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# Modulation of plant ethylene levels by the bacterial enzyme ACC deaminase

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**Abstract**

Soil microorganisms that produce the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase promote plant growth by sequestering and cleaving plant-produced ACC, and thereby lowering the level of ethylene in the plant. Decreased ethylene levels allows the plant to be more resistant to a wide variety of environmental stresses. Here, the biochemistry of ACC deaminase; the environmental distribution, regulation, evolution and expression of ACC deaminase genes; and information regarding the effect of this enzyme on different plants is documented and discussed.


*Keywords:* Plant growth-promoting bacteria; Ethylene; ACC deaminase

## 1. Ethylene

The plant hormone ethylene has played an important role in agricultural practice since antiquity. One of the oldest references to ethylene, albeit somewhat indirect, is in the Bible where one of the prophets is described as a ''scorer of figs'', referring to the ancient practice of scoring, or cutting, newly picked figs (and generating stress ethylene production) in an effort to hasten ripening. Nevertheless, ethylene was not recognized as a biologically active hormone until the work of Russian scientist, Dimitri Neljubov, with etiolated pea seedlings in greenhouses in the late 19th century [1].

Ethylene is one of the simplest organic molecules with biological activity, and can function as an efficient plant growth regulator at very low concentrations – biological effects attributable to ethylene are observed at concentrations as low as 0.05 μL/L [1]. It is important for normal development in plants as well as for their response to stress [2]. Many aspects of the growth of vegetative organs such as roots, stems and petioles, and all stages of development are affected by ethylene [2]. A variety of other plant processes involve ethylene including rhizobia nodulation of legumes, rooting of cuttings, and plant response to heavy metals, ozone, pathogens and flooding.

The production of ethylene is regulated by a large number of factors including temperature, light, gravity, nutrition, and other plant hormones. The term ''stress ethylene'' was coined [3] to describe the acceleration of ethylene biosynthesis associated with environmental and biological stresses including pathogen attack [4]. The increased level of ethylene formed in response to trauma inflicted by chemicals, temperature extremes, water stress, ultraviolet light, insect damage, disease, and mechanical wounding can be both the cause of some of the symptoms of stress, and the inducer of defense responses which help to enhance survival of the plant under adverse conditions.

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During much of a plant's growth and development ethylene levels are low (i.e., <0.05 μL/L), but during senescence and fruit ripening large quantities (~100 μL/L) are synthesized [1]. Many of the biological responses triggered by ethylene occur as a consequence of a small localized burst of ethylene that is sometimes difficult or even impossible to measure by gas chromatography (the standard means of assessing ethylene levels).

## 2. Plant growth-promoting bacteria

The interaction between bacteria and plants may be beneficial, harmful or neutral for the plant, and sometimes the effect of a particular bacterium may vary as the soil conditions change [5]. Bacteria that are beneficial to plants are of two general types: those that form a symbiotic relationship, which involves formation of specialized structures or nodules on host plant roots, and those that are free-living in the soil; the latter are often found near, on or even within the roots [6]. While numerous free-living soil bacteria are considered to be plant growth-promoting bacteria, not all bacterial strains of a particular genus and species have the same repertoire of metabolic capabilities so that only some *Pseudomonas putida* strains actively promote plant growth.

Bacteria that act by directly stimulating plant growth have received much less attention than biocontrol bacteria, reflecting the generally held view that in the field it is more difficult to reproducibly demonstrate the efficacy of these bacteria. There are several ways in which plant growth-promoting bacteria can directly facilitate plant proliferation [7]. They may: fix atmospheric nitrogen; synthesize siderophores which solubilize and sequester iron; produce phytohormones; solubilize minerals such as phosphorus; and synthesize some less well characterized low molecular mass compounds or enzymes that can modulate plant growth and development [7–9]. A particular bacterium may affect plant growth using any one, or more, of these mechanisms. Moreover, a bacterium may provide different benefits at various times during the life cycle of the plant.

The mechanism most often invoked to explain the direct effects of plant growth-promoting bacteria on plants is the production of phytohormones, including auxins such as indoleacetic acid, or IAA [8,10,11]. In addition, a number of plant growth-promoting bacteria contain the enzyme ACC deaminase and this enzyme can cleave the plant ethylene precursor ACC, and thereby lower the level of ethylene in a developing or stressed plant [7,12,13]. For many plants a burst of ethylene is required to break seed dormancy [14] but, following germination, a sustained high level of ethylene may inhibit root elongation [15]. Thus, plant growth-promoting bacteria that contain the enzyme ACC deaminase, when bound to the seed coat of a developing seedling, may act as a mechanism for ensuring that the ethylene level does not become elevated to the point where root growth is impaired. By facilitating the formation of longer roots, these bacteria may enhance the survival of some seedlings, especially during the first few days after the seeds are planted. Similarly, ACC deaminase-containing bacteria bound to the roots of plants can act as a sink for ACC and protect stressed plants from some of the deleterious effects of stress ethylene.

