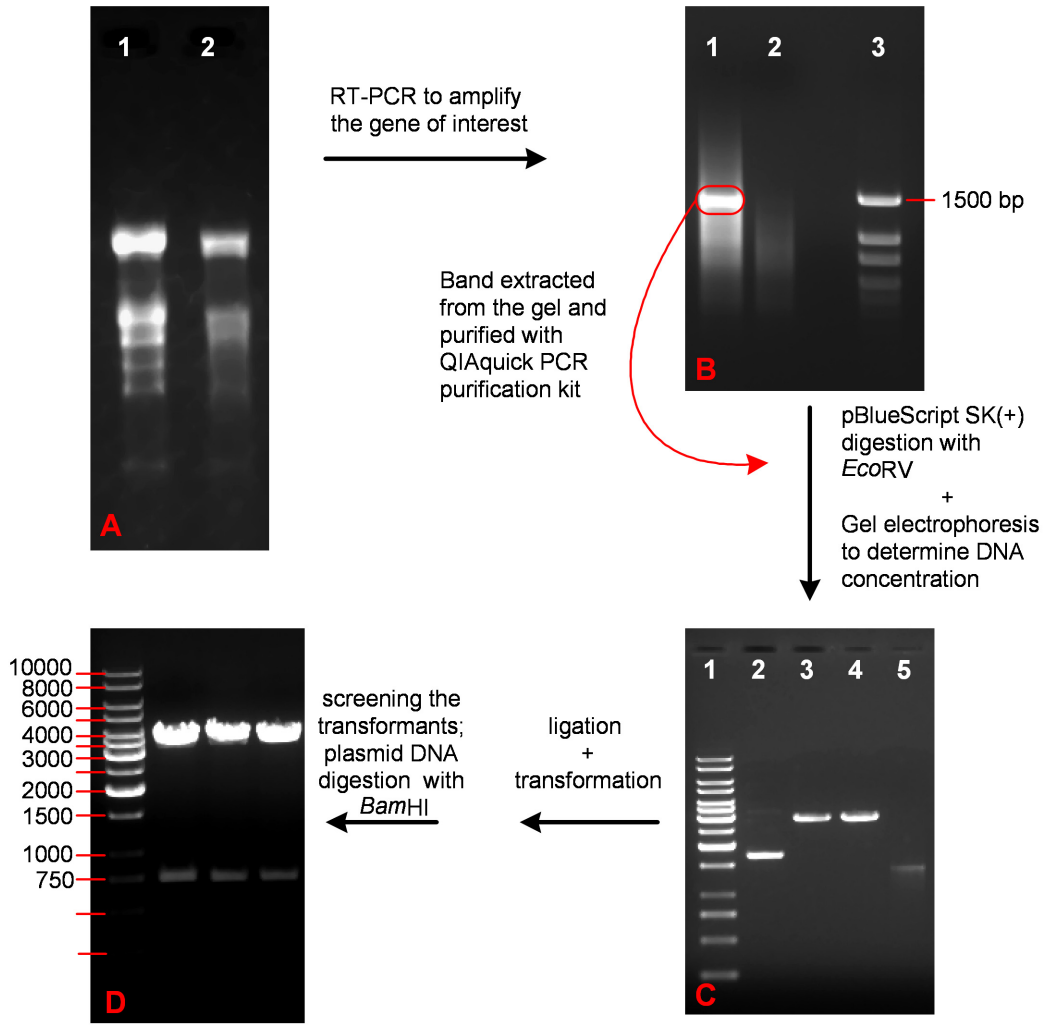


Figure 3-1. Overall cloning procedure to isolate a putative ACC deaminase from *S. lycopersicum*. *A*: formaldehyde RNA denaturing gel; 1: leaf tissue RNA; 2: breaker fruit RNA. *B*: 0.7% DNA agarose gel; 1: entire PCR reaction set up with F(BT013578) and B(BT013578)-1 primers and using cDNA from leaf tissue as the template; 2: negative control; 3: 100 bp ladder (Fermentas, Burlington, ON). *C*: 0.7% agarose gel; 1: 1 kb DNA ladder (Fermentas, Burlington, ON); 2: undigested pBluescript SK(+) plasmid DNA; 3 and 4: *Eco*RV digested pBluescript SK (+) plasmid DNA; 5: purified fragment from B. *D*: 1 kb ladder (Fermentas; same as in C) is marked on the left with the corresponding fragment size in bp; All three lanes are *Bam*HI digested plasmid DNA isolated from *E. coli* colonies carrying the potential construct after ligation and transformation. The appearance of two bands indicates the presence of the insert.

3.2 Analyzing and Comparing the pBS-acd Insert Sequence with the GenBank Published BT013578 Sequence

Comparing the isolated cDNA (pBS-acd insert) sequence with the GenBank published sequence for the putative ACC deaminase (BT013578), it is apparent that they are not identical. The expected product size based on the primer positions was 1408 bp, however, the sequence that was isolated and cloned is 1432 bp (Figure 3-2). At the nucleotide level, there appears to be a 7 bp gap starting at nucleotide 819 (counting based on the entire sequence that was cloned, not just the ORF), as well as an "extra" 31 bp that appear only in the pBS-acd insert sequence, but not in BT013578, and starting at nucleotide 885 (based on the BT013578 sequence; in pBS-acd this corresponds to the position of base pair 878) (Figure 3-2). As such, the isolated cDNA sequence has been deposited to GenBank with the Accession # EU639448.

When comparing these two sequences at the protein level (Figure 3-3), the gap and the "extra" sequence in EU639448 result in a different open reading frame, consequently changing the portion of the protein from amino acid 269 to 290 in BT013578 and 269 to 298 in pBS-acd (EU639448), with pBS-acd having an additional 8 amino acids in this region. Interestingly, beginning at the amino acid D291 for BT013578 and D299 for pBS-acd (EU639448) the sequences resume the same reading frame, resulting in the identical amino acid sequence thereafter. Hence, the protein that is encoded by BT013578 is predicted to be 417 amino acids long with a calculated molecular weight of 46.28 kDa; on the other hand, the pBS-acd (EU639448) encoded protein is predicted to be 425 amino acids long, with a predicted molecular weight of 46.51 kDa. When these two protein sequences are aligned with protein sequences for putative ACC deaminases from other

plants, in this case *Arabidopsis* and rice, it is apparent that pBS-acd, in the region where the difference between pBS-acd (EU639448) vs. BT013578 occurs, is more similar to the other plant sequences (Figure 3-4).

Additionally, the search of Expressed Sequence Tags (EST) databases and Tentative Consensus (TC) databases (TCs are clusters of ESTs assembled into a tentative consensus sequence) using the The Gene Index Project (http://compbio.dfci.harvard.edu/tgi/) database indicates that the pBS-acd (EU639448) version is in fact expressed, including in other tissues besides leaf, such as callus, flower and fruit. On the other hand, no ESTs were found that are the same as BT013578.

3.3 Determining if BT013578 Version is Expressed in *S. lycopersicum*, Bony Best Variety

The expression of the BT013578 version in *S. lycopersicum* Bony Best variety of tomato was tested by exploiting the differences between the sequence that was cloned, pBS-acd insert sequence (EU639448), and BT013578. A simple PCR strategy was developed where primers were designed to specifically bind to either BT013578 or pBS-acd (EU639448), if expressed. The primer design and rationale for primer design is explained in the Materials and Methods section and in Figure 2-1.

RNA was isolated from leaf tissue and from breaker fruit, reverse transcribed, and used as the template. The additional template, as a control, included plasmid DNA from pBS-acd. Each of these templates were set up with three different sets of primers. The reactions with Primers 1 and 3, which bind outside of the region that was different, were used as a positive control, amplifying both pBS-acd (EU639448) and BT013578

Figure 3-2. Nucleotide sequence alignment between putative ACC deaminase sequence from GenBank (Accession # BT013578) and the isolated and cloned putative ACC deaminase from *S. lycopersicum* Bony Best variety (i.e. insert from the pBS-acd construct). The predicted start and stop codons are marked with a box. The sequences are identical at the nucleotide level, except for the 7 bp gap and an "extra" 31 bp in the pBS-acd insert sequence (EU639448).

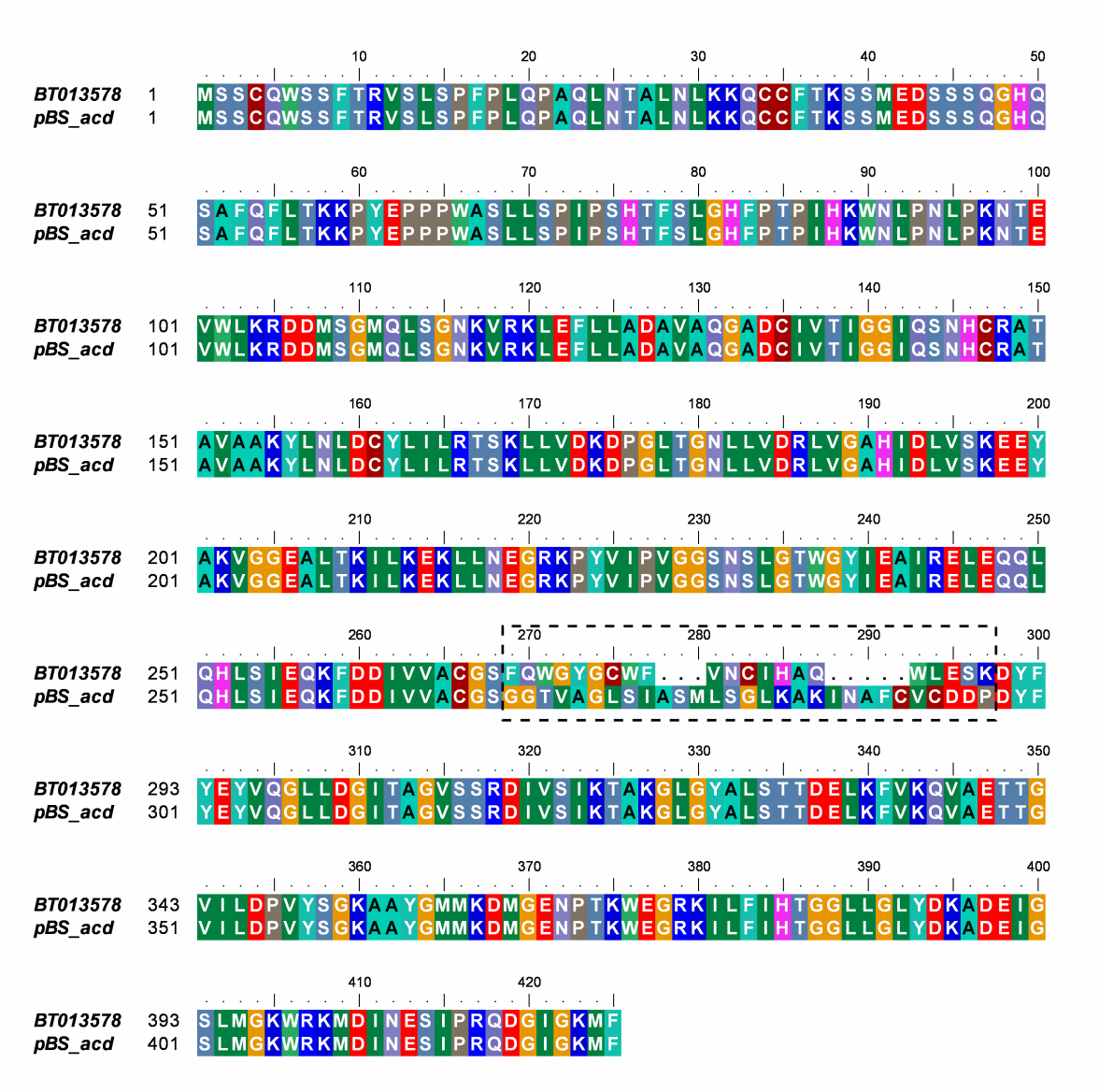


Figure 3-3. Protein sequence alignment between the predicted protein sequences for BT013578 and pBS-acd insert (EU639448). The region where amino acids do not align is indicated with a box. Before S268 and after D291 for BT013578 and D299 for pBS-acd, the reading frame is the same for both sequences, resulting in the identical amino acid sequence.

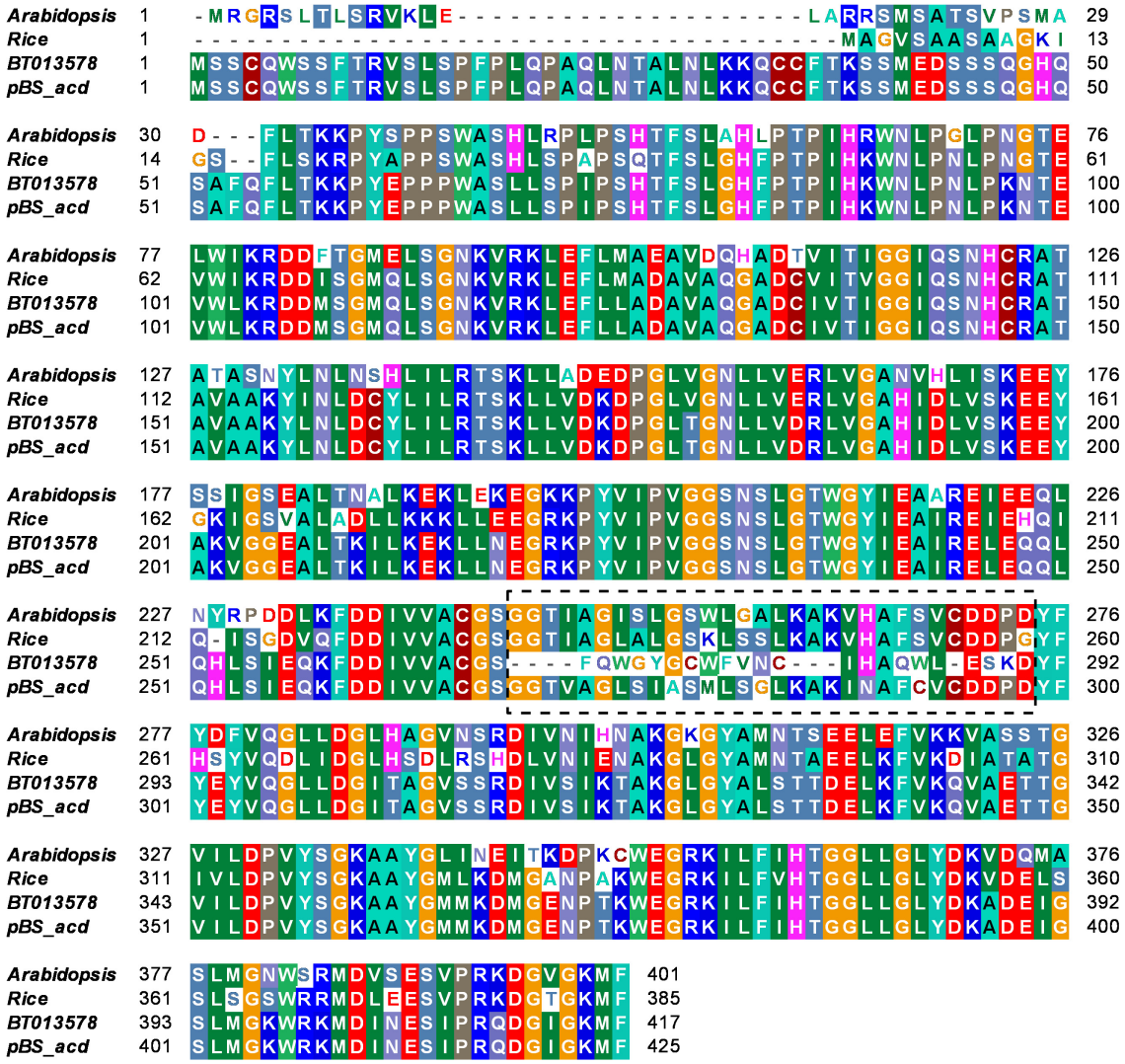


Figure 3-4. Protein alignment between predicted protein sequences for BT013578, isolated tomato sequence (pBS-acd), *Arabidopsis* (NM_103738) and rice (BAD16875) putative ACC deaminase. The region where BT013578 and pBS-acd (EU639448) differ is indicated with a box; pBS-acd (EU639448) sequence in this region is more conserved to the other plant putative ACC deaminase proteins than is BT013578.

whenever expressed. The PCR reactions with Primers 4 and 3 were set up to specifically test for the expression of BT013578, where a product of 143 bp was expected. RT-PCR reactions with Primers 1 and 5 specifically bind to the pBS-acd version (EU639448) (Figure 2-1).

As seen in Figure 3-5, the positive control (Primers 1 and 3) successfully amplifies a fragment of ~225 bp for all templates. If both versions were expressed, two bands would be visible on the gel, one product of 227 bp and the other 203 bp, where pBS-acd (EU639448) and BT013578 are amplified respectively. Additionally, this approach has illustrated that pBS-acd (EU639448) is expressed in both leaf and breaker fruit; when using Primers 1 and 5, a fragment of expected size (167 bp) is obtained for all templates.

The expression of the BT013578 version could not be determined in leaf or breaker fruit; however, BT013578 could be expressed in low levels and only in certain tomato tissues that were not tested here, or there may exist variety specific differences for this gene. Primer 4 was designed to bind in the region where there is a 7 bp gap in pBS-acd (EU639448), hence only amplifying BT013578 (Figure 2-1). As mentioned, the expected product size for PCR reactions with Primers 4 and 3 is 143 bp. A band of this size has not been obtained, but rather a much larger band of ~700 bp is seen for all three templates. This indicates that the BT013578 version is not expressed in leaf or breaker fruit, fruit being the tissue from which this cDNA sequence was originally obtained. The ~700 bp band that appears for these PCR reactions is due to nonspecific primer binding; the band of same size is also observed when pBS-acd plasmid DNA is used as the template. Increasing the annealing temperature for the PCR reaction does not avoid this problem, as test PCR reactions with gradient annealing temperature from 59-63°C were also performed (data not

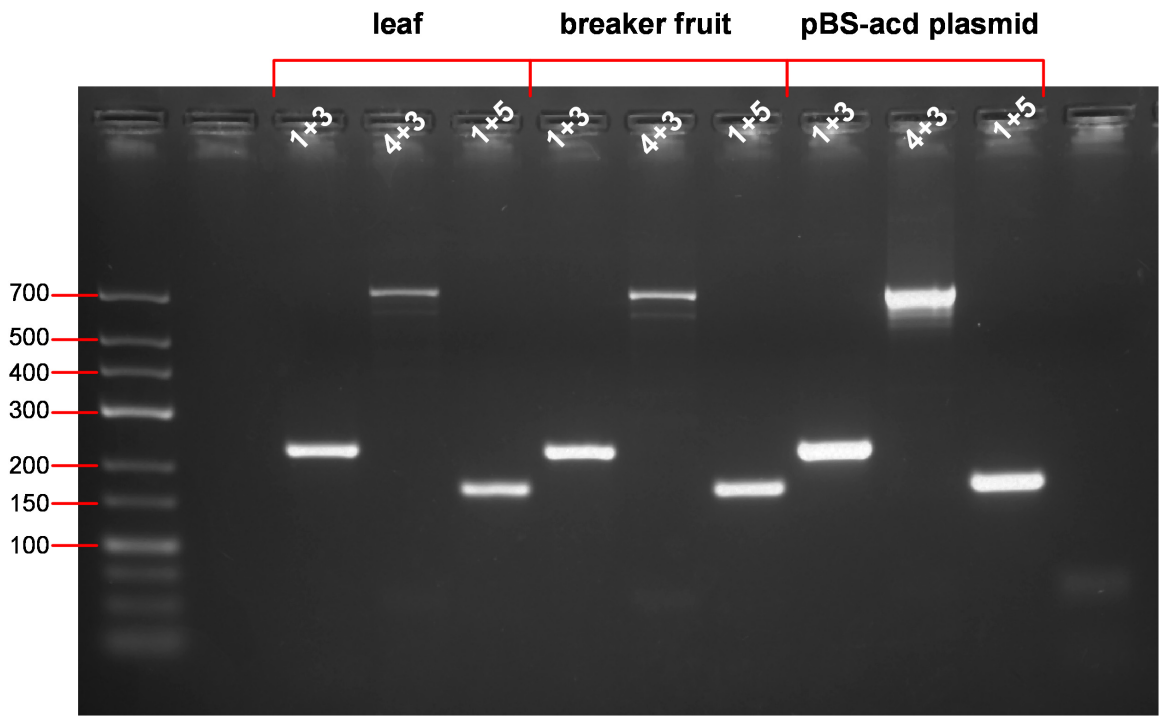


Figure 3-5. Determining BT013578 expression in *S. lycopersicum* Bony Best variety. 3% DNA agarose gel; DNA ladder is O'GeneRuler Low Range ladder from Fermentas (Burlington, ON). Numbers above each lane indicate the primer sets used in that reaction (e.g. 1+3 indicates Primer 1 and Primer 3); leaf, breaker fruit or pBS-acd plasmid are the respective templates used. For primer positions and primer design, please refer to Materials and Methods section and Figure 2-1. The same band pattern is obtained for all three templates, where in 1+3 a product of ~225 bp is observed; in 4+3, a product of ~700 bp is observed (whereas a band of 143 bp was expected if BT013578 is expressed). A 167 bp product is obtained for primer set 1+5. This indicates expression of the pBS-acd insert (EU639448), but not BT013578.

shown; performed initially for all primers). The most likely region where Primer 4 binds nonspecifically is shown in the Appendix.

3.4 Protein Expression and Solubility

For protein expression and purification purposes the isolated cDNA from the pBS-acd construct was sub-cloned into the expression vector pET30 Xa/LIC and this construct was then transformed into the expression host *E. coli* BL21(DE3). To determine the effective IPTG induction concentration, as well as the time required for protein expression, cultures were induced with IPTG concentrations of 0.1, 0.4 and 1.0 mM, and incubated for up to 6 h at 30°C. Samples were collected at several time points, and then analyzed by SDS-PAGE. SDS-PAGE protein gels for all analyzed time points are included in the Appendix. As seen in Figure 3-6, the recombinant protein is effectively expressed at all tested IPTG concentrations (0.1, 0.4, 1.0 mM), with no apparent difference between the samples. Additionally, the pET30 Xa/LIC expression vector and BL21(DE3) *E. coli* host seem to be an excellent expression system with observable protein expression even after one hour of induction.