## 3. The enzyme ACC deaminase

The bulk of the biochemical studies of ACC deaminase, its physical and chemical properties, and its mode of action have been performed by Honma and his co-workers [16–24] although a few other studies of the enzyme and its properties have been reported [12,25–28].

ACC deaminase is a multimeric enzyme with a monomeric subunit molecular mass of approximately 35–42 kDa (Table 1). It is a sulfhydryl enzyme that utilizes pyridoxal 5-phosphate as an essential co-factor. Pyridoxal phosphate is tightly bound to the enzyme in the amount of approximately one molecule per subunit; it displays a characteristic pyridoxaldimine visible absorbance at 418 nm. While several D-amino acids, notably D-serine and D-cysteine can act as substrates for ACC deaminase (albeit less efficiently than ACC), L-serine and L-alanine are effective competitive inhibitors of the enzyme. ACC deaminase catalyzes a cleavage of ACC that includes cyclopropane ring fragmentation, and deamination of ACC to form α-ketobutyrate and ammonia.

Despite the fact that its substrate ACC is plant-produced, in those instances where it has been examined ACC deaminase is not a secreted enzyme. Rather, it is localized within the cytoplasm of the microorganism that produces it [12]. In this case, the substrate, ACC is exuded by plant tissues [29–31] and is then taken up by the ACC deaminase-containing microbe [13].

X-ray crystallographic analysis reveals that ACC deaminase folds into two domains, each of which has an open twisted α/β structure that is similar to the β-subunit of the enzyme tryptophan synthase [32]. However, unlike other members of the β family of pyridoxal phosphate dependent enzymes, the pyridoxal phosphate co-factor of ACC deaminase is buried deep within the protein molecule. Two key amino acid residues have been located in the enzyme: a reactive thiol group at cysteine 162, located in the internal gap between the two domains of the molecule, and the pyridoxal phosphate binding site at lysine 51.

Table 1
Biochemical characteristics of some ACC deaminases

| Enzyme source | *Pseudomonas* sp. strain ACP | *Pseudomonas putida* GR12-2 | *Hansenula saturnus* | *Penicillium citrinum* | *Pseudomonas putida* UW4 |
|---|---|---|---|---|---|
| Native enzyme molecular mass (Daltons) | 104–112,000 | 105,000 | 69,000 | 68,000 | n.d. |
| Subunit molecular mass (Daltons) | 36,500 | 35,000 | 40,000 | 41,000 | 41,800 |
| Estimated number of subunits | 3 | 3 | 2 | 2 | n.d. |
| pH optimum | 8.0–8.5 | 8.5 | 8.5 | 8.5 | 8.0 |
| Temperature optimum | n.d. | 30 °C | n.d. | 35 °C | n.d. |
| $K_m$ for ACC (mM) | 1.5–9.2 | n.d. | 2.6 | 4.6 | 3.4 |
| $K_{cat}$ ($min^{-1}$) | 290 | n.d. | n.d. | n.d. | 146 |
| Melting temperature | n.d. | n.d. | n.d. | n.d. | 58–60 °C |
| Reference | [16–18] | [12] | [16,21] | [22] | [28] |

n.d. = not determined.

## 4. ACC deaminase genes in microorganisms and plants

### 4.1. Prevalence

All of the early studies of ACC deaminase focused on the enzyme from free-living soil bacteria (typically pseudomonads) and fungi [16,33–35]. More recently, ACC deaminase has been found in a wide range of Gram negative bacteria [36–39], Gram positive bacteria [37] rhizobia [40–42], endophytes [43] and fungi [22]. In addition, based on sequence similarity, many microorganisms have putative ACC deaminase genes; however, whether these are bona fide ACC deaminases remains to be demonstrated. In one instance, when an ACC deaminase homologue from *Pyrococcus horikoshii* was expressed in *Escherichia coli*, it did not show ACC deaminase activity, despite extensive sequence similarities, although it did have D-serine deaminase activity [24]. On the other hand, DNA sequence analysis has revealed the presence of putative ACC deaminase genes in the genomes of several plants, and there is evidence that *Arabidopsis* and poplar plants express ACC deaminase activity (McDonnell et al., submitted for publication).