Having ascertained effective protein expression, the recombinant protein was tested for solubility. For downstream application, activity testing and characterization, the recombinant protein must be expressed in a soluble, and hence enzymatically active form. The culture was induced, soluble and insoluble fractions separated, then examined by SDS-PAGE. It appeared that all of the expressed recombinant protein was present in the insoluble fraction (Figure 3-6). Other culture conditions were examined, including decreasing the induction temperature to 16°C, decreasing the IPTG concentration, and changing the host and expression vector. However, the recombinant protein always

appeared in the insoluble fraction (data not shown). Utilization of another expression vector (pQE30) with the *lac* promoter resulted in what appeared to be lower level of protein expression at the same IPTG concentration. Separated soluble and insoluble fractions for ET30-acd were also tested by Western blotting (using an anti-His primary antibody), however expression of the recombinant protein in soluble form was not detected (data not shown).

3.5 Protein Purification

Even though the recombinant protein appeared to be expressed entirely in the insoluble fraction, most likely occurring primarily as insoluble aggregates known as inclusion bodies, a procedure was optimized to partially purify the expressed protein. When expressed as an insoluble aggregate, typically recombinant proteins can efficiently be purified under denaturing conditions. However, to determine the enzymatic activity of the protein of interest, purification must be carried out under native non-denaturing conditions.

Utilizing the N-terminal 6X His-tag on the recombinant protein and 0.5 ml of Ni-NTA superflow resin, the 51.48 kDa recombinant protein (including the 6X His-tag and some vector-encoded sequence) was purified as seen in Figure 3-7. The typical purification fractions obtained are shown in Figure 3-7A. The column with the bound protein was washed with buffers of increasing imidazole concentrations, up to 60 mM, and then eluted with a 250 mM imidazole solution. The majority of the protein elutes in E2 and E3 fractions, with a typical recovery of ~600-700 µg of protein. However, the purification results in only a partially purified recombinant protein with two major contaminating proteins that appear above and below the recombinant protein on an SDS-PAGE gel, that is

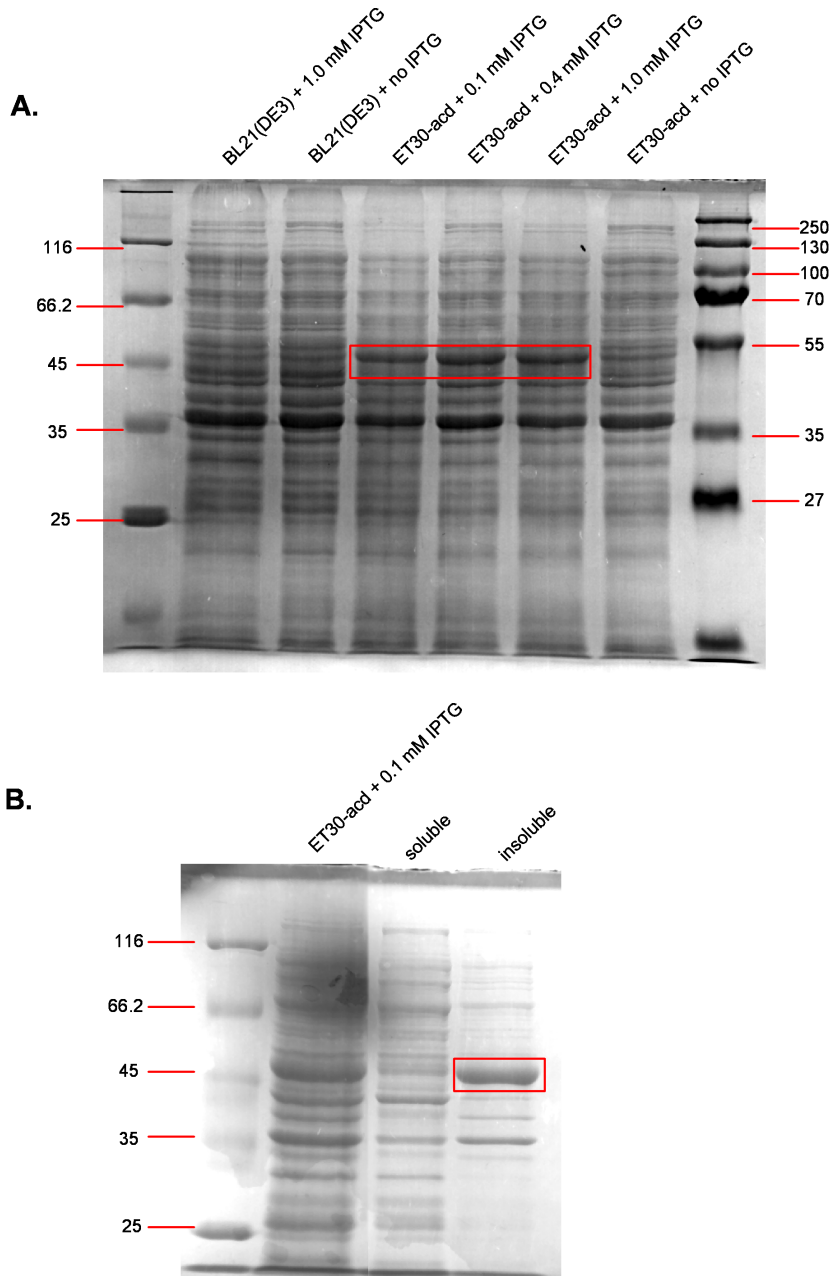


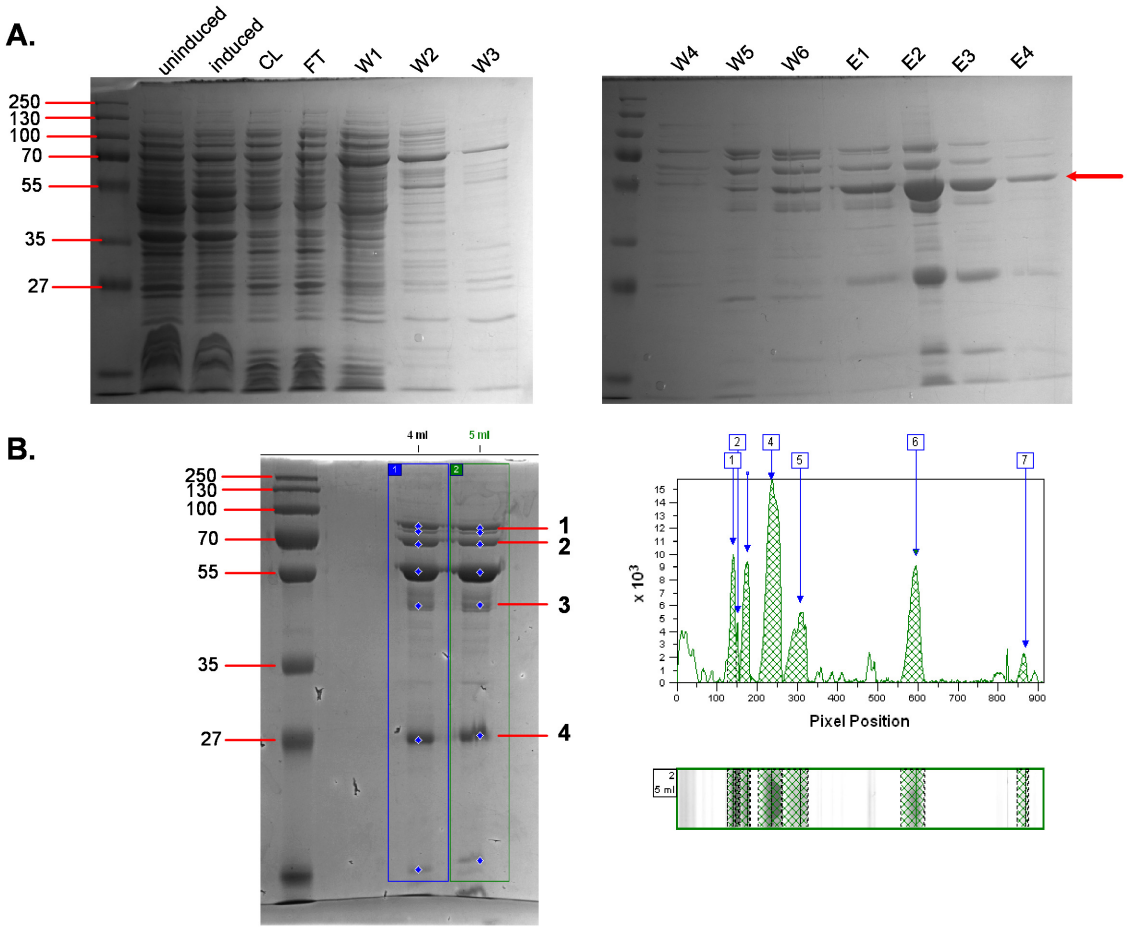
Figure 3-6. Examining protein expression and solubility with SDS-PAGE. *A*: induction with different IPTG concentrations of ET30-acd for 3h at 30°C. Protein is efficiently expressed at all IPTG concentrations. The protein ladder on the left and the right side is marked in kDa. *B*: testing for the solubility of the recombinant protein; based on SDS-PAGE analysis, the recombinant protein appears exclusively in the insoluble fraction.

to say, two larger and two smaller proteins than the recombinant protein. The purification procedure was tested with different buffer compositions, including the addition of ethanol and glycerol which decrease hydrophobic protein-protein interactions; however, the procedure used here resulted in the highest recombinant protein recovery, whereas other buffer compositions still only resulted in partial purification, but with somewhat reduced recovery (data not shown).

After purification by affinity chromatography, the purified protein was passed through a Sephadex-G25 gel filtration column to exchange the buffer; gel filtration was used instead of dialysis since this latter procedure was found to be inefficient for buffer exchange purposes. The recombinant protein eluted from the gel filtration column in 4 and 5 ml fractions (Figure 3-7C). Moreover, the overall purity of the protein of interest was determined using an SDS-PAGE image of the 4 and 5 ml protein fractions after gel filtration, then determining the percentage that each protein band constitutes via densitometry. After subtracting the background, the recombinant protein represents ~40% of each fraction (each purification procedure being slightly different).

The four major bands that co-purified with the recombinant protein were analyzed by mass spectrometry. The largest protein, indicated as "1" in Figure 3-7B was determined to be the host chaperone protein, heat shock protein 70 (hsp70). Additionally, the protein that appears right below the hsp70 on the SDS-PAGE gel, marked as "2", was determined to be Cpn60, a heat shock 60 protein. Based on the peptide sequences, the band below the recombinant protein, marked as "3," was shown to share identity with the putative ACC deaminase from rice. Thus, it is most likely that the appearance of this band is due to recombinant protein degradation during the process of purification. The last analyzed band,

Figure 3-7. Protein purification, densitometry and mass spectrometry. *A*: SDS-PAGE gel of purification fractions. CL=cleared lysate (soluble fraction), FT=flow-through, W1,2,3,4,5,6=wash 1-6, E1,2,3,4=elution 1-4. The recombinant protein is indicated with an arrow. *B*: Densitometry using the SDS-PAGE image with protein samples after gel filtration. 4 ml and 5 ml labels on the gel indicate the two different fractions in which the recombinant protein elutes after gel filtration. The dots on each protein band indicate that the band was considered in densitometry analysis. The pixel position plot on the right indicates the intensity of each band that was picked from the SDS-PAGE gel on the left. The largest peak indicated by 4 on the pixel plot is the recombinant protein. *C*: plot of protein concentration and conductivity for each gel filtration fraction; samples were collected as 1 ml fractions.


A.
uninduced
induced
CL
FT
W1
W2
W3
W4
W5
W6
E1
E2
E3
E4
250
130
100
70
55
35
27
B.
4 ml
5 ml
1
2
3
4
x 10^3
Pixel Position
C.
Protein, mg/ml
Conductivity, mS
volume, ml
[Protein], mg/ml
Conductivity, mS

"4," was determined to be another host chaperone protein, FKBP-type peptidyl-proly cis-trans isomerase.

3.6 Kinetic Characterization of Putative ACC Deaminase from Tomato

The purified protein was tested for both ACC deaminase activity, as well as D-cysteine desulfhydrase activity. Using the linked lactate dehydrogenase assay and various concentrations of the two substrates it was determined that this tomato enzyme utilizes only D-cysteine as a substrate. Hence, it was shown that the putative ACC deaminase that was isolated from tomato is not a functional ACC deaminase, but rather that it shows the same activity as determined by Riemenschneider and co-workers (2005) for an *Arabidopsis* ACC deaminase homologue (NM_103738). The specificity of this enzyme towards D-cysteine was also determined, based on the finding that it does not break down the optical isomer L-cysteine at all. Initial testing showed substrate inhibition above 1 mM D-cysteine; the same was previously observed for the *Arabidopsis* enzyme (Riemenschneider et al., 2005; Appendix).

Hence, the kinetic characterization of the enzyme, including the Km determination was performed at D-cysteine concentrations below 1 mM. Non-linear regression and data fitted to the Michaelis-Menten equation yielded a Km for D-cysteine of 0.21 ± 0.05 mM at pH 8.0 and 37°C (Figure 3-8). The turnover number, k_{cat}, was estimated to be 230 min^{-1}, and the second order rate constant k_{cat}/Km (=catalytic efficiency) of 1095 mM^{-1} min^{-1}. However, it should be noted that the turnover number and the second order rate constant are only estimates, since the protein was partially purified; hence, the turnover number is biased towards the densitometry analysis of protein purity. The calculated Km is comparable to that for the *Arabidopsis* homologue, where the Km was determined to be

0.25 mM, as well as to the *E. coli* enzyme where the Km was reported to be 0.15 or 0.3 mM in two different studies (Riemenschneider et al., 2005; Nagasawa et al., 1985; Soutourina et al., 2001).

The effects of pH and temperature on the activity of the enzyme were also investigated (Figure 3-9 and Figure 3-10). The enzyme shows the highest activity at pH 7.6; substantial decrease in activity, by almost 50%, is observed at pH 6.8 as well as on the other side of the curve, above pH 8.8. pKa_1 and pKa_2 of the recombinant protein were estimated to be 6.7 and 8.9, respectively. At D-cysteine concentration of 0.8 mM, below substrate inhibition, but well above the determined Km, the effects of temperature on the progress of the reaction were also examined. The temperature profile of the enzyme, from 10 to 70°C is shown in Figure 3-10. The optimum temperature of 30°C for this tomato enzyme agrees with that reported for the *Arabidopsis* homologue, where it was also found to be 30°C. However, contradictory to results reported by Riemenschneider et al. (2005) for the *Arabidopsis* homologue, the enzyme was found to be very stable with almost 80% of activity still retained at 50°C. The stability of the enzyme at higher temperatures is more in agreement with the reports for the *E. coli* D-cysteine desulfhydrase, where the optimum temperature was found to be 45°C (Nagasawa et al., 1985). Substantial loss in activity of the tomato enzyme was observed at temperature over 60°C, e.g. at 70°C only about 15% of maximum activity was observed.

Furthermore, the enzyme was predicted to be a pyridoxal phosphate (PLP) dependent enzyme; true ACC deaminase proteins and D-cysteine desulfhydrase proteins have been shown or predicted to bind PLP at a conserved lysine residue. Therefore, the dependence of the enzyme on PLP was investigated. A number of inhibitors of PLP

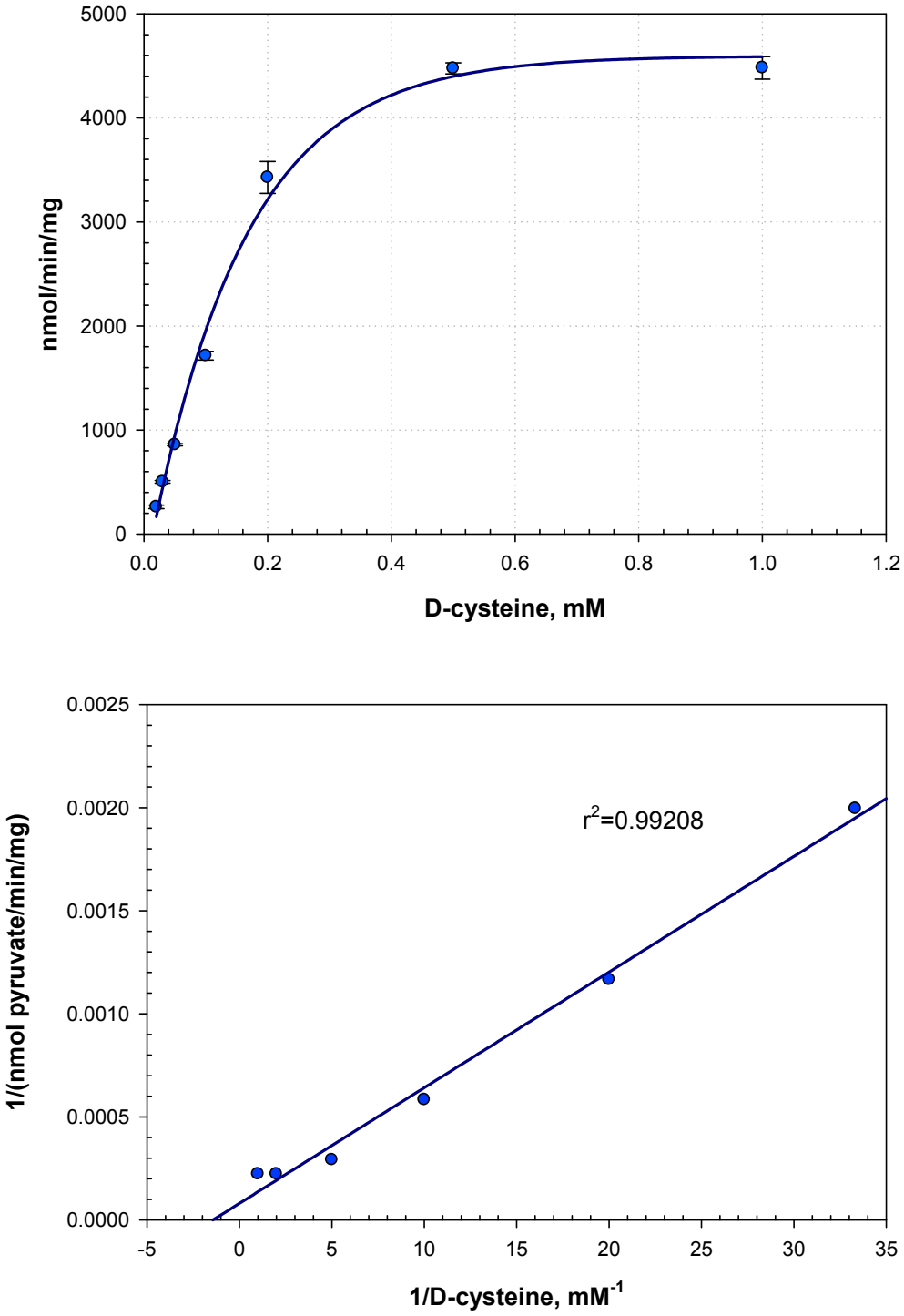


Figure 3-8. Kinetic data for *Solanum lycopersicum* enzyme. Top plot: Michaelis-Menten plot; effects of substrate concentration on the activity of the enzyme. Bottom plot: Lineweaver-Burk plot of the data.

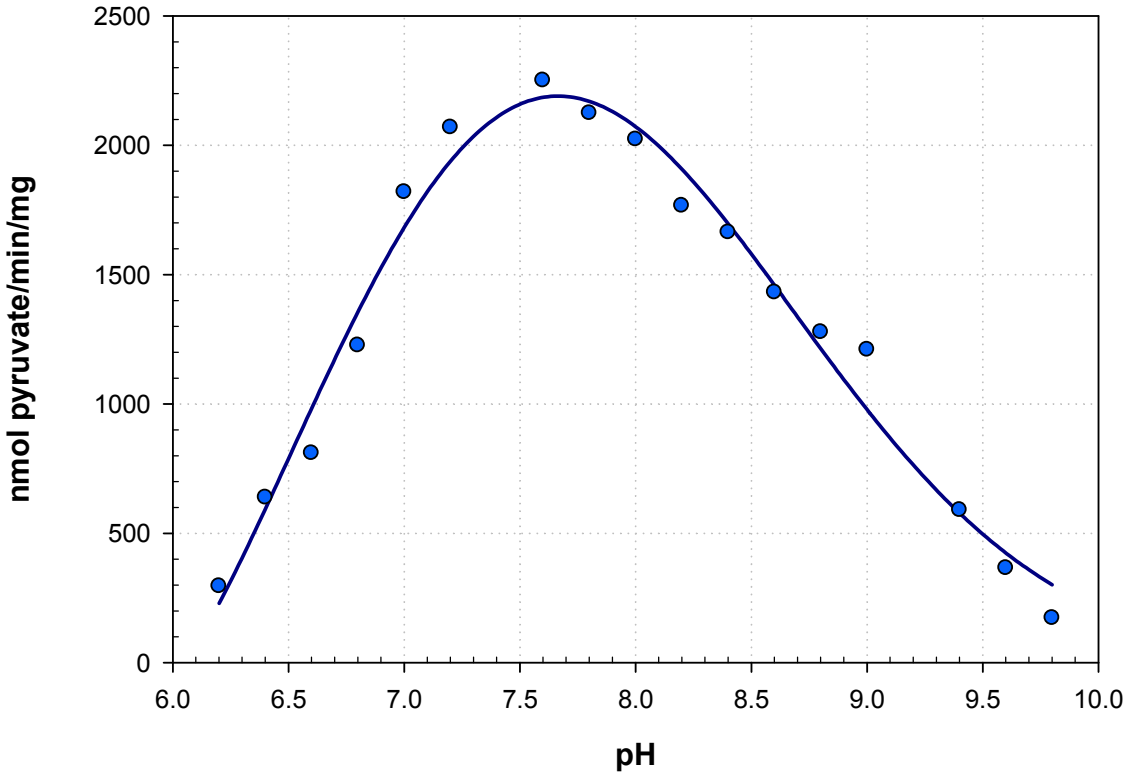


Figure 3-9. pH-rate profile of recombinant D-cysteine desulfhydrase from *Solanum lycopersicum.* The activity of the enzyme was determined at pH values of 6.2 – 9.8.

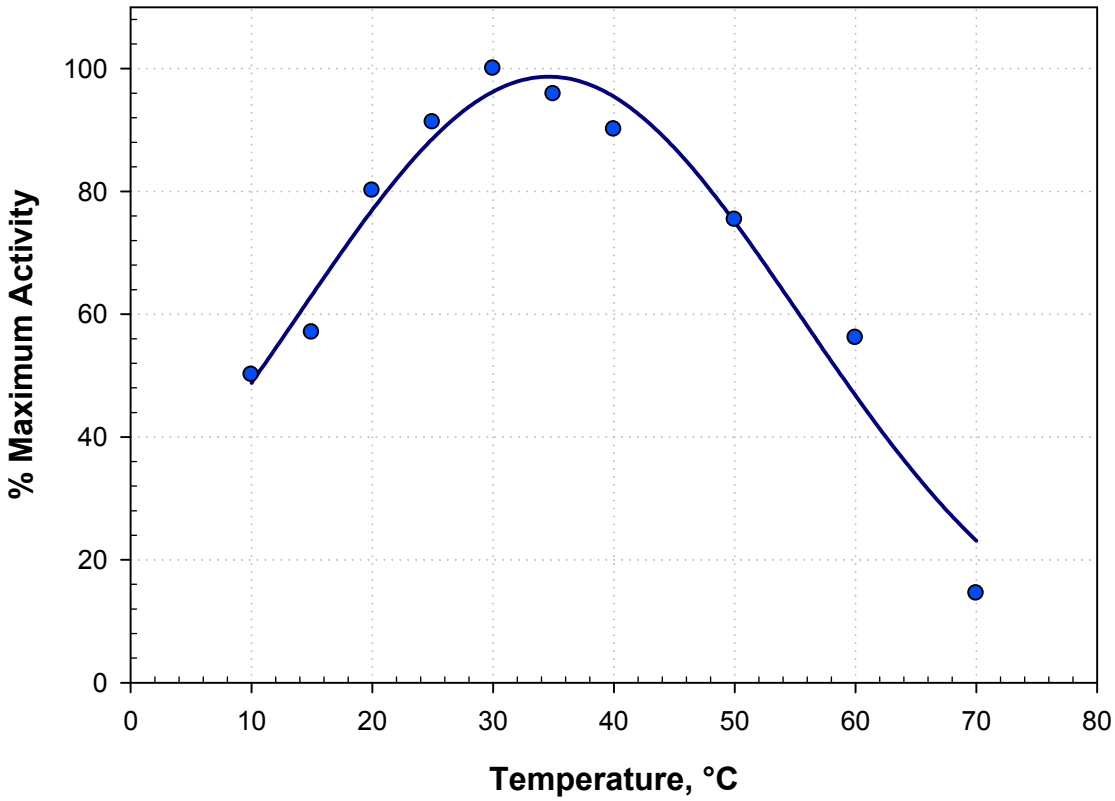


Figure 3-10. Temperature-rate profile of the recombinant D-cysteine desulfhydrase from *Solanum lycopersicum.* The activity of the enzyme was determined at temperatures of 10 – 70 °C.

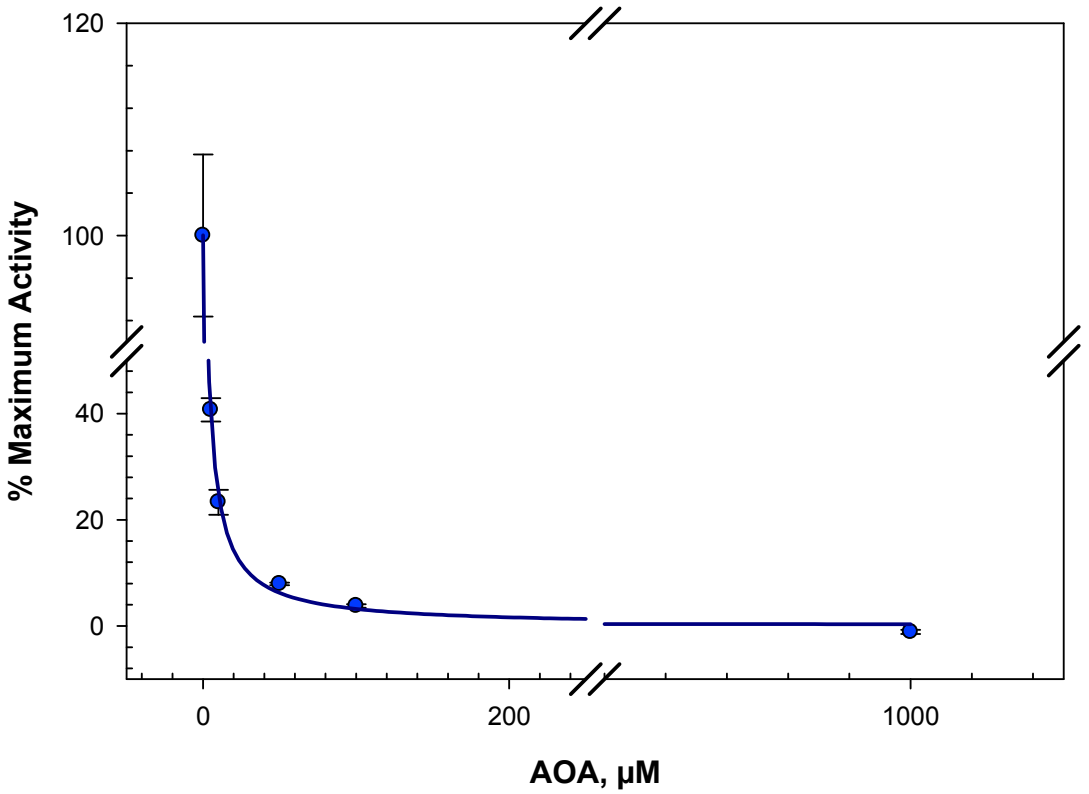


Figure 3-11. PLP dependence of recombinant D-cysteine desulfhydrase from *Solanum lycopersicum*. Aminooxyacetic acid (AOA) is a known inhibitor of PLP dependent enzymes. Incubation of the recombinant protein with various concentrations of AOA inhibits the activity of the enzyme.

dependent enzymes have been identified; one such inhibitor, aminooxyacetic acid (AOA) was used to show that the enzymatic activity is progressively inhibited as the AOA concentration is increased (Figure 3-11), rendering the enzyme PLP dependent. From this data it has been calculated based on non-linear regression that the Ki for AOA is 3.3 ± 0.17 μM.

3.7 3D Structure Prediction and Implications for Activity

To determine tertiary structure of D-cysteine desulfhydrase from tomato, a BLAST search was performed for proteins with similar sequence and known 3D structure. Similarities to bacterial and yeast ACC deaminase were found to be only ~20%, however the similarity to the *Pyrococcus horikoshii* ACC deaminase homologue was somewhat higher, showing an identity of ~30%. No significant similarities were found to any other proteins with known 3D structures. Based on the sequence identity, bacterial and yeast ACC deaminase structures were excluded as templates; since the *P. horikoshii* PH0054 ACC deaminase homologue has higher sequence identity in addition to features suitable for protein model construction, it was chosen as the template for the model presented herein (PDB ID 1J0A).

To assess the quality of the *P. horikoshii* homologue as the template, a multiple sequence alignment was performed, which included the *S. lycopersicum* enzyme and potential templates (yeast ACC deaminase and the *P. horikoshii* ACC deaminase homologue). Secondary structures were assigned to the primary sequence of each and compared based on the multiple sequence alignment. The experimentally observed position of secondary structures in the templates was also considered. Good agreement was found between the predicted secondary structure for the model and the experimentally determined

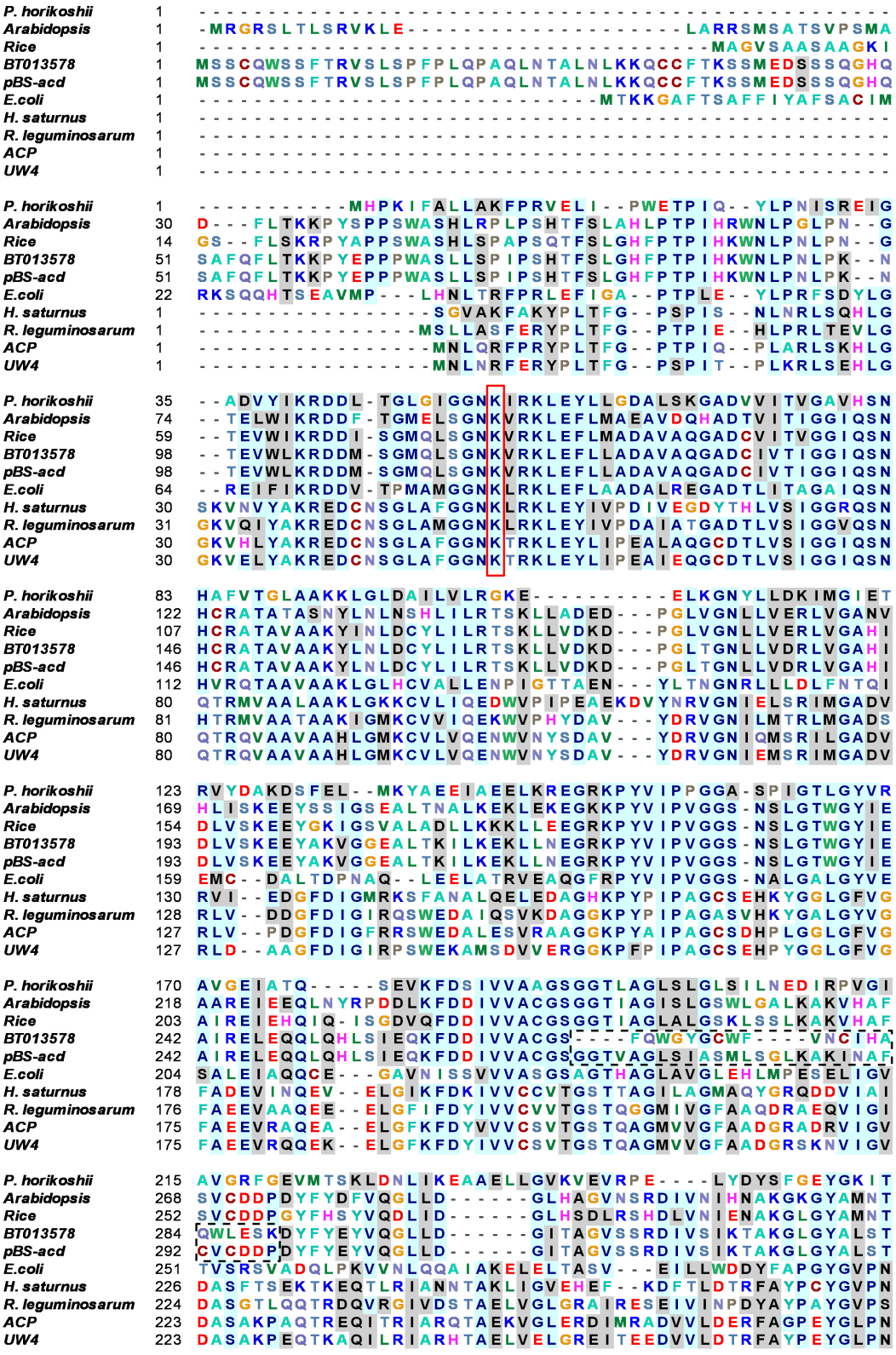
secondary structure position of the template, as well as the position of gaps, such that no refinements were necessary. The choice of the PH0054 *P. horikoshii* protein was confirmed as a suitable template for construction of 3D model.

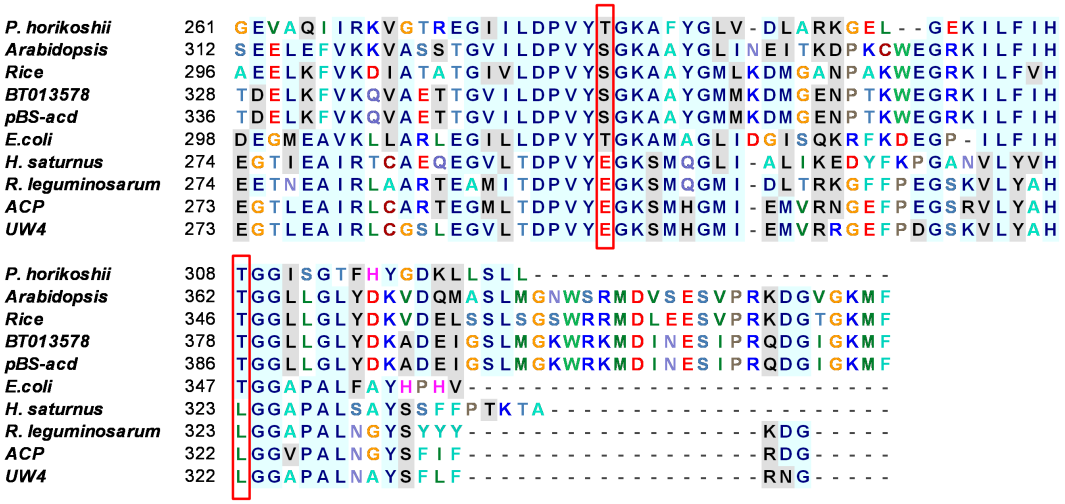
Once constructed, the 3D model was then subjected to analysis by PROCHECK which determines the stereochemical quality of the structure. The model was found to have 81.2% of its residues in most favored regions and only 2.2% of its residues in disallowed regions. A good quality model is expected to have over 90% of residues in most favored regions, however, considering that for the template only 85.9% of residues are in most favoured regions, the model at 81.2% is considered to be of high quality based on the stereochemical features.

The quality of the model was also evaluated by comparing the position of secondary structure elements after the model construction (i.e. from 3D coordinates). The positions were compared to that of the template as well as to the position in other structures that were identified by the BLAST search of the PDB protein bank. The position of these elements was identified by a DSSP analysis on the model. Again, these are in agreement with each other, confirming the good quality of the model, as there are very few gaps, and those that are present fall into loop regions. The positions are also in agreement with those predicted by PHD from the primary sequence of the *S. lycopersicum* enzyme.

The 3D structure of the model is represented in Figure 3-13A. The modeled protein folds as an open twisted α/β structure consisting of 42.0% helical, 13% β-strand, 45% other structures. Comparing the modeled structure with the structure of the template, it appears that this enzyme is also composed of two domains: the small domain and the large or PLP-binding domain. The small domain consist of a central four-stranded parallel β-sheet and

Figure 3-12. Multiple sequence alignment of annotated and putative ACC deaminase proteins. The *Pyrococcus horikoshii* homologue, plant putative ACC deaminases including the cloned tomato cDNA sequence (pBS-acd, EU639448) and the GenBank published putative ACC deaminase sequence from tomato (BT013578), *E. coli* putative ACC deaminase, and true ACC deaminases from *Hansenula saturnus, Rhizobium leguminosarum, Pseudomonas* sp. ACP and *Pseudomonas putida* UW4. The lysine residue that binds PLP is marked as well as the two amino acids that are the targets of site-directed mutagenesis. The coloring of the alignment was adjusted such that when at least 50% of residues are identical, the colour appears blue.


P. horikoshii
Arabidopsis
Rice
BT013578
pBS-acd
E.coli
H. saturnus
R. leguminosarum
ACP
UW4


P. horikoshii 261 GEVAQIIRKVGTREGIILDPVYTGKAFYGLV-DLARKGEL--GEKILFIH
Arabidopsis 312 SEELEFVKKVASSTGVILDPVYSGKAAYGLINEITKDPKCWEGRKILFIH
Rice 296 AEELKFVKDIATATGIVLDPVYSGKAAYGMLKDMGANPAKWEGRKILFVH
BT013578 328 TDELKFVKQVAETTGVILDPVYSGKAAYGMMKDMGENPTKWEGRKILFIH
pBS-acd 336 TDELKFVKQVAETTGVILDPVYSGKAAYGMMKDMGENPTKWEGRKILFIH
E.coli 298 DEGMEAVKLLARLEGILLDPVYTGKAMAGLIDGISQKRFKDEGP-ILFIH
H. saturnus 274 EGTIEAIRTCAEQEGVLTDPVYEGKSMQGLI-ALIKEDYFKPGANVLYVH
R. leguminosarum 274 EETNEAIRLAARTEAMITDPVYEGKSMQGMI-DLTRKGFFPEGSKVLYAH
ACP 273 EGTLEAIRLCARTEGMLTDPVYEGKSMHGMI-EMVRNGEFPEGSRVLYAH
UW4 273 EGTLEAIRLCGSLEGVLTDPVYEGKSMHGMI-EMVRRGEFPDGSKVLYAH
P. horikoshii 308 TGGISGTFHYGDKLLSLL----------
Arabidopsis 362 TGGLLGLYDKVDQMASLMGNWSRMDVSESVPRKDGVGKMF
Rice 346 TGGLLGLYDKVDELSSLSGSWRRMDLEESVPRKDGTGKMF
BT013578 378 TGGLLGLYDKADEIGSLMGKWRKMDINESIPRQDGIGKMF
pBS-acd 386 TGGLLGLYDKADEIGSLMGKWRKMDINESIPRQDGIGKMF
E.coli 347 TGGAPALFAYHPHV----------
H. saturnus 323 LGGAPALSAYSSFFPTKTA----------
R. leguminosarum 323 LGGAPALNGYSYYY----------KDG-----
ACP 322 LGGVPALNGYSFIF----------RDG-----
UW4 322 LGGAPALNAYSFLF----------RNG-----

four surrounding helices. The PLP-binding domain contains seven helices and four β-strands of which three are parallel, but one short strand is anti-parallel.

It must be mentioned, however, that the model was constructed excluding some of the amino acid residues at the N- and C-terminal regions, which would have most likely resulted in an additional α-helix at the N-terminal of the protein, and additional β-strands in the PLP-binding domain. These conclusions are based on the comparison to the yeast and PH0054 structures (Figure 3-13). The structural organization of the model is in perfect agreement with SCOP and CATH analyses performed on crystallographic structures of yeast and bacterial ACC deaminase and PH0054 protein.

Furthermore, the overall topology of the predicted structure is very similar to the template, as well as to the yeast ACC deaminase. Comparing the structures, the model also seems to form a crevice between the two domains. The co-enzyme PLP is expected to bind within the crevice with a Schiff base to Lys117. This residue is conserved between all examined sequences (Figure 3-12), implying that the D-cysteine desulfhydrase from tomato is also a PLP-dependent enzyme. The most obvious difference in overall structure between yeast and PH0054 proteins is the lack of two loop regions in PH0054 (residues 104-116 and 168-171 in the yeast ACC deaminase); these also appear to be lacking in the predicted structure. These loops have been described as important in stabilizing the two domains through hydrogen bonding. In addition, the loops that bury the PLP deep into the interior of the molecule in the yeast ACC deaminase appear to be lacking in the constructed model, whereas PH0054 appears to contain only one of the loops. These are the loops formed by residues 101-116, 132-141, 262-273 (numbered according to the yeast ACC deaminase); PH0054 contains the loop corresponding to position of 262-273 residues in

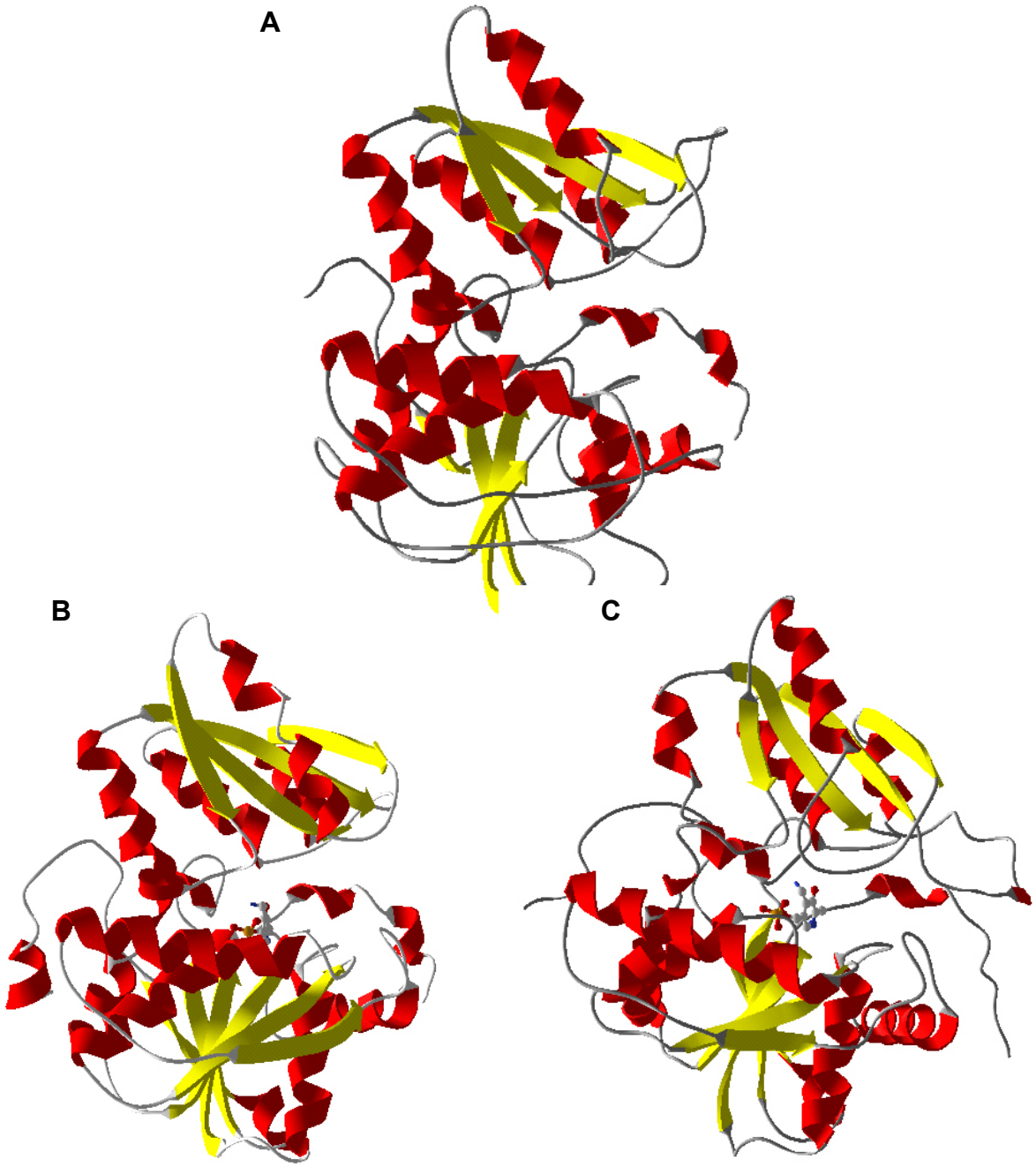


Figure 3-13. Predicted 3D structure of tomato enzyme, known structures of *H. saturnus* and *P. horikoshii* enzymes. Ribbon diagram of *A*: predicted structure of putative ACC deaminase from tomato, *B*: *P. horikoshii* homologue (PDB 1J0A) of ACC deaminase (PH0054) and *C*: *H. saturnus* (yeast) ACC deaminase (PDB 1F2D). The structures are coloured according to the secondary structure; helices in red, sheets in yellow and coils in gray. The PLP molecule in *B* and *C* is shown as ball-and-stick. Only monomers of PH0054 and yeast ACC deaminase are shown.

yeast, but not the other two (Fujino et. al., 2004). This observation suggest that the active site of yeast ACC deaminase is less accessible, perhaps accessible only to smaller molecules, and that the active site of the modeled enzyme and the homologue PH0054 are less buried and could be accessed by larger molecules.