### 4.2. Regulation

A study of ACC deaminase activity in *Pseudomonas putida* GR12-2 indicated that full induction took much longer than the generation time of the bacterium [12] suggesting that the mechanism of ACC deaminase induction and hence its mode of regulation in this bacterium is relatively complex. Subsequently, analysis of DNA sequence data for the region upstream of the ACC deaminase structural gene from *P. putida* UW4 indicated that this DNA segment contained a CRP (cyclic AMP receptor protein) binding site, an FNR (fumarate–nitrate reduction regulatory protein) binding site (which is a known anaerobic transcriptional regulator), an Lrp (leucine-responsive regulatory protein) binding site and an open reading frame encoding an Lrp protein [44,45]. All of these features were shown to be involved in the transcriptional regulation of the ACC deaminase gene, with the *P. putida* UW4 ACC deaminase structural gene (*acdS*) promoter under the transcriptional control of the regulatory Lrp protein (encoded by *acdR*). More recently, in *P. putida* UW4, a protein that interacts with ACC, the Lrp protein and the region of DNA upstream of *acdS* was identified and characterized (Cheng et al., submitted for publication). Although all of the details of the above mentioned mechanism of transcriptional regulation are not yet completely understood, genes encoding Lrp proteins have been found immediately upstream from a number of bacterial ACC deaminase structural genes (see below) suggesting that this mode of transcriptional regulation is a central feature of the functioning of many bacterial ACC deaminases.

In addition to regulation by Lrp, an *acdS* gene from *Mesorhizobium loti* MAFF303099 has been shown to be under the transcriptional regulatory control of the *nifA* promoter [46], and to be expressed within legume nodules [42]. Although it is entirely speculative at this point, the expression of ACC deaminase genes within nitrogen fixing nodules might decrease the rate of nodule senescence – as nitrogen fixation with its high energy demand could activate stress ethylene synthesis – and thereby effectively increase the amount of fixed nitrogen.

The affinity of an enzyme for a particular substrate (i.e., the $K_m$ value) reflects more than the tightness of substrate binding, rather it has a profound effect on the kinetics of conversion of substrate into product. When the $K_m$ values for the binding of ACC by ACC deaminase were determined for enzyme extracts of several different microorganisms at pH 8.5, the values ranged from 1.5 to 17.4 mM, indicating that the enzyme does not have a particularly high affinity for ACC. There are two significant consequences of the low affinity of ACC deaminase for ACC. First, because the enzyme ACC oxidase (which catalyzes ethylene formation from ACC) has a much greater affinity for ACC than does ACC deaminase, the only way that ACC deaminase can effectively compete with ACC oxidase for ACC and thereby lower plant ethylene levels is for the amount of ACC deaminase to be much greater (~100- to 1000-fold) than the amount of ACC oxidase.

This is likely to often be the case since ACC oxidase is an induced enzyme that is normally present in non-senescent and non-stressed tissues in only very low levels. Second, since plant ACC levels are typically in the micromolar range and the $K_m$ is in the millimolar range, Michaelis–Menton kinetics indicate that every increase in ACC concentration will be accompanied by a parallel increase in the rate of ACC cleavage (i.e., when $K_m > S$ then $v \propto S$), independent of the level of enzyme present. Thus, the enzyme will immediately respond to a 2- to 3-fold increase in ACC levels (e.g., following environmental stress) by increasing, by 2- to 3-fold, the rate of conversion of ACC to ammonia and α-ketobutyrate.

### 4.3. Inheritance

One model for the origin of ACC deaminase genes suggests that they may have arisen by convergent evolution following modification and/or duplication of bacterial genes encoding pyridoxal phosphate-requiring amino acid deaminases or aminotransferases. This model predicts the existence of a limited number of ACC deaminase gene motifs because the possible number of pre-existing genes that could be mutated, without a large number of changes, to encode ACC deaminase, is likely to be small. However, soil bacteria may acquire ACC deaminase genes by mechanisms other than fortuitous mutation, and the transfer of such genes from one soil bacterium to another is not uncommon [47].

When the 16S rDNA sequence phylogenetic tree for a number of ACC deaminase-containing bacteria was compared with a phylogenetic tree based on the ACC deaminase gene sequences from these bacteria, it is observed that the ACC deaminase phylogenetic tree did not mirror the 16S rDNA phylogenetic tree [39]. For example, the *Pseudomonas* encoded ACC deaminases were distributed throughout the tree, and the yeast and fungal ACC deaminases did not form outliers. This suggests the possibility that bacterial ACC deaminase genes are not inherited vertically and at least some bacterial ACC deaminase genes may have evolved through lateral (horizontal) gene transfer. Based on similar evidence, prokaryotic genes involved in photosynthesis, aerobic respiration, nitrogen fixation, sulphate reduction, quorum sensing, methylotrophy, isoprenoid biosynthesis, and extremophily, have all been suggested to have evolved through lateral gene transfer [48].