Essential residues for ACC deaminase catalytic activity were described based on the three-dimensional structures of the yeast and bacterial ACC deaminases and the *P. horikoshii* ACC deaminase homologue, together with mutational studies of some of the residues that were thought to be necessary for activity. Based on the yeast ACC deaminase numbering, these residues are Lys51 (binds PLP), Lys54, Ser78, Asn79, Gln80, Tyr269, Tyr295, Gln296 and Leu323 (Figure 3-14). Only three of these amino acids are different for PH0054, which resulted in different enzymatic activity. These include Thr283 (Glu296 in yeast), Thr308 (Leu323 in yeast) and His80 (Gln77 in yeast). The presence of His80 is a feature of PH0054 protein; in the modeled structure of the tomato enzyme, this position is occupied by a glutamine residue, as is the case for true ACC deaminases (Figure 3-14).

Residues important in substrate recognition, Ser78 and Asn79 are conserved in all examined sequences (Figure 3-12). The positions occupied by Lys54, Tyr269 and Tyr295 residues are also fully conserved when examined in the multiple sequence alignment (Figure 3-12). In the 3D structure, the phenol group of Tyr295 is stacked to the plane of the pyridinium ring, such that it prevents movement of the pyridine ring so as not to over-rotate, whereas Tyr269 is thought to play a role in the reaction mechanism as part of the charge relay system. However, the corresponding residue for Tyr269 in the predicted model from tomato (Tyr331) is found away from the active site, such that the charge relay system would not be possible (Figure 3-14).

The key difference in the putative active site of the modeled tomato structure is at the positions of Glu296 and Leu323 (based on yeast numbering). At the Glu296 position, examined plant sequences including the tomato enzyme, have a serine residue, while the putative *E. coli* ACC deaminase and *P. horikoshii* homologue have a threonine (Figure 3-12). Additionally, genuine ACC deaminases seem to have a leucine residue conserved at the position of Leu323 in yeast, whereas the homologue PH0054 and the predicted modeled protein both have a threonine. In the tertiary structure, the leucine residue provides space for the long side-chain of the glutamate (i.e. Glu296 in yeast) residue by orienting itself in the opposite direction.

Based on the data examined herein, the modeled structure from tomato appears to have the overall topology of ACC deaminase conserved, but the analysis of the putative active site suggests that this enzyme has more features in common with the *P. horikoshii* PH0054 ACC deaminase homologue, and appears to be more like other members of TRPSβ family than a true ACC deaminase. It is therefore, most likely that the two amino acid residues at the position where true ACC deaminases have a glutamate and leucine (e.g. at the position of Glu296 and Leu323 in yeast ACC deaminase) make the major difference between whether the enzyme can or cannot cleave the cyclopropane ring of ACC (Figure 3-12). Hence, these two amino acid residues were the targets of the site-directed mutagenesis studies described below.

3.8 Analysis of the Mutants

To determine what makes the ACC deaminase reaction unique and to investigate why the putative ACC deaminase from *S. lycopersicum* does not utilize the amino acid ACC as its substrate, but instead uses D-cysteine, the two C-terminal amino acids of

interest were changed (Figure 3-12) and the activity of the resultant recombinant proteins was examined. The *S. lycopersicum* enzyme was modified to resemble a true ACC deaminase at the two amino acid positions, whereas the *P. putida* UW4 ACC deaminase was mutated in the same two amino acids such that its active site should look more like that of the *P. horikoshii* ACC deaminase homologue, or the *S. lycopersicum* D-cysteine desulfhydrase.

In Figure 3-15A the relative activities of the *S. lycopersicum* D-cysteine desulfhydrase and the two single mutants, in addition to the double mutant, is illustrated. This data indicates that both residues Ser358 and Thr384 are important for activity. The Ser358 appears to be more important, since the substitution at this position causes a complete loss of activity; the same being observed for the double mutant, ET30-Tm3. Nevertheless, changing the threonine residue reduces the activity of the enzyme substantially (to ~11.5% of the wild-type recombinant enzyme), although it does not cause complete loss of activity.

As has been previously reported for true ACC deaminase proteins, substitution at the glutamate residue leads to complete loss of activity (Ose et al., 2003; Figure 3-12). Now, the same has been demonstrated with the mutation of Glu295 to a Ser residue in *P. putida* UW4 ACC deaminase (Figure 3-15B). Not surprisingly, the double mutant (ET30-Um2) where both the Glu295 residue is mutated to a Ser and Leu322 is changed to a Thr shows complete loss of activity (Figure 3-15B).

All of the recombinant proteins of the constructed mutants were tested for the ability to utilize a new substrate, in this case ACC for the tomato mutants, and D-cysteine for the *P. putida* UW4 mutants. The double mutant, ET30-Tm3 whose active site should

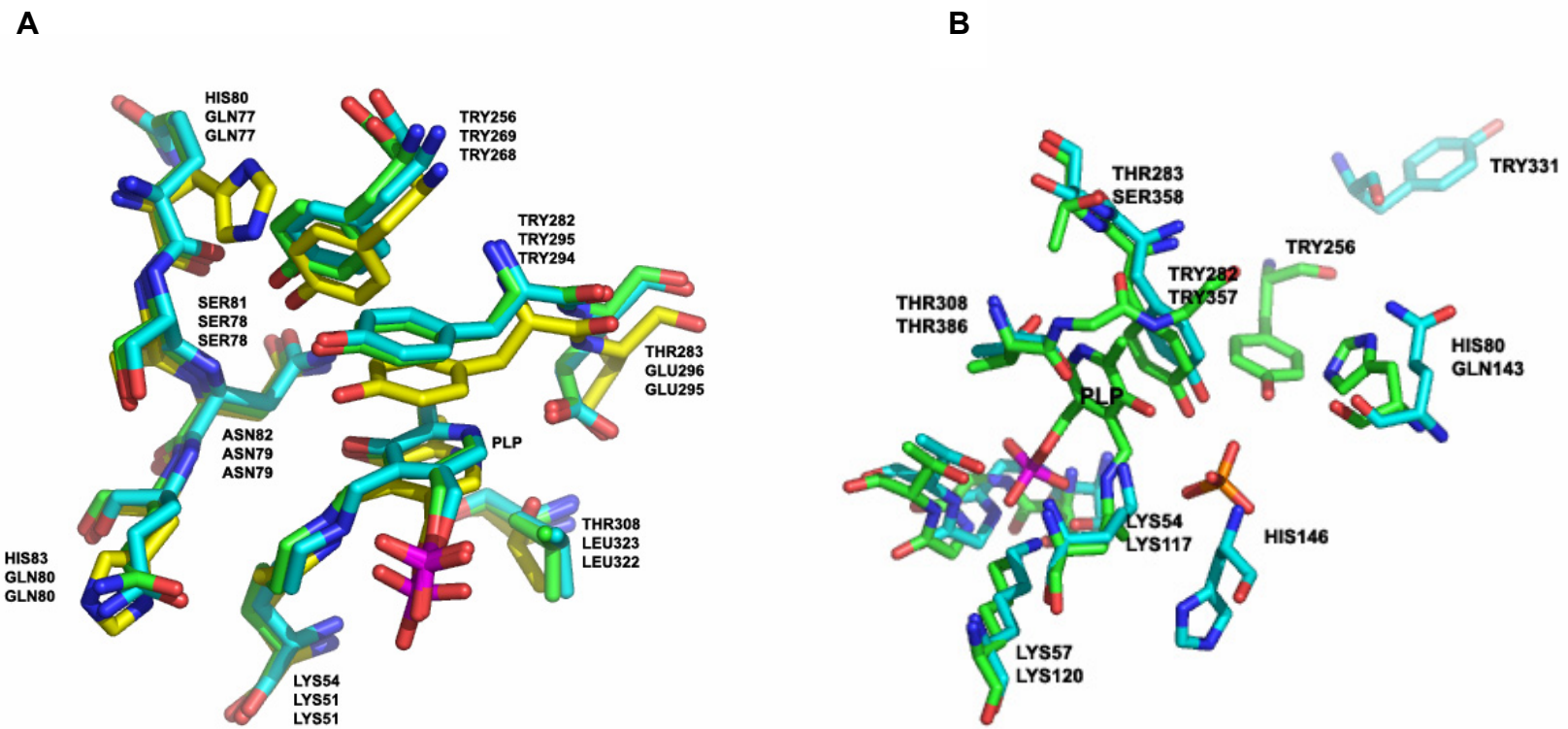


Figure 3-14. Superimposed active site residues. *A: P. horikoshii* ACC deaminase homologue (carbon atoms are yellow), *H. saturnus* (carbon atoms are green), and *Pseudomonas* sp. ACP (carbon atoms are cyan) ACC deaminase. The amino acid residues are labeled in that order. *B: P. horikoshii* ACC deaminase homologue (carbon atoms are green) and predicted active site residues for tomato D-cysteine desulfhydrase (carbon atoms are cyan); amino acid residues are labeled in that order.

resembles that of a true ACC deaminases, loses its ability to use D-cysteine as the substrate, but acquires a low level of activity when 10 mM ACC is provided as the substrate (Figure 3-16, Table 4) and the reaction carried out at 30°C and pH 8.0.

The data for the *P. putida* UW4 mutants also illustrates that they lose the ability to break down the original substrate, ACC, but they acquire the ability to use D-cysteine as the substrate and break it down as a true D-cysteine desulfhydrase (Figure 3-17), albeit at a slower reaction rate than native. The single mutant, ET30-Um1 shows a slight increase in activity as compared to the wild-type UW4 ACC deaminase when D-cysteine is provided as the substrate (Table 4); however, a more significant activity towards D-cysteine as the substrate is observed in the double mutant, ET30-Um2 (Figure 3-17, Table 4). This UW4 mutant was also further characterized, such that from non-linear regression the Km for D-cysteine was determined to be 0.34 ± 0.1 mM, and the k_{cat} value of 10.9 min^{-1}. Interestingly, the ET30-Um2 mutant also shows the characteristic substrate inhibition above 1 mM D-cysteine as observed with other true D-cysteine desulfhydrase enzymes (Figure 3-18).

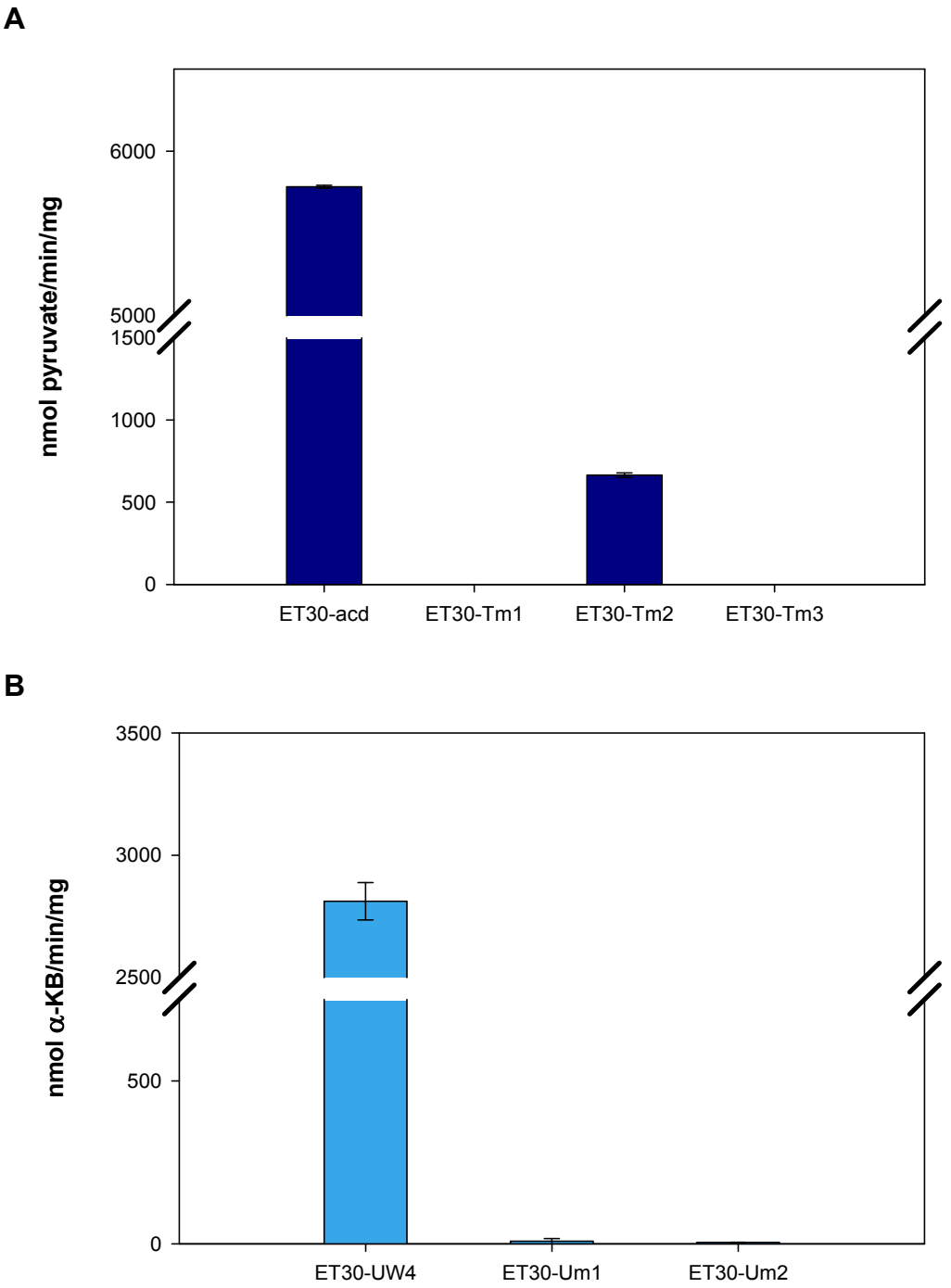


Figure 3-15. Activity of wild-type recombinant proteins and their mutants. *A*: ability of *S. lycopersicum* D-cysteine desulfhydrase (ET30-acd) and the mutants ET30-Tm1(S358E), ET30-Tm2 (T386L) and ET30-Tm3 (S358E + T386L) to use D-cysteine as the substrate. *B*: ability of *P. putida* UW4 ACC deaminase and ET30-Um1 (E295S) and ET30-Um2 (E295S + L322T) mutants to use ACC as the substrate; α-KB= α-ketobutyrate.

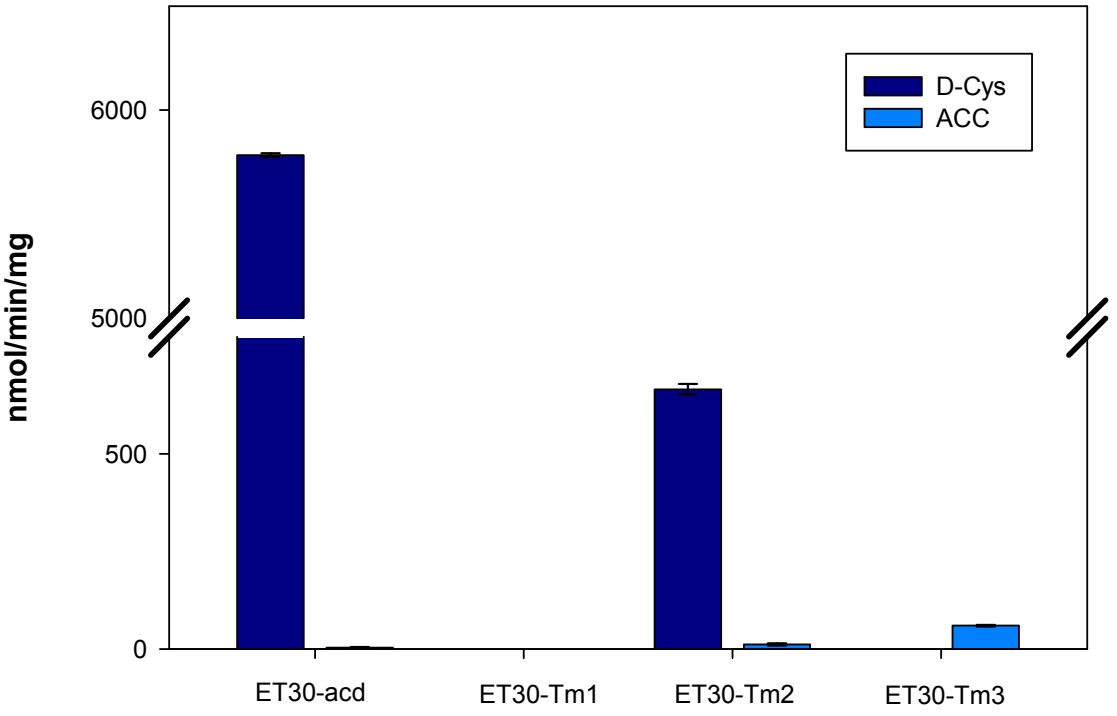


Figure 3-16. Comparison of activities of *S. lycopersicum* enzyme and its mutants towards both substrates, D-cysteine and ACC. ET30-acd, wild-type recombinant D-cysteine desulfhydrase; ET30-Tm1 contains a point mutation S358E; ET30-Tm2 contains a point mutation T386L; and ET30-Tm3 contains two point mutations S358E and T386L. D-cysteine was used at 0.8 mM concentration and ACC at 10 mM. The reactions were carried out at 30°C and pH 8.0.

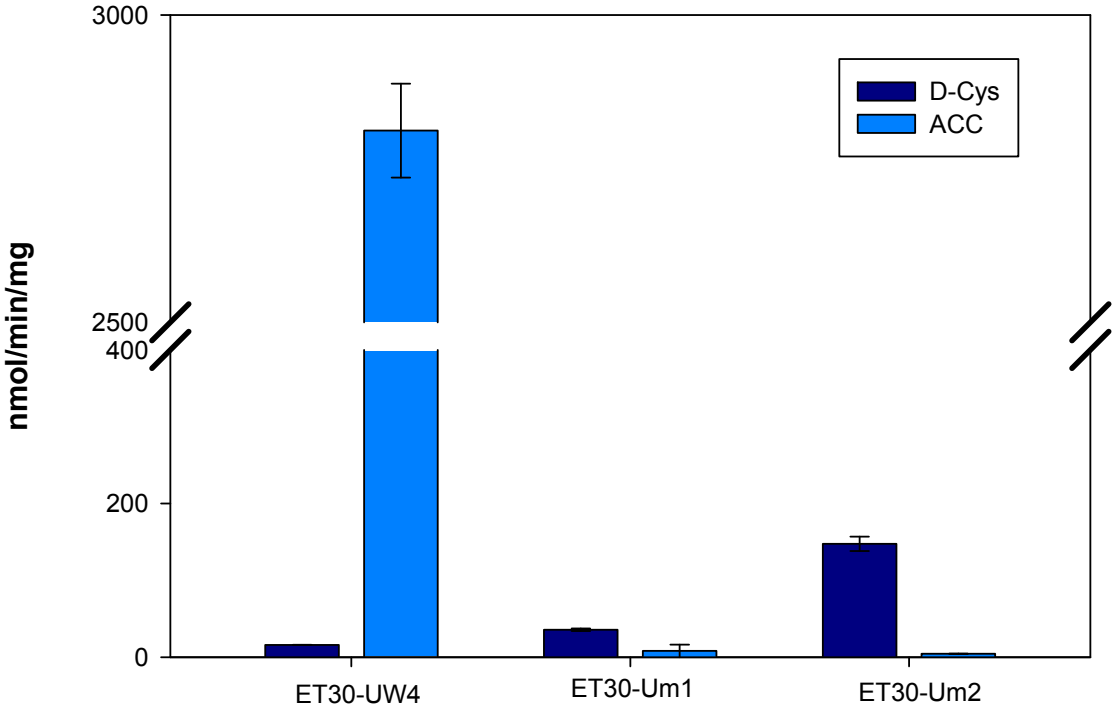


Figure 3-17. Comparison of activities of *P. putida* UW4 ACC deaminase and its mutants towards both substrates, ACC and D-cysteine. ET30-UW4 is the wild-type recombinant ACC deaminase; ET30-Um1 contains the point mutation E295S; and ET30-Um2 contains two point mutations E295S and L322T. ACC was used at 10 mM concentration and D-cysteine at 0.8 mM. The reactions were carried out at 30°C and pH 8.0.

Table 3-1. Activity of native ACC deaminase from *P. putida* UW4, native D-cysteine desulfhydrase from tomato and the mutants of both enzymes.

	ACC deaminase activity (nmol α-ketobutyrate/min/mg)	**D-cysteine desulfhydrase activity (nmol pyruvate/min/mg)**
ET30-acd	Activity below detection	5784.20 ± 9.0
ET30-Tm1 (S358E)	Activity below detection	Activity below detection
ET30-Tm2 (T386L)	11.43 ± 2.75	665.33 ± 13.4
ET30-Tm3 (S358E/T386L)	59.88 ± 2.00	Activity below detection
ET30-UW4	2811.75 ± 76.42	15.94 ± 0.09
ET30-Um1 (E295S)	8.19 ± 0.45	35.6 ± 1.68
ET30-Um2 (E295S/L322T)	Activity below detection	147.47 ± 9.42

	Km (mM)	**k_{cat} (min^{-1})**	**k_{cat}/Km (mM^{-1} min^{-1})**
ET30-acd + D-Cys	0.21	230	1095
ET30-UW4 + ACC[†]	3.4	146	42.9
ET30-Um2 (E295S/L322T) + D-Cys	0.34	10.9	32.1

[†] Hontzeas et al., 2004

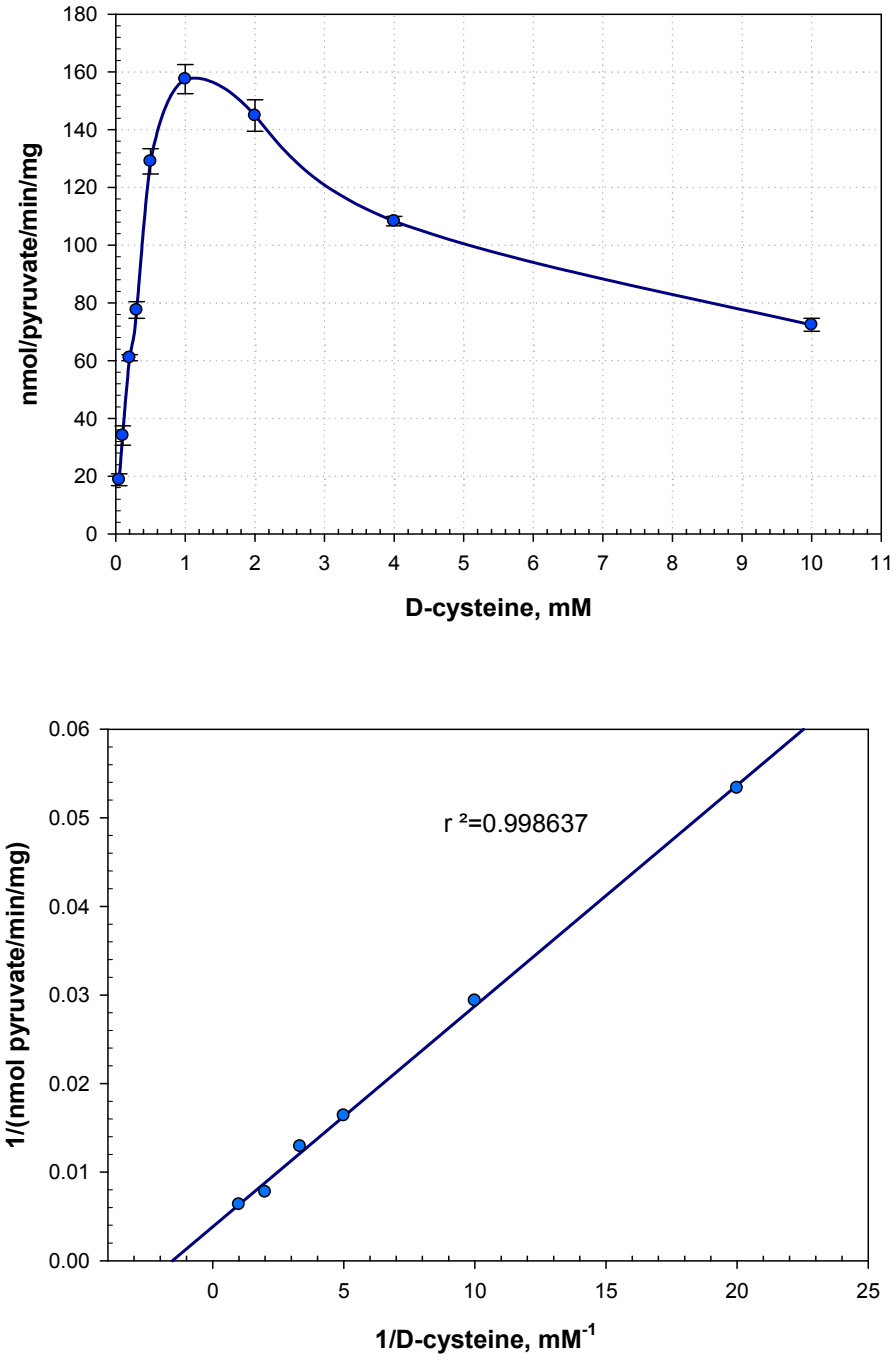


Figure 3-18. Kinetic analysis of the ET30-Um2 mutant (E295S + L322T) of *P. putida* UW4 ACC deaminase. Top plot: Michaelis-Menten plot; effects of substrate concentration on the activity of the enzyme. Bottom plot: Lineweaver-Burk plot of the data.

Chapter 4
Discussion

The possibility that plants encode a functional 1-aminocyclopropane-1-carboxylate deaminase (ACC deaminase) has been investigated previously (McDonnell et al., in press). In that instance the focus was on the model plant *Arabidopsis thaliana*, and a gene that showed some sequence similarity to bacterial ACC deaminases (Accession # NM_103738). Riemenschneider and co-workers (2005) isolated what appeared to be the ACC deaminase gene and found that the enzyme encoded by this gene does not have the ability to break the cyclopropane ring of ACC, but rather it catalyzes the conversion of D-cysteine into pyruvate, ammonia and hydrogen sulfide. On the other hand, the gene isolated by Riemenschneider et al., (2005) differs from the putative *Arabidopsis* ACC deaminase gene by five amino acids (McDonnell et al., in press). Moreover, McDonnell et al. (in press) demonstrated that transgenic *Arabidopsis* lines that over-express this gene no longer respond with the characteristic triple-response when seedlings are grown in presence of the ethylene precursor ACC. This finding has suggested that either *Arabidopsis* contains two enzymes with similar sequences but different activities or that besides having the D-cysteine desulfhydrase activity, the enzyme in question may also have a low level of ACC deaminase activity. To determine whether other plants have a functional ACC deaminase, we have focused on the tomato (*Solanum lycopersicum*) homologue of the above mentioned *Arabidopsis* enzyme.

4.1 The Gene

Here, a tomato (Bony Best variety) cDNA was isolated using primers that were designed based on the GenBank published sequence for a putative tomato ACC deaminase (Accession # EU639448). The isolated cDNA has an ORF of 1278 bp encoding a predicted protein of 425 amino acids and 46.51 kDa. Comparison between the GenBank published sequence (Accession # BT013578) and the isolated sequence (Accession # EU639448) reveals some differences (Figure 3-3). Starting with Bony Best tomato and using a PCR protocol where primers were designed to specifically amplify, from leaves or breaker fruit, either the BT013578 version or the version isolated (Figure 2-1), only the expression of the isolated version was observed (Figure 3-5). Whether the BT013578 version of the gene is expressed in other tomato tissues is unknown, but unlikely, since BT013578 cDNA was originally isolated from fruit tissue. The expression of this gene may be variety specific.

The use of available EST databases has also proven a useful tool in deciphering the expression patterns of the two versions. When GenBank, EMBL and DDBJ databases are searched, ESTs identical to the isolated sequence (in the region where the difference between BT013578 and EU639448 occurs) are present and come from callus, flower, leaf and fruit tissue. On the other hand, ESTs that are identical to cDNA for the putative ACC deaminase from GenBank (BT013578; with the extra 7 bp and lacking 31 bp that occur in EU639448 (Figure 3-2)) were not found. Moreover, a multiple sequence alignment has illustrated that the isolated version of the cDNA (EU639448) is more similar to homologues from other plants, both at the nucleotide and protein level (Figure 3-4).

4.2 Kinetic Characterization of the Recombinant Enzyme

It has been reported that the *Arabidopsis* homologue of the tomato recombinant enzyme has the ability to break down the ethylene precursor ACC. This is based on the ability of transgenic plants over-expressing the cDNA to grow on medium supplemented with ethylene precursor ACC without displaying the ethylene triple response (McDonnell et al., in press).

Given the uncertainty that exists regarding the nature of putative ACC deaminase from plants, the recombinant protein for putative ACC deaminase from tomato was tested for both ACC deaminase and D-cysteine desulfhydrase activity. Both enzymatic activities were determined through a coupled assay with lactate dehydrogenase (LDH), where the disappearance of NADH (the co-enzyme for LDH) was monitored at 340 nm. With the enzyme from tomato, when ACC was provided as the substrate at various concentrations, no change in absorbance at 340 nm was observed, indicating that the enzyme does not have the ability to break the cyclopropane ring of ACC and thus is not a functional ACC deaminase. However, when D-cysteine is used as a substrate, a rapid decrease in absorbance at 340 nm was observed, indicating that the enzyme was a D-cysteine desulfhydrase, breaking down D-cysteine into pyruvate, ammonia and hydrogen sulfide.

The catalytic activity of the His-tagged recombinant enzyme compares well with other D-cysteine desulfhydrase enzymes from eukaryotes as well as microorganisms (Nagasawa et al., 1985; Riemenschneider et al., 2005; Soutourina et al., 2001). The observed Km value of 0.21 ± 0.05 mM is similar to that reported for the homologue from *Arabidopsis* (Km=0.25 mM), and to the *E. coli* D-cysteine desulfhydrase where the Km was reported to

be 0.15 mM or 0.3 mM for the recombinant and purified enzyme, respectively (Nagasawa et al., 1985; Soutourina et al., 2001). As for the k_{cat}, D-cysteine desulfhydrase from tomato is similar to its *Arabidopsis* homologue. The reported value for the *Arabidopsis* enzyme is 360 min^{-1}, and that estimated for the tomato enzyme is 230 min^{-1}.

Optimal activity for tomato D-cysteine desulfhydrase was observed at pH 7.6 in phosphate buffer, with almost complete loss of activity at the lowest tested pH of 6.2 and the highest tested pH of 9.8 (Figure 3-9). The pH optima for the D-cysteine desulfhydrase-catalyzed reaction has been reported as pH 9.0 in *E. coli*, whereas pH of 8.5 for the *Chlorella fusca* and *Spinacia oleracea* (Nagasawa et al., 1985; Schmidt, 1982; Schmidt and Erdle, 1983). The tomato enzyme is again most similar to the *Arabidopsis* homologue which has a pH optimum of 8.0 (Riemenschneider et al., 2005). The data from different organisms indicates that this reaction is carried out at various pH values.

Tomato D-cysteine desulfhydrase is a stable enzyme as evident by a broad temperature-rate profile (Figure 3-10). The enzyme has a temperature optimum of 30°C, with relatively high activity remaining at 50°C. The stability of the enzyme correlates well with the reports for the *E. coli* D-cysteine desulfhydrase, where only 11%, 24% and 40% of activity was lost when the enzyme was incubated at 65, 70 and 75°C, respectively (Nagasawa et al., 1985). Contradictory results were reported for the *Arabidopsis* homologue, where complete loss of activity occurs at 60°C (Riemenschneider et al., 2005). Perhaps, the method of storage of purified enzyme may play a role in the different observations on enzymatic stability. Both *E. coli* and tomato enzymes were stored in a similar buffer containing glycerol, whereas the *Arabidopsis* enzyme was stored without glycerol.

The tomato enzyme was predicted to require pyridoxal phosphate as a co-factor. Hence, the activity of the enzyme was investigated in presence of the PLP inhibitor, aminooxyacetic acid (AOA). AOA is a competitive inhibitor, serving as a α-methyl analogue of the substrate. Incubation of the enzyme with various concentrations of AOA for 15 min at 30°C inhibits the activity (Figure 3-11); a Ki value of 3.3 μM was observed. This data is consistent with the enzyme being classified as a pyridoxal phosphate dependent enzyme.

4.3 D-cysteine Desulfhydrase: Its Role in Microorganisms and Plants

The breakdown of L-cysteine by an L-cysteine desulfhydrase in higher plants was first reported by Harrington and Smith (1980). They used cultured tobacco cells and found that sulfide and pyruvate were produced from L-cysteine. The existence of L-cysteine desulfhydrase was later observed in other plant species (Rennenberg, 1983; Rennenberg et al., 1987; Rennenberg and Filner, 1983; Schutz et al., 1991). Additionally, in several organisms D-cysteine desulfhydrase activity, converting D-cysteine into pyruvate, ammonium and hydrogen sulfide has also been measured (Nagasawa et al., 1985; Nagasawa et al., 1988). A similar activity was detected in the green alga *Chlorella fusca*, and in several plant species including *Spinacia oleracae, Cucurbita pepo, Cucumis sativus* and *Nicotiana tabacum* (Rennenberg, 1983; Rennenberg et al., 1987; Schmidt, 1982; Schmidt and Erdle, 1983).

The *E. coli* D-cysteine desulfhydrase is one of the best characterized enzymes with this activity; it is a PLP-dependent enzyme, catalyzing the α,β-elimination reaction of D-cysteine and of several D-cysteine derivatives (Nagasawa et al., 1985; Soutourina et al.,

2001). The enzyme also catalyzes the β-replacement reaction of β-chloro-D-alanine in the presence of a high concentration of various thiols or from *O*-acetyl-D-serine and H_2S to form D-cysteine or D-cysteine-related amino acids (Nagasawa et al., 1985). *E. coli* growth is impaired in the presence of micromolar amounts of D-cysteine (Soutourina et al., 2001). Over-expression of D-cysteine desulfhydrase protects *E. coli* against D-cysteine, whereas its inactivation renders the bacterium hypersensitive to this D-amino acid. Therefore, the involvement of the enzyme in enabling the bacterium to thrive in the presence of D-cysteine has been established. It has been suggested that D-cysteine exerts its toxicity through inhibition of threonine deaminase, the key enzyme in isoleucine, leucine and valine biosynthesis pathway. The presence of this enzyme also stimulates cell growth when D-cysteine is provided as a sole sulfur source, where the expression of the enzyme is induced under sulfur limiting conditions.

Some studies have examined the distribution of D-cysteine desulfhydrase activity in other bacterial strains. Nagasawa et al. (1985) tested 10 strains of *E. coli* and 52 other bacterial strains for their ability to catalyze the α,β-elimination of D-cysteine to form pyruvate and the β-replacement reaction of β-chloro-D-alanine and sodium hydrosulfide to form D-cysteine. These two activities were found in *E. coli*, *Citrobacter freundii*, *Klebsiella pneumoniae*, and *Enterbacter cloacae*. On the other hand, no activity was found in the bacteria of genera *Arthrobacter*, *Alcaligenes*, *Agrobacterium*, *Bacillus*, *Brevibacterium*, *Corynebacterium*, *Erwinia*, *Flavobacterium*, *Micrococcus*, *Proteus*, *Pseudomonas*, *Salmonella*, *Sarcina*, *Serratia*, and *Santhomonas* (Nagasawa et al., 1985).

The first report of D-cysteine desulfhydrase activity in tomato has been described in this work. The same activity has been detected in several other plant species, however, the role of this enzyme in plants is not clear. It has been well documented that in general, amino acids are used in the L-form, and the enzymes involved in their metabolism are specific for the L-enantiomers. However, D-amino acids are distributed widely in living organisms, e.g. in unprocessed vegetables and fruit about 0.5-3% of D-amino acids are present relative to L-enantiomers (Bruckner and Westhauser, 2003; Friedman, 1999). Nevertheless, synthesis of D-amino acids in plant tissues has not been described, but is assumed to occur (Riemenschneider et al., 2005). It is thought that several enzymes might be synthesizing D-amino acids from L-amino acids such as racemases, transaminases and amino acid oxidases. The occurrence of D-amino acid aminotransferase has been reported in pea seedlings(Ogawa et al., 1973).

Information on the possible role of this enzyme in plants comes from the studies with the *Arabidopsis* homologue (Riemenschneider et al., 2005). It has been shown that the enzyme is a nuclearly encoded protein transported into the mitochondria. Previous experiments have demonstrated the highest specific activity in cytoplasm and mitochondria (Burandt et al., 2001). In *Cucurbita pepo* plants the highest D-cysteine desulfhydrase activity was localized in the cytoplasm, with small amounts of activity present in mitochondria and chloroplasts (Rennenberg et al., 1987). Localization of L-cysteine desulfhydrase activity was also reported exclusively to mitochondria and chloroplasts. Based on a computer analysis predicting the intracellular localization of proteins in plant cells (iPSORT, TargetP, Predotar,

MITOPROT and ChlorP from the http://www.expacy.ch/tools website), the localization of the tomato D-cysteine desulfhydrase enzyme is unclear.

To gain more insight into the role of D-cysteine desulfhydrase in *Arabidopsis*, Riemenschneider et al. (2005) examined RNA and protein levels in developing plants. The RNA levels were shown to increase in developing *Arabidopsis*, but decreasing levels in senescent plants were observed. The protein levels remained unchanged, with the specific activity highest in senescent plants. Plants grown under low sulfate concentrations showed increased D-cysteine desulfhydrase RNA and protein levels. Similar to the role of *E. coli* D-cysteine desulfhydrase, the plant encoded enzymes may be involved in providing the plant with essential sulfur when the environmentally provided concentrations are too low.

Based on limited data, a number of other functions of this enzyme have been proposed. For instance, the biosynthesis of cysteine may be specific for the L-isomer and degradation might occur via the corresponding D-amino acid. The separation could facilitate the regulation of synthesis and degradation by compartmentalization of amino acid concentration without a special compartment (Schmidt, 1982). Other possible roles such as the involvement of the enzyme in certain biosynthetic routes which use D-amino acids have been proposed; certain D-amino acids could act as signals for specific regulatory mechanisms, then be degraded by enzymes such as D-cysteine desulfhydrase (Schmidt, 1982). In addition, the enzyme could also be involved in detoxification mechanisms. For instance, the phytotoxic peptide malformin, produced by *Aspergillus niger*, contains D-cysteine, such that D-cysteine desulfhydrase may be involved in detoxification of malformin

and its components (Riemenschneider et al., 2005). The tomato D-cysteine desulfhydrase may therefore, have a similar function.

It has also been hypothesized that cysteine desulfhydrase enzymes may be a part of what is known as sulfur-induced resistance (SIR). The role of sulfur in the resistance of crops to disease is apparent, where a number of field trials have demonstrated that an adequate sulfur supply through fertilization can increase the resistance of plants to fungal pathogens (Papenbrock et al., 2007). The term sulfur-induced resistance was coined to describe the phenomenon, however, the molecular basis of SIR is still largely unknown. Sulfur-containing compounds in plants which might be involved in SIR range from elemental sulfur to complex proteins; thiols, glucosinolates, cysteine-rich proteins, phytoalexins, elemental sulfur and H_2S may all be involved. (Bohlmann and Apel, 1991; Giamoustaris and Mithen, 1997; Kliebenstein, 2004). All of these most likely employ different mechanisms in defense of the plant against pathogens and herbivores. For example, it has been demonstrated that H_2S is cytotoxic to some pathogens and that a relationship between increasing H_2S emission and resistance of crops to pests and disease is possible (Beauchamp et al., 1984). Cysteine desulfhydrases have been proposed to be good candidates as H_2S-relasing enzymes which may, therefore, be involved in the pathways of plant defense (Papenbrock et al., 2007).

Bloem and co-workers (2004) have used canola plants (*Brassica napus*) to demonstrate a correlation between sulfur status, pathogen infection, and cysteine desulfhydrase activity. In field trials, they have shown that sulfur fertilization increases the contents of total sulfur, sulfate, organic sulfur, cysteine and glutathione in plants, but decreases the L-cysteine desulfhydrase activity. Additionally, infection of canola plants with

Pyrenopeziza brassicae, pathogenic fungi, increases cysteine and glutathione content and increases L-cysteine desulfhydrase activity. Hence, canola plants react to a fungal infection by increasing their potential to release H_2S (Bloem et al., 2004; Papenbrock et al., 2007). The molecular and enzymatic basis for cysteine degradation and H_2S release are mainly attributed to the activity of L-cysteine desulfhydrase, and possibly D-cysteine desulfhydrase.

4.4 PLP-dependent Enzymes: ACC Deaminase and D-Cysteine Desulfhydrase

Both ACC deaminase and D-cysteine desulfhydrase have been shown to belong to a diverse pyridoxal phosphate family of enzymes. The two enzymes share sequence similarity, but have different activities. Enzymes that are PLP dependent catalyze a diverse number of reactions, acting upon amino acids and their derivatives as substrates. They are essential in linking the carbon and nitrogen metabolism, they are principally involved in biosynthesis of amino acids and are also found in biosynthetic pathways of amino sugars and other amine-containing compounds. PLP is one of the most versatile co-enzymes, comparable to zinc. Even though this group of enzymes carry out diverse reactions, they all share mechanistic features. In all of these enzymes, PLP is bound covalently via an imine bond to the ε-amino group of a lysine residue, forming what is referred to as "internal" aldimine. As a first step of all PLP enzyme catalyzed reactions, the amino group of the incoming substrate replaces the ε-amino group to form a co-enzyme substrate imine. This complex is referred to as an "external" aldimine and is common to all enzymatic and non-enzymatic reactions of PLP with amino acids. In the following step, different reaction pathways diverge; reaction types

can be divided according to the position on the substrate where the reaction occurs. Reactions at the α position include transamination, decarboxylation, racemization, and elimination and replacement of an electrophilic R group (Eliot and Kirsch, 2004). Those at the β and γ position include elimination or replacement. Exception to the common types of reactions includes the formation of cyclopropane ring from S-adenosyl-methionine, catalyzed by ACC synthase (Adams and Yang, 1979), and the cleavage of ACC to α-ketobutyrate and ammonia catalyzed by ACC deaminase. In all of the above mentioned reactions the co-enzyme acts as an electron sink, storing electrons from cleaved substrate bonds, then dispensing them for the formation of new linkages with incoming protons or second substrates.

PLP-dependent enzymes are classified into four major groups based on their structure. Early on it was postulated that the structures of PLP enzymes would correlate with the reaction type (Alexander et al., 1994), but it has since been found that each of the structural classes contains representatives of multiple reaction types. These are: *aspartate aminotransferase family*, the largest and most diverse group; *tryptophan synthase family* (TRPSβ) which is similar to the aspartate aminotransferase family but is evolutionarily distinct; *alanine racemase family*, strikingly different from other groups; and *D-amino acid aminotransferase family*, which is similar to the aspartate aminotransferase family and trypophan synthase family. According to this classification, ACC deaminase fits into the tryptophan synthase (TRPSβ) family; structural information for D-cysteine desulfhydrase enzymes is not available and hence no absolute classification can be made. However, based on the sequence similarity to ACC deaminases and an ACC deaminase homologue (Fujino et

al., 2004; Karthikeyan et al., 2004b; Yao et al., 2000), it is likely that this enzyme also belongs to the TRPSβ family.

Evolutionary relationships among PLP-dependent enzymes have been extensively examined (Christen and Mehta, 2001). It is believed that reaction types generally evolved first within each fold type, followed by narrowing substrate specificity. Due to its many diverse member enzymes, the evolutionary pedigree of the aspartate aminotransferase family is most informative for deducing how functional specialization occurred during molecular evolution. Analysis of this family clearly shows that the ancestor protein first diverged into reaction-specific enzymes, followed by the last and shortest phase in the development of the modern enzymes with specialization for substrate specificity (Christen and Mehta, 2001). Evolutionary preference for reaction specificity over substrate specificity is also observed in other cases, for instance, in families of proteinases or sugar kinases (Perona and Craik, 1997).

Researchers have used site-directed mutagenesis and directed molecular evolution to successfully simulate the specialization for substrate and reaction specificity that may occur. For instance, the conversion of tyrosine phenol-lyase (aspartate aminotransferase family) to glutamate/aspartate β-lyase, an enzyme not found in nature, was achieved by a double point mutation in the active site (Mouratou et al., 1999). The activity of the newly generated enzyme is orders of magnitude lower than that of an average PLP-dependent enzyme, with the k_{cat} of 0.2 s^{-1}(or 12 min^{-1}). Further optimization of other amino acid residues, including non-active site residues would be required to improve the catalytic potency of the enzymes. This example is only one of several that have been recently published, demonstrating how mutagenesis of a few important amino acid residues in an active site of a PLP-dependent

enzyme can result in a change of reaction type and substrate specificity (Graber et al., 1999; Oue et al., 1999). This kind of change may have occurred during evolution, such that an ancestor protein either evolved into an ACC deaminase or D-cysteine desulfhydrase. Hence, we have also utilized site-directed mutagenesis as a tool to establish why certain enzymes predicted to be an ACC deaminase encode a functional ACC deaminase and others, as in the case of tomato D-cysteine desulfhydrase, do not.

4.4.1 ACC Deaminase Reaction and Requirements for Activity

The first step of the ACC deaminase reaction is common with all other PLP-dependent enzymes. However, after the "external" aldimine formation between PLP and the substrate, the common mechanisms cannot be applied to the ACC deaminase reaction since ACC as a substrate does not have an α-hydrogen and the reaction must be initiated without the accessibility to an α-carbanionic intermediate (reviewed in Hontzeas et al., 2006). The mechanism of ACC cleavage is still not fully understood, with two proposed pathways, either nucleophilic addition to open the ring followed by β-proton abstraction or direct β-proton abstraction leading to ring cleavage (Walsh et al., 1981). In the nucleophilic addition followed by β-proton abstraction mechanism, it is proposed that nucleophilic attack by an amino acid residue occurs on the pro-S β-carbon of ACC which initiates the cyclopropane ring opening, followed by β-proton abstraction at the pro-R carbon by a basic active site residue, such as the lysine residue that binds PLP (Zhao et al., 2003). The model for direct β-proton abstraction was proposed by Ose and co-workers (2003) where following direct β-proton abstraction a quinonoid intermediate undergoes rearrangements, which eventually

leads to hydrolysis into the respective products. There seems to be more experimental data, and more methodical explanation in terms of the chemistry and thermodynamic favorability for the nucleophilic addition and β-proton abstraction mechanism (Hontzeas et al., 2006).

Comparison of ACC deaminase with other PLP-dependent enzymes of TRPSβ family whose structures have been solved reveals similarities, such as the overall fold topology, despite the fundamentally different chemistries of the reactions carried out (Yao et al., 2000). However, the environment surrounding PLP is different from those of other PLP-dependent enzymes. For instance, at the front side of the PLP pyridine of the *H. saturuns* ACC deaminase, the extra loops bury the PLP deep in the interior of the molecule. Another major difference between ACC deaminase and other members of TRPSβ family is the stacking of the phenol group of Tyr295 (according the *H. saturnus* numbering) to the plane of the pyridinium ring at an angle of about 20° (Yao et al., 2000; Figure 3-14). This kind of stacking is the first occurrence in the TRPSβ family, however it has been found in other types of PLP-dependent enzymes.

4.4.2 Comparing ACC Deaminase to the *P. horikoshii* homologue

Most of our understanding of the mechanisms employed by ACC deaminase to break the cyclopropane ring of ACC comes from the structural studies of true ACC deaminases from the bacterium *Pseudomonas* sp. ACP and the yeast *Hansenula saturnus* (Karthikeyan et al., 2004b; Yao et al., 2000). The essential residues for the catalysis and ACC recognition were derived from these structures, in addition to site-specific mutagenesis studies to confirm the essential residues (Ose et al., 2003). Most of the important residues are conserved

between the bacterial and yeast ACC deaminase (Figure 3-14); for instance, the lysine residue that binds PLP, the tyrosine residue that stacks with the pyridine ring, and residues important in recognizing ACC are all conserved. The bacterial and yeast active sites are thus virtually identical (Figure 3-14).

The protein PH0054 from *Pyrococcus horikoshii* OT3 was predicted to be an ACC deaminase due to its sequence similarity to other ACC deaminase enzymes. PH0054 preserves the key amino acid residues; however, it does not show ACC deaminase activity, but rather has deaminase activity toward D- and L-serine (Fujino et al., 2004). The three-dimensional structure of this ACC deaminase homologue has also been reported and the comparison between the yeast and PH0054 structures provides information on the structural requirements for the enzymatic activity of ACC deaminase. The overall topology of the PH0054 structure is very similar to that of yeast ACC deaminase, the main difference being the lack of loop regions that are thought to play a role in stabilizing the small and PLP-binding domain of the yeast ACC deaminase (Fujino et al., 2004). The environment around PLP is similar in the *P. horikoshii* homologue to the true ACC deaminases; e.g. the pyridine ring of PLP is stably stacked by the aromatic ring of Tyr282, a feature of a true ACC deaminase. The substitutions around the active site are only His80(*P. horikoshii*)-Gln77(*H. saturnus*), Thr283(*P. horikoshii*)-Glu296(*H. saturnus*) and Thr308(*P. horikoshii*)-Leu323(*H. saturnus*) (Figure 3-14). The side chain directions of His80 and Gln77 are different; the Thr308 residue is located within hydrogen bonding distance of the pyridine nitrogen atom of PLP. The corresponding *H. saturnus* residue, Leu323, has completely different characteristics and is positioned differently. The carboxylate oxygen atom of Glu296 in the

H. saturnus structure makes a hydrogen bond with pyridine nitrogen atom. The existence of the carboxylate group at this position is widely seen in PLP-dependent enzymes, except in other members of the TRPSβ family. In the TRPSβ family, a hydroxyl group such as a serine or threonine side-chain is always found within hydrogen-bonding distance of the pyridine nitrogen atom. The side-chain of the corresponding residue in the homologue, Thr283, is oriented in the opposite direction such that the hydroxyl group cannot interact with the pyridine nitrogen atom (Figure 3-12, Figure 3-14)

4.5 3D Modeling of Tomato Enzyme and Implications for Activity

There is no structural information available for D-cysteine desulfhydrase from tomato or any other organism. Therefore, the amino acid residues that form the active site have not been described. It is, however, known that the catalyzed reaction to form pyruvate, ammonia and H_2S from D-cysteine involves an α,β-elimination. We have therefore used proteins which share sequence similarity to D-cysteine desulfhydrase and whose tertiary structures are known, to predict the structure and active site residues for the tomato enzyme characterized in this work. True ACC deaminase proteins from bacteria and fungi share ~20% sequence identity at the amino acid level to the tomato enzyme; the above described ACC deaminase homologue from *P. horikoshii* is ~30% identical at the protein level. Therefore, the homologue was chosen as the template for structural predictions.

A sequence identity between 20 and 40% is considered low or borderline when constructing protein models, however, with careful assessment and treatment of individual cases, protein structures may be successfully predicted (Kuiper et al., 2001; Marabotti et al.,

2004). The largest problem with low sequence identity between the template and subject is that these sequences may be aligned in different ways with similar scores, and gaps may fall in the middle of secondary structure elements or represent loops, leading to a high probability of producing incorrect models. Even so, when proteins used for alignment and modeling belong to the same family, where the structure is well conserved, functional information and overall structure similarity can overcome the low sequence identity and a good sequence alignment suitable for structure modeling can be built. Furthermore, information such as the position of secondary structure elements may be used to verify the quality of the alignment, and if necessary to optimize the position of any gaps. Based on a multiple sequence alignment and the position of secondary structure elements, the *P. horikoshii* homologue was confirmed as a good template for structural modeling.

The structure of D-cysteine desulfhydrase from tomato is shown in Figure 3-13. The overall topology of the enzyme is predicted to be well conserved compared both to the template (*P. horikoshii* ACC deaminase homologue) and to the true ACC deaminase from *H. saturnus*. This suggests that the enzyme, as mentioned previously, is most likely a member of the tryptophan synthase family (TRPSβ). More notably, an analysis of the putative active site reveals the possible changes from the true ACC deaminase enzymes that may have an impact on the activity (Figure 3-14). Based entirely on an analysis of the primary sequence, important residues appear to be conserved. These include the Lys117 residue that binds the co-enzyme, Lys120 (Lys57 in *P. horikoshii*), and the active site tyrosine residues Tyr331 and Tyr357 (Tyr256 and Tyr282 in *P. horikoshii*). One major difference is the position of His80 of *P. horikoshii*; at this position the tomato D-cysteine desulfhydrase appears to be more like

a true ACC deaminase with Gln143 (Figure 3-14). Additionally, the tomato enzyme shares comparable differences to true ACC deaminase enzymes as does the *P. horikoshii* homologue; at the position of Glu296 and Leu323 in *H. saturnus*, the *P. horikoshii* enzyme contains two threonine residues (Thr282 and Thr308). The tomato enzyme contains a serine residue (Ser358) at the position of Glu296 (from yeast), and a threonine (Thr384) residue at the position of Leu323.

Tertiary structure modeling has revealed that the above described conserved residues indeed occur in the putative active site (Figure 3-14). However, one difference is predicted to occur at the position of Tyr331. This residue is found somewhat further away from the active site, such that it should not play a role in catalysis. When this residue was mutated in a true ACC deaminase, the enzyme lost 90% of its activity (Ose et al., 2003). Considering that residue positioning is based on protein structure modeling, it cannot be ruled out that this observation may be an artifact of the structural modeling procedure.

Researchers have used structural data from the *P. horikoshii* homologue as a means to suggest why this enzyme is inert towards ACC as a substrate (Fujino et al., 2004). Having established that D-cysteine desulfhydrase catalyzes a similar reaction to the *P. horikoshii* homologue, and that the two are predicted to have similar active sites (more similar to each other than either is to a true ACC deaminase), the same principles can be applied to the inertness of the tomato enzyme. Fujino and co-workers (2004) have shown that the common step to all PLP enzymatic reaction, the formation of "external" aldimine occurs between the inert enzyme and ACC. They have also established through the analysis of the enzyme-ACC complex that conformational changes or domain closure occur upon ACC binding, which is

common to many enzymes. This suggests that the homologue, and most likely D-cysteine desulfhydrase, binds ACC but cannot abstract the β-proton from the pro-R/S carbon atom of ACC. Rather, these enzymes have the ability to abstract an α-proton from L- or D-serine in the case of the homologue and the α-proton of D-cysteine in the case of D-cysteine desulfhydrase. It has been proposed that the electron density of the ACC cyclopropane ring is influenced by the pyridine ring of PLP through an external aldimine group and is different in the homologue of ACC deaminase than in yeast or bacterial ACC deaminase. The charge density of the pyridine ring is strictly modulated by various neighboring amino acids. The nitrogen atom of the pyridine ring in a true ACC deaminase is within hydrogen bonding distance of the side-chain carboxyl oxygen atom of glutamate (Glu296 in yeast), which is not the case in the homologue or in D-cysteine desulfhydrase. This creates a different environment in the active site, such that D-cysteine desulfhydrase and the homologue are more similar to the other members of the TRPSβ family such as cystathionine β-synthase, threonine deaminase or threonine synthase. Additionally, true ACC deaminase enzymes have a leucine residue in the active site (Leu323 in yeast) which provides space for the long side-chain of the glutamate residue by orienting itself in the opposite direction (Fujino et al., 2004). It is therefore likely that the major difference that occurred during the evolutionary process to distinguish between ACC deaminase activity and D-cysteine desulfhydrase activity is at the position of these two amino acids. Hence, mutating the two positions of a true ACC deaminase (Glu296 and Leu323 in *H. saturnus*, for instance) to resemble the predicted active site of a D-cysteine desulfhydrase should result in this enzyme becoming a D-cysteine desulfhydrase. In addition, mutating these residues in D-cysteine desulfhydrase

(Ser358 and Thr384 to glutamate and leucine, respectively) should enable the enzyme to acquire ACC deaminase activity.

4.6 Changing the Activity of the ACC Deaminase and D-cysteine Desulfhydrease

Five different mutants were constructed with the aim of obtaining double mutants of both D-cysteine desulfhydrase and *P. putida* UW4 ACC deaminase. S358E, T386L and the double mutant S358E/T386L of the tomato D-cysteine desulfhydrase was constructed through site-directed mutagenesis. In addition, two *P. putida* UW4 ACC deaminase mutants were created; these include E295S and a double mutant E295S/L322T. The active site of the double mutant of the D-cysteine desulfhydrase is predicted to resemble an ACC deaminase, whereas, the mutation of the glutamate and leucine to a serine and a threonine residue should create an enzyme whose active site looks like that of D-cysteine desulfhydrase. The double mutant of the *P. putida* UW4 ACC deaminase should be more similar to the D-cysteine desulfhydrase than the *P. horikoshii* homologue, due to the mutation of the glutamate residue to a serine, instead of a threonine that is present in the *P. horikoshii* homologue.

The mutated recombinant proteins were purified and tested for their ability to use either D-cysteine or ACC as the substrate. As seen in Figure 3-15, the S358E mutation of the tomato enzyme results in complete loss of activity towards D-cysteine as the substrate; this is true for both the single S358E mutant, and the double mutant S358E/T386L. The change of the characteristic serine residue common to other members of TRPSβ family renders the enzyme completely inactive. This may indicate that unlike the Thr283 residue of the *P.*

horikoshii homologue, the serine of the tomato enzyme residue may be involved in hydrogen bonding with the nitrogen atom of the pyridine ring of PLP. Mutation of the threonine residue that is characteristic of both tomato D-cysteine desulfhydrase and the *P. horikoshii* homologue (involved in hydrogen bond formation between pyridine ring nitrogen atom in the homologue), to a leucine residue characteristic of ACC deaminase enzymes causes a loss of activity without completely abolishing it. Approximately 11.5% of the original activity is retained. Therefore, the serine residue most likely influences the pyridine ring through hydrogen bonding, and hence the electron density of the substrate, whereas the threonine residue is most likely not hydrogen bonded with the co-enzyme, but it still influences activity.

It has previously been shown that mutation of Glu296 in yeast ACC deaminase results in a complete loss of activity (Ose et al., 2003). The same is true for the *P. putida* UW4 ACC deaminase, since the E295S mutant shows no significant activity (Figure 3-15). Again, this illustrates the necessity of the hydrogen bonding between the glutamate side-chain and the nitrogen atom of the pyridine ring. The double mutant (E295S/L322T) of the UW4 enzyme also shows undetectable activity towards ACC as the substrate (Figure 3-15).

When analysis of the mutant proteins for the ability to perform a new catalytic activity was examined, the double mutant of tomato D-cysteine desulfhydrase (S358E/T386L mutation) shows over a 50-fold increase in activity towards ACC as the substrate (Figure 3-16, Table 4). The same mutant shows no detectable activity towards the original D-cysteine substrate. Additionally, only a very low and insignificant level of activity was detected when D-cysteine was used in a reaction with the wild-type UW4 ACC deaminase, but an activity

increase of approximately 10-fold was observed with the double mutant E295S/L322T (Figure 3-17, Table 4). The same double mutant lost the ability to break the cyclopropane ring of ACC, illustrating that these two amino acids represent the major difference between ACC deaminase and D-cysteine desulfhydrase activity.

The UW4 double mutant was further characterized to determine whether the enzyme shows typical Michaels-Menten kinetics. Based on non-linear regression, the Km of the enzyme for D-cysteine was determined to be 0.34 ± 0.1 mM, which is stronger than the binding of the native ACC deaminase for ACC, where the Km is 3.4 mM (Hontzeas et al., 2004). In fact, the Km of the mutant is comparable to the Km of a true D-cysteine desulfhydrase enzyme (Nagasawa et al., 1985; Soutourina et al., 2001; Riemenschneider et la., 2005). The inefficiency of the UW4 mutant enzyme is illustrated by k_{cat} value of 10.9 min^{-1}. Similar observations have been made in other studies where the k_{cat} of a newly created activity was low, which suggests that during the evolutionary process changes in other amino acid residues need to occur to render the enzyme more active. The UW4 ACC deaminase catalytic efficiency with ACC as the substrate has previously been determined to be 42.9 mM^{-1} min^{-1} (Hontzeas et al., 2004). The double mutant of UW4 ACC deaminase has a similar catalytic efficiency for D-cysteine of 32 mM^{-1} min^{-1}.

The change in the reaction type and substrate specificity of D-cysteine desulfhydrase and ACC deaminase through mutation of just two amino acids, as shown in this work, illustrates that through evolution the change in the two residues may have occurred in an ancestral protein, such that two different activities arose. Other changes must have subsequently occurred to improve upon the efficiency of the enzyme.

4.7 Conclusion

With the availability of sequences from both microorganisms and plants, many have been annotated as a putative ACC deaminase. However, only a portion of these have been shown to have ACC deaminase activity, and others such as the enzyme from tomato characterized in this study use other substrates. The question arises why certain organisms have ACC deaminase activity and others do not despite the fact that they both have genes encoding for putative ACC deaminase.

The puzzling reaction of ACC deaminase has led researchers to determine its tertiary structure, and the structure of an ACC deaminase homologue that does not have the activity. With the availability of structural data, amino acid residues necessary for activity have been described, yet the exact mechanism of the reaction is still unknown. We have shown that two important amino acid residues may distinguish between ACC deaminase and D-cysteine desulfhydrase activity. Since other plant putative ACC deaminase enzymes share virtually identical amino acid residues that are predicted to be important, especially the serine residue at the position of glutamate in a true ACC deaminase and threonine at the position of leucine in a true ACC deaminase (Figure 3-12), these enzymes are all predicted to be unable to break the cyclopropane bond of ACC. They also share high sequence similarity to the tomato D-cysteine desulfhydrase, and hence will most likely encode a functional D-cysteine desulfhydrase. As for other organisms predicted to have a functional ACC deaminase, all that lack the glutamate or leucine will most likely also lack this activity. These enzymes should have the ability to deaminate other amino acids such as serine or cysteine.

Appendix

Figure 6-1. The insert sequence from pBS-acd construct (Accession # EU639448). The insert was sequenced from both directions, using vector specific primers (T7 and T3 promoter primers) as well as sequence specific primers.

>gi|47104993|gb|BT013578.1| Lycopersicon esculentum clone 132323R, mRNA sequence

GAAAGGGGAAAGGTTAAAA**ATG**TCGAGTTGCCAATGGAGTAGCTTCACTAGAGTATCACTATCTCCATTT
CCCTTGCAGCCAGCACAACTCAATACGGCATTAAACTTGAAGAAACAGTGTTGCTTTACCAAATCATCGA
TGGAGGATTCCAGTTCCCAGGGTCACCAATCGGCCTTTCAGTTTCTGACGAAGAAGCCTTACGAGCCTCC
TCCATGGGCTTCGCTTCTTAGCCCAATTCCCTCTCACA*CCTTTTCGCTTGGTCATTTTCCG*ACTCCAATT
CACAAGTGGAACCTGCCTAATTTACCGAAGAACACCGAGGTTTGGTTAAAGCGTGATGATATGTCAGGAA
TGCAATTAAGTGGAAACAAGGTCAGAAAGC*TGGAGTTCTTGTTGGCAGATGCT*GTAGCACAGGGTGCTGA
CTGCATAGTGACTATAGGTGGCATACAAAGTAATCACTGTCGTGCTACTGCTGTCGCTGCCAAGTACTTG
AACCTTGACTGCTATCTCATCTTACGCACTTCAAAGTTACTTGTAGATAAAGATCCTGGATTAACAGGGA
ACCTCCTTGTTGACCGTTTAGTTGGAGCACACATTGATCTTGTTTCAAAAGAAGAATATGCAAAAGTTGG
CGGTGAGGCTCTTACCAAAATATTGAAAGAAAAGCTGTTAAATGAAGGGAGAAAGCCATATGTCATCCCT
GTTGGTGGATCCAATTCTCTAGGAACCTGGGGCTATATTGAGGCAATTAGGGAATTGGAGCAACAACTTC
AGCACTTGAGCATTGAACAGAAATTCGACGACAT**TGTTGTAGCTTGTGGCAGTTTCC**AGTGGGGGTACGG
TTGCTGGTTTGTCAATTGCATCCATGCTCAGTGGCTTGAAAGCAAAGATTACTTTTATGAATATGTTCAA
GGCCTACTTG**ACGGAATCACTGCTGGAGTTAGCTC**CCGTGATATTGTTAGCATCAAAACTGCAAAAGGCC
TTGGGTATGCTTTGAGCACCACTGATGAGCTTAAATTTGTGAAGCAAGTTGCTGAAACCACAGGTGTTAT
TCTTGACCCTGTCTACAGTGGTAAAGCAGCTTATGGAATGATGAAAGACATGGGCGAGAATCCAACAAAG
TGGGAGGGAAGAAAGATTCTGTTCATACACACAGGTGGGCTACTAGGTTTGTATGACAAAGCTGATGAAA
TAGGGTCACTAATGGGCAAATGGCGTAAAATGGATATCAATGAATCTATCCCTAGACAAGATGGCATCGG
CAAAATGTTC**TGA**AATTGACGAAAAAGGTGATACCAATTGTGTTGTCATTTATCAACGCTTACAAGATTA
AAAATCAAATTCCAAACATTTCTAGATTCAGATGAATAAAAAAAAGATGTTTTCTGCCTACATCCAAGAC
TAGTTGAGTTATGGATGCATGGATAGTCTTGTGCTCCCCTCGATTATTTCATTGTATACATGTTACTGTT
ACTACTGAAAAAAAAAAAAAA

PRIMER 4: **TGTTGTAGCTTGTGGCAGTTTCC**

PRIMER 3: **ACGGAATCACTGCTGGAGTTAGCTC**

Figure 6-2. Nonspecific binding of Primer 4. The major product observed on the gel when Primer 4 and Primer 3 are used in a PCR reaction is approximately 700 bp; this is true for both reverse-transcriptase PCR reactions and PCR reactions where pBS-acd plasmid DNA was used as a template. The expected size of the produce if BT013578 version is expressed is 143 bp; the observed PCR product of just over 700 bp is due to unspecific primer binding. Analysis of BT013578 sequence and pBS-acd insert sequence (EU639448) has revealed that there exist regions in both sequences where Primer 4 may be binding nonspecifically. Indicated in pink, underlined and italicized is the region where Primer 4 shares significant overlap, and is most likely binding and amplifying the EU639448 version. The first region (starting from the ATG start codon) would result in a product of 721 bp when pBS-acd is amplified. The second region would result in a product of 591 bp.

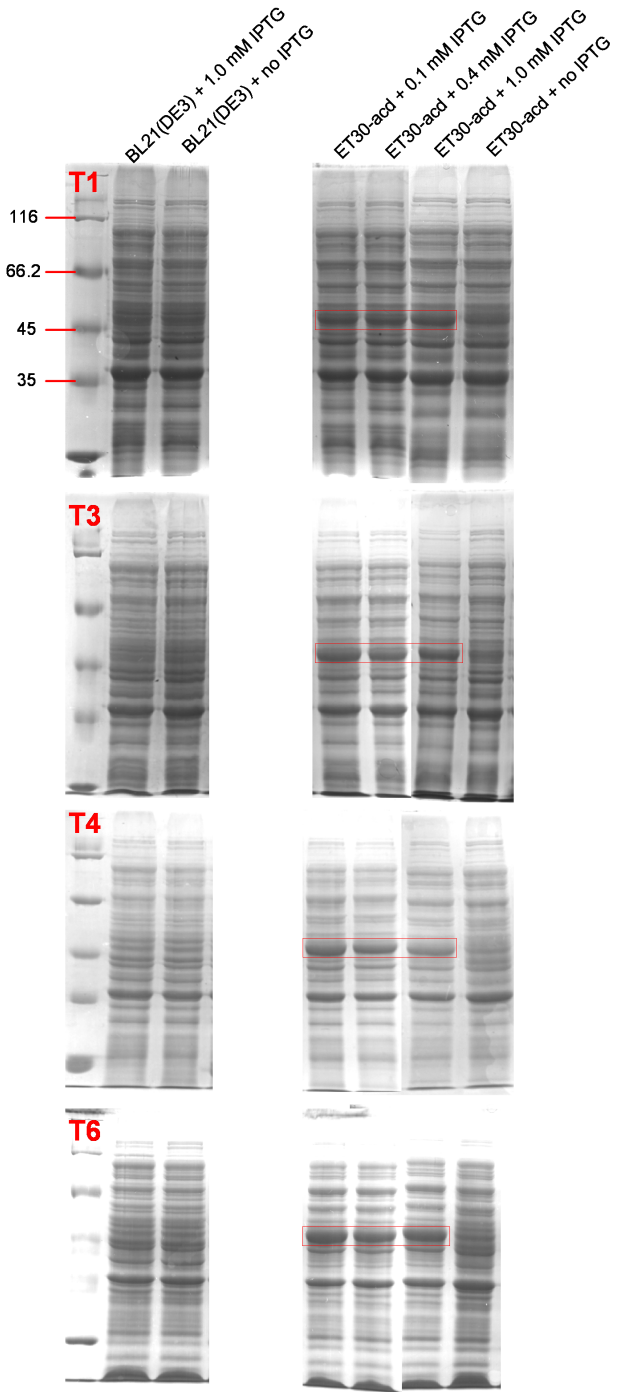


Figure 6-3. Putative ACC deaminase recombinant protein expression over time. T1-T6 indicates time points at which expression was examined (e.g. T1 indicates 1 h after induction). Recombinant protein is expressed after an hour of induction.

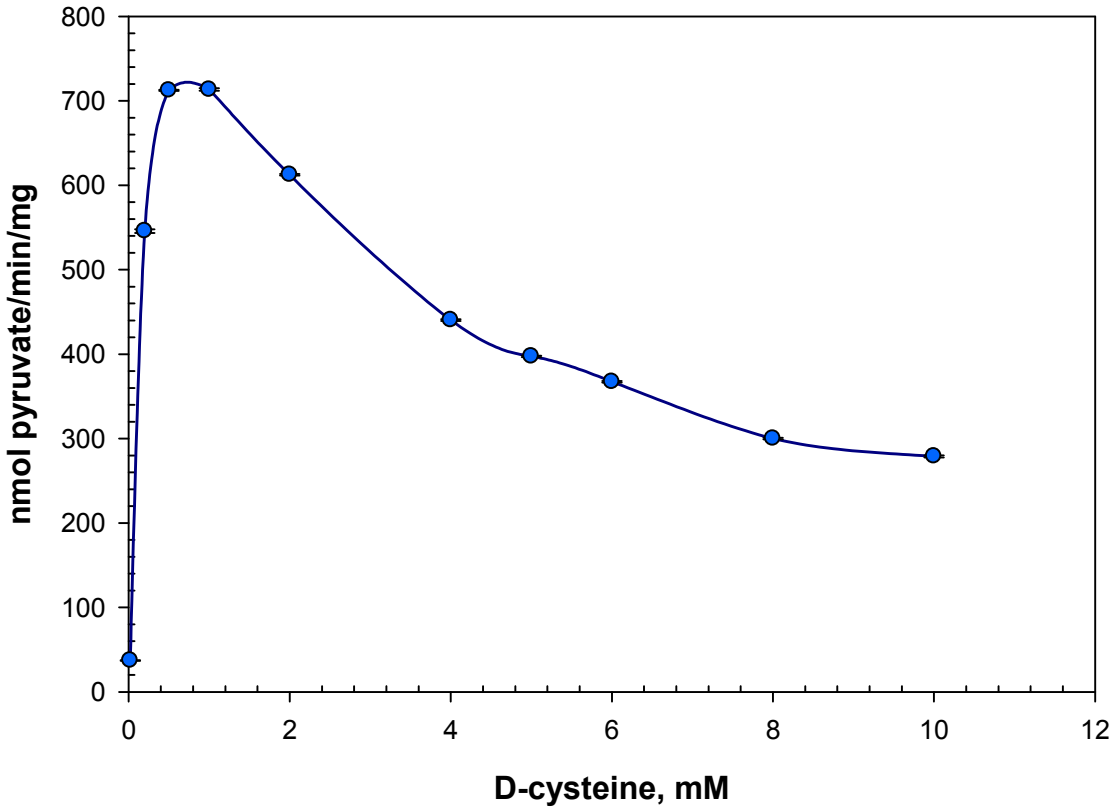


Figure 6-4. Substrate inhibition of D-cysteine desulfhydrase from tomato. Above 1 mM D-cysteine, the activity of the enzyme decreases; Km values were determined based on D-cysteine concentration below 1 mM.

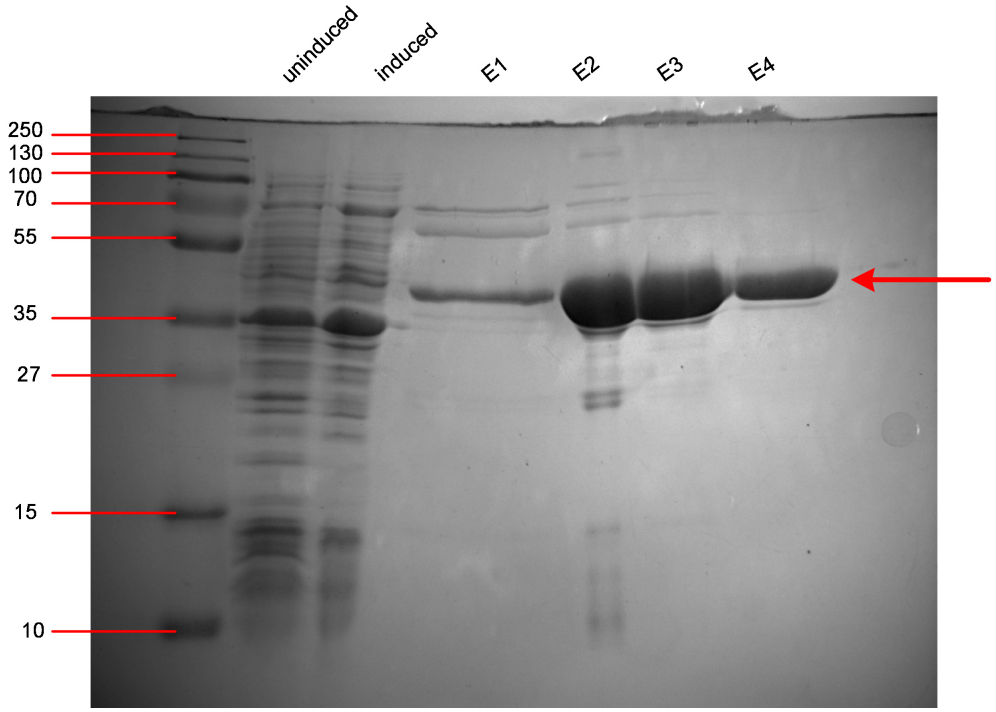
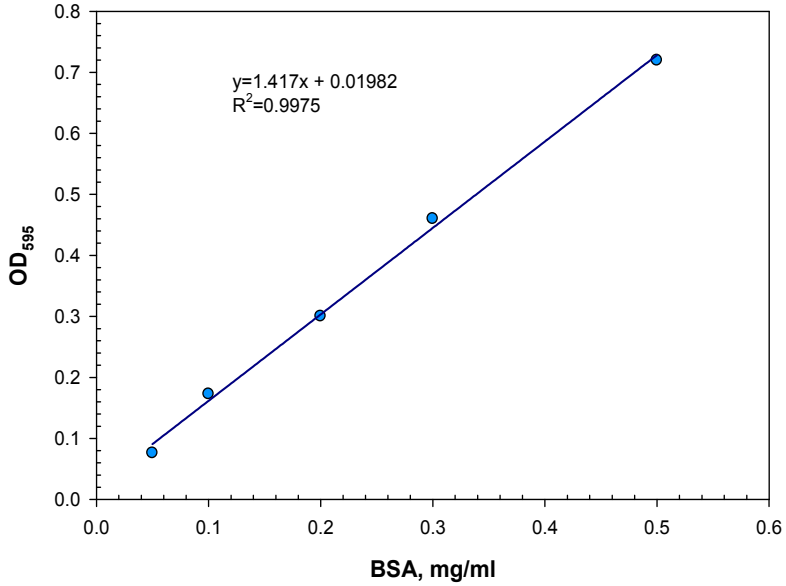


Figure 6-5. Protein purification of recombinant *P. putida* UW4 ACC deaminase. The gel illustrates typical elution fractions for the wild-type recombinant UW4 ACC deaminase and mutants of the enzyme. The recombinant protein is 42.17 kDa, whereas the enzyme without the 6X His-tag is 36.88 kDa, based on the amino acid sequence.E1-E4: elution fractions 1-4.



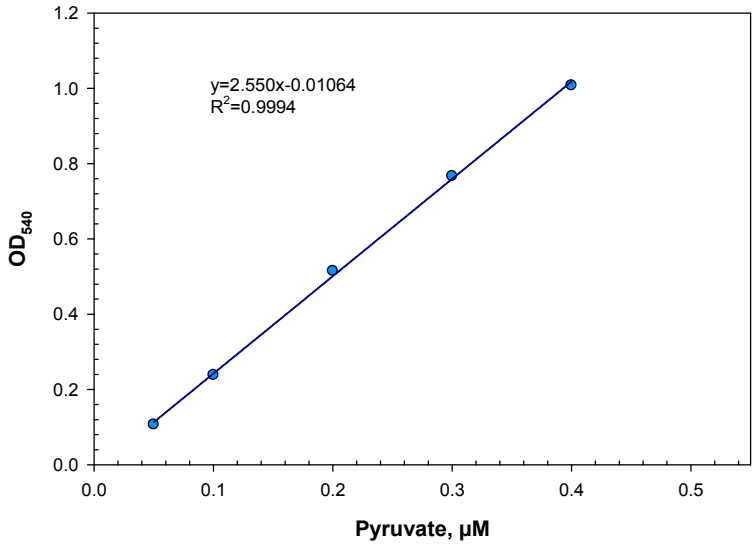


Figure 6-6. Typical standard curves. Top: standard curve for the BioRad protein assay. Bottom: pyruvate standard curve for the D-cysteine desulfhydrase assay.

Figure 6-7. Nucleotide sequence alignment between D-cysteine desulfhydrase from tomato and the constructed mutants.

```
ET30-Tm3        ATGCACCATCATCATCATCATTCTTCTGGTCTGGTGCCACGCGGTTCTGGTATGAAAGAA
ET30-acd        ATGCACCATCATCATCATCATTCTTCTGGTCTGGTGCCACGCGGTTCTGGTATGAAAGAA
ET30-Tm1        ATGCACCATCATCATCATCATTCTTCTGGTCTGGTGCCACGCGGTTCTGGTATGAAAGAA
ET30-Tm2        ATGCACCATCATCATCATCATTCTTCTGGTCTGGTGCCACGCGGTTCTGGTATGAAAGAA
                ************************************************************

ET30-Tm3        ACCGCTGCTGCTAAATTCGAACGCCAGCACATGGACAGCCCAGATCTGGGTACCGGTGGT
ET30-acd        ACCGCTGCTGCTAAATTCGAACGCCAGCACATGGACAGCCCAGATCTGGGTACCGGTGGT
ET30-Tm1        ACCGCTGCTGCTAAATTCGAACGCCAGCACATGGACAGCCCAGATCTGGGTACCGGTGGT
ET30-Tm2        ACCGCTGCTGCTAAATTCGAACGCCAGCACATGGACAGCCCAGATCTGGGTACCGGTGGT
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ET30-Tm3        GGCTCCGGTATTGAGGGTCGCATGTCGAGTTGCCAATGGAGTAGCTTCACTAGAGTATCA
ET30-acd        GGCTCCGGTATTGAGGGTCGCATGTCGAGTTGCCAATGGAGTAGCTTCACTAGAGTATCA
ET30-Tm1        GGCTCCGGTATTGAGGGTCGCATGTCGAGTTGCCAATGGAGTAGCTTCACTAGAGTATCA
ET30-Tm2        GGCTCCGGTATTGAGGGTCGCATGTCGAGTTGCCAATGGAGTAGCTTCACTAGAGTATCA
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ET30-Tm3        CTATCTCCATTTCCCTTGCAGCCAGCACAACTCAATACGGCATTAAACTTGAAGAAACAG
ET30-acd        CTATCTCCATTTCCCTTGCAGCCAGCACAACTCAATACGGCATTAAACTTGAAGAAACAG
ET30-Tm1        CTATCTCCATTTCCCTTGCAGCCAGCACAACTCAATACGGCATTAAACTTGAAGAAACAG
ET30-Tm2        CTATCTCCATTTCCCTTGCAGCCAGCACAACTCAATACGGCATTAAACTTGAAGAAACAG
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ET30-Tm3        TGTTGCTTTACCAAATCATCGATGGAGGATTCCAGTTCCCAGGGTCACCAATCGGCCTTT
ET30-acd        TGTTGCTTTACCAAATCATCGATGGAGGATTCCAGTTCCCAGGGTCACCAATCGGCCTTT
ET30-Tm1        TGTTGCTTTACCAAATCATCGATGGAGGATTCCAGTTCCCAGGGTCACCAATCGGCCTTT
ET30-Tm2        TGTTGCTTTACCAAATCATCGATGGAGGATTCCAGTTCCCAGGGTCACCAATCGGCCTTT
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ET30-Tm3        CAGTTTCTGACGAAGAAGCCTTACGAGCCTCCTCCATGGGCTTCGCTTCTTAGCCCAATT
ET30-acd        CAGTTTCTGACGAAGAAGCCTTACGAGCCTCCTCCATGGGCTTCGCTTCTTAGCCCAATT
ET30-Tm1        CAGTTTCTGACGAAGAAGCCTTACGAGCCTCCTCCATGGGCTTCGCTTCTTAGCCCAATT
ET30-Tm2        CAGTTTCTGACGAAGAAGCCTTACGAGCCTCCTCCATGGGCTTCGCTTCTTAGCCCAATT
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ET30-Tm3        CCCTCTCACACCTTTTCGCTTGGTCATTTTCCGACTCCAATTCACAAGTGGAACCTGCCT
ET30-acd        CCCTCTCACACCTTTTCGCTTGGTCATTTTCCGACTCCAATTCACAAGTGGAACCTGCCT
ET30-Tm1        CCCTCTCACACCTTTTCGCTTGGTCATTTTCCGACTCCAATTCACAAGTGGAACCTGCCT
ET30-Tm2        CCCTCTCACACCTTTTCGCTTGGTCATTTTCCGACTCCAATTCACAAGTGGAACCTGCCT
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ET30-Tm3        AATTTACCGAAGAACACCGAGGTTTGGTTAAAGCGTGATGATATGTCAGGAATGCAATTA
ET30-acd        AATTTACCGAAGAACACCGAGGTTTGGTTAAAGCGTGATGATATGTCAGGAATGCAATTA
ET30-Tm1        AATTTACCGAAGAACACCGAGGTTTGGTTAAAGCGTGATGATATGTCAGGAATGCAATTA
ET30-Tm2        AATTTACCGAAGAACACCGAGGTTTGGTTAAAGCGTGATGATATGTCAGGAATGCAATTA
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ET30-Tm3        AGTGGAAACAAGGTCAGAAAGCTGGAGTTCTTGTTGGCAGATGCTGTAGCACAGGGTGCT
ET30-acd        AGTGGAAACAAGGTCAGAAAGCTGGAGTTCTTGTTGGCAGATGCTGTAGCACAGGGTGCT
ET30-Tm1        AGTGGAAACAAGGTCAGAAAGCTGGAGTTCTTGTTGGCAGATGCTGTAGCACAGGGTGCT
ET30-Tm2        AGTGGAAACAAGGTCAGAAAGCTGGAGTTCTTGTTGGCAGATGCTGTAGCACAGGGTGCT
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ET30-Tm3        GACTGCATAGTGACTATAGGTGGCATACAAAGTAATCACTGTCGTGCTACTGCTGTCGCT
ET30-acd        GACTGCATAGTGACTATAGGTGGCATACAAAGTAATCACTGTCGTGCTACTGCTGTCGCT
ET30-Tm1        GACTGCATAGTGACTATAGGTGGCATACAAAGTAATCACTGTCGTGCTACTGCTGTCGCT
ET30-Tm2        GACTGCATAGTGACTATAGGTGGCATACAAAGTAATCACTGTCGTGCTACTGCTGTCGCT
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ET30-Tm3        GCCAAGTACTTGAACCTTGACTGCTATCTCATCTTACGCACTTCAAAGTTACTTGTAGAT
ET30-acd        GCCAAGTACTTGAACCTTGACTGCTATCTCATCTTACGCACTTCAAAGTTACTTGTAGAT
ET30-Tm1        GCCAAGTACTTGAACCTTGACTGCTATCTCATCTTACGCACTTCAAAGTTACTTGTAGAT
ET30-Tm2        GCCAAGTACTTGAACCTTGACTGCTATCTCATCTTACGCACTTCAAAGTTACTTGTAGAT
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ET30-Tm3        AAAGATCCTGGATTAACAGGGAACCTCCTTGTTGACCGTTTAGTTGGAGCACACATTGAT
ET30-acd        AAAGATCCTGGATTAACAGGGAACCTCCTTGTTGACCGTTTAGTTGGAGCACACATTGAT
ET30-Tm1        AAAGATCCTGGATTAACAGGGAACCTCCTTGTTGACCGTTTAGTTGGAGCACACATTGAT
ET30-Tm2        AAAGATCCTGGATTAACAGGGAACCTCCTTGTTGACCGTTTAGTTGGAGCACACATTGAT
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ET30-Tm3        CTTGTTTCAAAAGAAGAATATGCAAAAGTTGGCGGTGAGGCTCTTACCAAAATATTGAAA
ET30-acd        CTTGTTTCAAAAGAAGAATATGCAAAAGTTGGCGGTGAGGCTCTTACCAAAATATTGAAA
ET30-Tm1        CTTGTTTCAAAAGAAGAATATGCAAAAGTTGGCGGTGAGGCTCTTACCAAAATATTGAAA
ET30-Tm2        CTTGTTTCAAAAGAAGAATATGCAAAAGTTGGCGGTGAGGCTCTTACCAAAATATTGAAA
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ET30-Tm3        GAAAAGCTGTTAAATGAAGGGAGAAAGCCATATGTCATCCCTGTTGGTGGATCCAATTCT
ET30-acd        GAAAAGCTGTTAAATGAAGGGAGAAAGCCATATGTCATCCCTGTTGGTGGATCCAATTCT
ET30-Tm1        GAAAAGCTGTTAAATGAAGGGAGAAAGCCATATGTCATCCCTGTTGGTGGATCCAATTCT
ET30-Tm2        GAAAAGCTGTTAAATGAAGGGAGAAAGCCATATGTCATCCCTGTTGGTGGATCCAATTCT
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ET30-Tm3        CTAGGAACCTGGGGCTATATTGAGGCAATTAGGGAATTGGAGCAACAACTTCAGCACTTG
ET30-acd        CTAGGAACCTGGGGCTATATTGAGGCAATTAGGGAATTGGAGCAACAACTTCAGCACTTG
ET30-Tm1        CTAGGAACCTGGGGCTATATTGAGGCAATTAGGGAATTGGAGCAACAACTTCAGCACTTG
ET30-Tm2        CTAGGAACCTGGGGCTATATTGAGGCAATTAGGGAATTGGAGCAACAACTTCAGCACTTG
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ET30-Tm3        AGCATTGAACAGAAATTCGACGACATTGTTGTAGCTTGTGGCAGTGGGGGTACGGTTGCT
ET30-acd        AGCATTGAACAGAAATTCGACGACATTGTTGTAGCTTGTGGCAGTGGGGGTACGGTTGCT
ET30-Tm1        AGCATTGAACAGAAATTCGACGACATTGTTGTAGCTTGTGGCAGTGGGGGTACGGTTGCT
ET30-Tm2        AGCATTGAACAGAAATTCGACGACATTGTTGTAGCTTGTGGCAGTGGGGGTACGGTTGCT
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ET30-Tm3        GGTTTGTCAATTGCATCCATGCTCAGTGGCTTGAAAGCAAAGATTAATGCATTTTGTGTC
ET30-acd        GGTTTGTCAATTGCATCCATGCTCAGTGGCTTGAAAGCAAAGATTAATGCATTTTGTGTC
ET30-Tm1        GGTTTGTCAATTGCATCCATGCTCAGTGGCTTGAAAGCAAAGATTAATGCATTTTGTGTC
ET30-Tm2        GGTTTGTCAATTGCATCCATGCTCAGTGGCTTGAAAGCAAAGATTAATGCATTTTGTGTC
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ET30-Tm3        TGCGACGATCCAGATTACTTTTATGAATATGTTCAAGGCCTACTTGACGGAATCACTGCT
ET30-acd        TGCGACGATCCAGATTACTTTTATGAATATGTTCAAGGCCTACTTGACGGAATCACTGCT
ET30-Tm1        TGCGACGATCCAGATTACTTTTATGAATATGTTCAAGGCCTACTTGACGGAATCACTGCT
ET30-Tm2        TGCGACGATCCAGATTACTTTTATGAATATGTTCAAGGCCTACTTGACGGAATCACTGCT
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ET30-Tm3        GGAGTTAGCTCCCGTGATATTGTTAGCATCAAAACTGCAAAAGGCCTTGGGTATGCTTTG
ET30-acd        GGAGTTAGCTCCCGTGATATTGTTAGCATCAAAACTGCAAAAGGCCTTGGGTATGCTTTG
ET30-Tm1        GGAGTTAGCTCCCGTGATATTGTTAGCATCAAAACTGCAAAAGGCCTTGGGTATGCTTTG
ET30-Tm2        GGAGTTAGCTCCCGTGATATTGTTAGCATCAAAACTGCAAAAGGCCTTGGGTATGCTTTG
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ET30-Tm3        AGCACCACTGATGAGCTTAAATTTGTGAAGCAAGTTGCTGAAACCACAGGTGTTATTCTT
ET30-acd        AGCACCACTGATGAGCTTAAATTTGTGAAGCAAGTTGCTGAAACCACAGGTGTTATTCTT
ET30-Tm1        AGCACCACTGATGAGCTTAAATTTGTGAAGCAAGTTGCTGAAACCACAGGTGTTATTCTT
ET30-Tm2        AGCACCACTGATGAGCTTAAATTTGTGAAGCAAGTTGCTGAAACCACAGGTGTTATTCTT
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ET30-Tm3        GACCCTGTCTACGAAGGTAAAGCAGCTTATGGAATGATGAAAGACATGGGCGAGAATCCA
ET30-acd        GACCCTGTCTACAGTGGTAAAGCAGCTTATGGAATGATGAAAGACATGGGCGAGAATCCA
ET30-Tm1        GACCCTGTCTACGAAGGTAAAGCAGCTTATGGAATGATGAAAGACATGGGCGAGAATCCA
ET30-Tm2        GACCCTGTCTACAGTGGTAAAGCAGCTTATGGAATGATGAAAGACATGGGCGAGAATCCA
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ET30-Tm3        ACAAAGTGGGAGGGAAGAAAGATTCTGTTCATACACCTGGGTGGGCTACTAGGTTTGTAT
ET30-acd        ACAAAGTGGGAGGGAAGAAAGATTCTGTTCATACACACAGGTGGGCTACTAGGTTTGTAT
ET30-Tm1        ACAAAGTGGGAGGGAAGAAAGATTCTGTTCATACACACAGGTGGGCTACTAGGTTTGTAT
ET30-Tm2        ACAAAGTGGGAGGGAAGAAAGATTCTGTTCATACACCTGGGTGGGCTACTAGGTTTGTAT
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ET30-Tm3        GACAAAGCTGATGAAATAGGGTCACTAATGGGCAAATGGCGTAAAATGGATATCAATGAA
ET30-acd        GACAAAGCTGATGAAATAGGGTCACTAATGGGCAAATGGCGTAAAATGGATATCAATGAA
ET30-Tm1        GACAAAGCTGATGAAATAGGGTCACTAATGGGCAAATGGCGTAAAATGGATATCAATGAA
ET30-Tm2        GACAAAGCTGATGAAATAGGGTCACTAATGGGCAAATGGCGTAAAATGGATATCAATGAA
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ET30-Tm3        TCTATCCCTAGACAAGATGGCATCGGCAAAATGTTCTGA
ET30-acd        TCTATCCCTAGACAAGATGGCATCGGCAAAATGTTCTGA
ET30-Tm1        TCTATCCCTAGACAAGATGGCATCGGCAAAATGTTCTGA
ET30-Tm2        TCTATCCCTAGACAAGATGGCATCGGCAAAATGTTCTGA
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ET30-Tm3        MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTGGGSGIEGRMSSCQWSSFTRVS
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ET30-Tm1        MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTGGGSGIEGRMSSCQWSSFTRVS
ET30-Tm2        MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTGGGSGIEGRMSSCQWSSFTRVS
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ET30-Tm3        LSPFPLQPAQLNTALNLKKQCCFTKSSMEDSSSQGHQSAFQFLTKKPYEPPPWASLLSPI
ET30-acd        LSPFPLQPAQLNTALNLKKQCCFTKSSMEDSSSQGHQSAFQFLTKKPYEPPPWASLLSPI
ET30-Tm1        LSPFPLQPAQLNTALNLKKQCCFTKSSMEDSSSQGHQSAFQFLTKKPYEPPPWASLLSPI
ET30-Tm2        LSPFPLQPAQLNTALNLKKQCCFTKSSMEDSSSQGHQSAFQFLTKKPYEPPPWASLLSPI
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ET30-Tm3        PSHTFSLGHFPTPIHKWNLPNLPKNTEVWLKRDDMSGMQLSGNKVRKLEFLLADAVAQGA
ET30-acd        PSHTFSLGHFPTPIHKWNLPNLPKNTEVWLKRDDMSGMQLSGNKVRKLEFLLADAVAQGA
ET30-Tm1        PSHTFSLGHFPTPIHKWNLPNLPKNTEVWLKRDDMSGMQLSGNKVRKLEFLLADAVAQGA
ET30-Tm2        PSHTFSLGHFPTPIHKWNLPNLPKNTEVWLKRDDMSGMQLSGNKVRKLEFLLADAVAQGA
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ET30-Tm3        DCIVTIGGIQSNHCRATAVAAKYLNLDCYLILRTSKLLVDKDPGLTGNLLVDRLVGAHID
ET30-acd        DCIVTIGGIQSNHCRATAVAAKYLNLDCYLILRTSKLLVDKDPGLTGNLLVDRLVGAHID
ET30-Tm1        DCIVTIGGIQSNHCRATAVAAKYLNLDCYLILRTSKLLVDKDPGLTGNLLVDRLVGAHID
ET30-Tm2        DCIVTIGGIQSNHCRATAVAAKYLNLDCYLILRTSKLLVDKDPGLTGNLLVDRLVGAHID
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ET30-Tm3        LVSKEEYAKVGGEALTKILKEKLLNEGRKPYVIPVGGSNSLGTWGYIEAIRELEQQLQHL
ET30-acd        LVSKEEYAKVGGEALTKILKEKLLNEGRKPYVIPVGGSNSLGTWGYIEAIRELEQQLQHL
ET30-Tm1        LVSKEEYAKVGGEALTKILKEKLLNEGRKPYVIPVGGSNSLGTWGYIEAIRELEQQLQHL
ET30-Tm2        LVSKEEYAKVGGEALTKILKEKLLNEGRKPYVIPVGGSNSLGTWGYIEAIRELEQQLQHL
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ET30-Tm3        SIEQKFDDIVVACGSGGTVAGLSIASMLSGLKAKINAFCVCDDPDYFYEYVQGLLDGITA
ET30-acd        SIEQKFDDIVVACGSGGTVAGLSIASMLSGLKAKINAFCVCDDPDYFYEYVQGLLDGITA
ET30-Tm1        SIEQKFDDIVVACGSGGTVAGLSIASMLSGLKAKINAFCVCDDPDYFYEYVQGLLDGITA
ET30-Tm2        SIEQKFDDIVVACGSGGTVAGLSIASMLSGLKAKINAFCVCDDPDYFYEYVQGLLDGITA
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ET30-Tm3        GVSSRDIVSIKTAKGLGYALSTTDELKFVKQVAETTGVILDPVYEGKAAYGMMKDMGENP
ET30-acd        GVSSRDIVSIKTAKGLGYALSTTDELKFVKQVAETTGVILDPVYSGKAAYGMMKDMGENP
ET30-Tm1        GVSSRDIVSIKTAKGLGYALSTTDELKFVKQVAETTGVILDPVYEGKAAYGMMKDMGENP
ET30-Tm2        GVSSRDIVSIKTAKGLGYALSTTDELKFVKQVAETTGVILDPVYSGKAAYGMMKDMGENP
                ******************************************.*****************

ET30-Tm3        TKWEGRKILFIHLGGLLGLYDKADEIGSLMGKWRKMDINESIPRQDGIGKMF
ET30-acd        TKWEGRKILFIHTGGLLGLYDKADEIGSLMGKWRKMDINESIPRQDGIGKMF
ET30-Tm1        TKWEGRKILFIHTGGLLGLYDKADEIGSLMGKWRKMDINESIPRQDGIGKMF
ET30-Tm2        TKWEGRKILFIHLGGLLGLYDKADEIGSLMGKWRKMDINESIPRQDGIGKMF
                ************ ************************************
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Figure 6-8. Protein sequence alignment between D-cysteine desulfhydrase from tomato and the mutants of the enzyme.

Figure 6-9. Nucleotide sequence alignment between ACC deaminase from *P. putida* UW4 and the constructed mutants.

```
ET30-UW4        ATGCACCATCATCATCATCATTCTTCTGGTCTGGTGCCACGCGGTTCTGGTATGAAAGAA
ET30-Um2        ATGCACCATCATCATCATCATTCTTCTGGTCTGGTGCCACGCGGTTCTGGTATGAAAGAA
ET30-Um1        ATGCACCATCATCATCATCATTCTTCTGGTCTGGTGCCACGCGGTTCTGGTATGAAAGAA
                ************************************************************

ET30-UW4        ACCGCTGCTGCTAAATTCGAACGCCAGCACATGGACAGCCCAGATCTGGGTACCGACGAC
ET30-Um2        ACCGCTGCTGCTAAATTCGAACGCCAGCACATGGACAGCCCAGATCTGGGTACCGACGAC
ET30-Um1        ACCGCTGCTGCTAAATTCGAACGCCAGCACATGGACAGCCCAGATCTGGGTACCGACGAC
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ET30-UW4        GACGACAAGGCCATGGCTGATATCATGAACCTGAATCGTTTTGAACGTTATCCGTTGACC
ET30-Um2        GACGACAAGGCCATGGCTGATATCATGAACCTGAATCGTTTTGAACGTTATCCGTTGACC
ET30-Um1        GACGACAAGGCCATGGCTGATATCATGAACCTGAATCGTTTTGAACGTTATCCGTTGACC
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ET30-UW4        TTCGGTCCATCCCCCATCACTCCCTTGAAACGCCTCAGCGAGCACCTGGGCGGCAAGGTG
ET30-Um2        TTCGGTCCATCCCCCATCACTCCCTTGAAACGCCTCAGCGAGCACCTGGGCGGCAAGGTG
ET30-Um1        TTCGGTCCATCCCCCATCACTCCCTTGAAACGCCTCAGCGAGCACCTGGGCGGCAAGGTG
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ET30-UW4        GAACTGTATGCCAAGCGTGAAGACTGCAATAGCGGCCTGGCCTTCGGCGGGAACAAAACG
ET30-Um2        GAACTGTATGCCAAGCGTGAAGACTGCAATAGCGGCCTGGCCTTCGGCGGGAACAAAACG
ET30-Um1        GAACTGTATGCCAAGCGTGAAGACTGCAATAGCGGCCTGGCCTTCGGCGGGAACAAAACG
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ET30-UW4        CGCAAGCTCGAATATCTGATTCCCGAGGCCATCGAGCAAGGCTGCGACACCTTGGTGTCC
ET30-Um2        CGCAAGCTCGAATATCTGATTCCCGAGGCCATCGAGCAAGGCTGCGACACCTTGGTGTCC
ET30-Um1        CGCAAGCTCGAATATCTGATTCCCGAGGCCATCGAGCAAGGCTGCGACACCTTGGTGTCC
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ET30-UW4        ATCGGCGGTATCCAGTCGAACCAGACCCGCCAGGTCGCTGCGGTCGCCGCCCACTTGGGT
ET30-Um2        ATCGGCGGTATCCAGTCGAACCAGACCCGCCAGGTCGCTGCGGTCGCCGCCCACTTGGGT
ET30-Um1        ATCGGCGGTATCCAGTCGAACCAGACCCGCCAGGTCGCTGCGGTCGCCGCCCACTTGGGT
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ET30-UW4        ATGAAGTGTGTGCTTGTGCAGGAAAACTGGGTGAACTACTCCGACGCTGTATATGACCGC
ET30-Um2        ATGAAGTGTGTGCTTGTGCAGGAAAACTGGGTGAACTACTCCGACGCTGTATATGACCGC
ET30-Um1        ATGAAGTGTGTGCTTGTGCAGGAAAACTGGGTGAACTACTCCGACGCTGTATATGACCGC
                ************************************************************

ET30-UW4        GTCGGCAACATCGAGATGTCGCGGATCATGGGAGCGGATGTGCGGCTTGATGCTGCAGGT
ET30-Um2        GTCGGCAACATCGAGATGTCGCGGATCATGGGAGCGGATGTGCGGCTTGATGCTGCAGGT
ET30-Um1        GTCGGCAACATCGAGATGTCGCGGATCATGGGAGCGGATGTGCGGCTTGATGCTGCAGGT
                ************************************************************

ET30-UW4        TTCGACATTGGAATTCGGCCGAGCTGGGAAAAGGCCATGAGCGATGTCGTGGAGCGCGGC
ET30-Um2        TTCGACATTGGAATTCGGCCGAGCTGGGAAAAGGCCATGAGCGATGTCGTGGAGCGCGGC
ET30-Um1        TTCGACATTGGAATTCGGCCGAGCTGGGAAAAGGCCATGAGCGATGTCGTGGAGCGCGGC
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ET30-UW4        GGCAAACCGTTTCCAATTCCGGCGGGCTGTTCCGAGCATCCCTATGGAGGGCTCGGGTTT
ET30-Um2        GGCAAACCGTTTCCAATTCCGGCGGGCTGTTCCGAGCATCCCTATGGAGGGCTCGGGTTT
ET30-Um1        GGCAAACCGTTTCCAATTCCGGCGGGCTGTTCCGAGCATCCCTATGGAGGGCTCGGGTTT
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ET30-UW4        GTCGGCTTCGCTGAGGAAGTGCGGCAGCAGGAAAAGGAGTTGGGCTTCAAGTTTGACTAC
ET30-Um2        GTCGGCTTCGCTGAGGAAGTGCGGCAGCAGGAAAAGGAGTTGGGCTTCAAGTTTGACTAC
ET30-Um1        GTCGGCTTCGCTGAGGAAGTGCGGCAGCAGGAAAAGGAGTTGGGCTTCAAGTTTGACTAC
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ET30-UW4        ATCGTGGTCTGCTCGGTGACCGGCAGTACCCAGGCCGGCATGGTCGTCGGTTTCGCGGCT
ET30-Um2        ATCGTGGTCTGCTCGGTGACCGGCAGTACCCAGGCCGGCATGGTCGTCGGTTTCGCGGCT
ET30-Um1        ATCGTGGTCTGCTCGGTGACCGGCAGTACCCAGGCCGGCATGGTCGTCGGTTTCGCGGCT
                ************************************************************

ET30-UW4        GACGGTCGCTCGAAAAACGTGATCGGGGTCGATGCTTCGGCGAAACCGGAGCAAACCAAG
ET30-Um2        GACGGTCGCTCGAAAAACGTGATCGGGGTCGATGCTTCGGCGAAACCGGAGCAAACCAAG
ET30-Um1        GACGGTCGCTCGAAAAACGTGATCGGGGTCGATGCTTCGGCGAAACCGGAGCAAACCAAG
                ************************************************************

ET30-UW4        GCGCAGATCCTGCGTATCGCTCGACATACCGCTGAACTGGTGGAGCTGGGGCGCGAAATC
ET30-Um2        GCGCAGATCCTGCGTATCGCTCGACATACCGCTGAACTGGTGGAGCTGGGGCGCGAAATC
ET30-Um1        GCGCAGATCCTGCGTATCGCTCGACATACCGCTGAACTGGTGGAGCTGGGGCGCGAAATC
                ************************************************************

ET30-UW4        ACTGAAGAGGATGTGGTGCTCGATACGCGTTTCGCCTATCCGGAATATGGCTTGCCCAAC
ET30-Um2        ACTGAAGAGGATGTGGTGCTCGATACGCGTTTCGCCTATCCGGAATATGGCTTGCCCAAC
ET30-Um1        ACTGAAGAGGATGTGGTGCTCGATACGCGTTTCGCCTATCCGGAATATGGCTTGCCCAAC
                ************************************************************

ET30-UW4        GAAGGGACGCTGGAAGCGATTCGCCTGTGCGGCAGTCTTGAGGGGGTGTTGACCGATCCG
ET30-Um2        GAAGGGACGCTGGAAGCGATTCGCCTGTGCGGCAGTCTTGAGGGGGTGTTGACCGATCCG
ET30-Um1        GAAGGGACGCTGGAAGCGATTCGCCTGTGCGGCAGTCTTGAGGGGGTGTTGACCGATCCG
                ************************************************************

ET30-UW4        GTCTACGAGGGCAAATCCATGCACGGCATGATTGAAATGGTACGCCGCGGGGAATTCCCT
ET30-Um2        GTCTACAGCGGCAAATCCATGCACGGCATGATTGAAATGGTACGCCGCGGGGAATTCCCT
ET30-Um1        GTCTACAGCGGCAAATCCATGCACGGCATGATTGAAATGGTACGCCGCGGGGAATTCCCT
                ******   ************************************************

ET30-UW4        GACGGCTCCAAAGTTCTTTATGCCCACCTGGGCGGCGCACCTGCGTTGAACGCCTACAGC
ET30-Um2        GACGGCTCCAAAGTTCTTTATGCCCACACCGGCGGCGCACCTGCGTTGAACGCCTACAGC
ET30-Um1        GACGGCTCCAAAGTTCTTTATGCCCACCTGGGCGGCGCACCTGCGTTGAACGCCTACAGC
                **************************   ****************************

ET30-UW4        TTCCTGTTTCGCAACGGCTGA
ET30-Um2        TTCCTGTTTCGCAACGGCTGA
ET30-Um1        TTCCTGTTTCGCAACGGCTGA
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ET30-UW4        MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAMADIMNLNRFERYPLT
ET30-Um2        MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAMADIMNLNRFERYPLT
ET30-Um1        MHHHHHHSSGLVPRGSGMKETAAAKFERQHMDSPDLGTDDDDKAMADIMNLNRFERYPLT
                ************************************************************

ET30-UW4        FGPSPITPLKRLSEHLGGKVELYAKREDCNSGLAFGGNKTRKLEYLIPEAIEQGCDTLVS
ET30-Um2        FGPSPITPLKRLSEHLGGKVELYAKREDCNSGLAFGGNKTRKLEYLIPEAIEQGCDTLVS
ET30-Um1        FGPSPITPLKRLSEHLGGKVELYAKREDCNSGLAFGGNKTRKLEYLIPEAIEQGCDTLVS
                ************************************************************

ET30-UW4        IGGIQSNQTRQVAAVAAHLGMKCVLVQENWVNYSDAVYDRVGNIEMSRIMGADVRLDAAG
ET30-Um2        IGGIQSNQTRQVAAVAAHLGMKCVLVQENWVNYSDAVYDRVGNIEMSRIMGADVRLDAAG
ET30-Um1        IGGIQSNQTRQVAAVAAHLGMKCVLVQENWVNYSDAVYDRVGNIEMSRIMGADVRLDAAG
                ************************************************************

ET30-UW4        FDIGIRPSWEKAMSDVVERGGKPFPIPAGCSEHPYGGLGFVGFAEEVRQQEKELGFKFDY
ET30-Um2        FDIGIRPSWEKAMSDVVERGGKPFPIPAGCSEHPYGGLGFVGFAEEVRQQEKELGFKFDY
ET30-Um1        FDIGIRPSWEKAMSDVVERGGKPFPIPAGCSEHPYGGLGFVGFAEEVRQQEKELGFKFDY
                ************************************************************

ET30-UW4        IVVCSVTGSTQAGMVVGFAADGRSKNVIGVDASAKPEQTKAQILRIARHTAELVELGREI
ET30-Um2        IVVCSVTGSTQAGMVVGFAADGRSKNVIGVDASAKPEQTKAQILRIARHTAELVELGREI
ET30-Um1        IVVCSVTGSTQAGMVVGFAADGRSKNVIGVDASAKPEQTKAQILRIARHTAELVELGREI
                ************************************************************

ET30-UW4        TEEDVVLDTRFAYPEYGLPNEGTLEAIRLCGSLEGVLTDPVYEGKSMHGMIEMVRRGEFP
ET30-Um2        TEEDVVLDTRFAYPEYGLPNEGTLEAIRLCGSLEGVLTDPVYSGKSMHGMIEMVRRGEFP
ET30-Um1        TEEDVVLDTRFAYPEYGLPNEGTLEAIRLCGSLEGVLTDPVYSGKSMHGMIEMVRRGEFP
                ****************************************.*******************

ET30-UW4        DGSKVLYAHLGGAPALNAYSFLFRNG
ET30-Um2        DGSKVLYAHTGGAPALNAYSFLFRNG
ET30-Um1        DGSKVLYAHLGGAPALNAYSFLFRNG
                ********* ****************
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Figure 6-10. Protein sequence alignment between ACC deaminase from *P. putida* UW4 and the mutants of the enzyme.

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