If ACC deaminase genes have been acquired by some bacteria by lateral gene transfer, then it is possible that the transcriptional regulatory elements for this gene were acquired in a similar manner. The juxtaposition of *acdS* and *acdR* genes has also been observed for *Agrobacterium tumefaciens* d3, *Bradyrhizobium japonicum* USDA110, *Rhizobium leguminosarum* bv. viciae 128C53K, *Variovarax paradoxus* 5C2, and *Achromobacter xylosoxidans* A551 [39,41,49,50], and is consistent with the notion that the entire region of DNA containing the ACC deaminase structural and regulatory genes was acquired by lateral gene transfer.

Additional evidence in support of the possibility of lateral gene transfer may be found in the fact that the presence of functional ACC deaminases has been demonstrated in both poplar and *Arabidopsis* (McDonnell et al., submitted for publication). These and other plants may have acquired the gene from soil bacteria with which they normally associate.

### 4.4. Expression levels

There is a wide range (>100-fold) in the level of ACC deaminase activity that is observed in nature from one organism to another so that these organisms may be conceptually divided into two groups, those with either high or low enzyme activity. High ACC deaminase-expressing organisms typically bind relatively non-specifically to a variety of plant surfaces. This group includes most, if not all, rhizosphere and phyllosphere organisms as well as endophytes, all of which can act as a sink for ACC produced as a consequence of plant stress. These organisms display little preference for one particular plant over another. On the other hand, low deaminase-expressing organisms bind only to specific plants or are expressed only in certain tissues, and they do not lower the overall level of ethylene in the plant, but rather prevent a localized rise in ethylene levels. This group includes most, if not all, rhizobia as well as plant ACC deaminases. Rhizobia infection and nodulation causes only a very small rise in plant ethylene levels so that only a low level of enzyme activity is required to prevent this from occurring. Low plant ACC deaminase activity expressed only in specific tissues (e.g., phloem but not xylem) de facto ''targets'' ACC deaminase to where it is needed.

### 4.5. Transgenic plants

Increased ethylene levels in plants exposed to various types of stress including chilling, heat, wounding, pathogen infection and nutritional stress, with increased damage as the result has been documented [51]. In stressed plant tissues there is an initial very small peak of ethylene which initiates a protective response by the plant and then a second much larger peak some time later which initiates processes such as senescence, chlorosis and abscission, all of which are inimical to plant survival [52]. By expressing a bacterial gene for ACC deaminase in transgenic plants, it is possible to delay fruit ripening [33] or protect plants from damage caused by pathogens [53,54] flooding [55], heavy metals [56] and the effects of high salt concentrations (Sergeeva et al.,

submitted for publication). Interestingly, the treatment of plants with ACC deaminase-containing plant growth-promoting bacteria is an even more effective means of decreasing the ethylene mediated damage to plants, than is the creation of transgenic plants, from a variety of environmental stresses [31,57–60].

## 5. Affect of ACC deaminase on plants

### 5.1. Stress responses

Treatment of plant seeds or roots with ACC deaminase-containing bacteria typically reduces ACC and ethylene levels about 2- to 4-fold [29,31,59]. ACC and/or ethylene levels are generally reduced to a similar extent in transgenic plants that express a bacterial ACC deaminase under the control of either the 35*S* (constitutive) or *rolD* (root-specific) promoter [55], although ethylene levels have been reported to be decreased by more than 95% in some ripening transgenic tomato fruit [33]. Notwithstanding the often small reduction in ACC and ethylene levels, the protection afforded stressed plants through the action of ACC deaminase is often quite dramatic. While most of the experiments where a protective effect has been demonstrated have been conducted in either a greenhouse or growth chamber setting, significant protection against flooding (Glick et al., unpublished data), the presence of high levels of nickel [57] and polycyclic aromatic hydrocarbons (Huang et al., unpublished data) has been observed in field trials.

### 5.2. Plant gene expression

In a study designed to elucidate some of the genetic changes in plants caused by plant growth-promoting bacteria [61], *Paenibacillus polymyxa* was added to *Arabidopsis thaliana* roots and, using differential display PCR, a number of genes whose expression was altered significantly were identified. Subsequently, RNA arbitrarily primed PCR was used [62] to identify several genes in canola roots whose expression was affected differentially by the addition of an ACC deaminase-containing plant growth-promoting bacterium and an ACC deaminase negative mutant of that strain. The ACC deaminase-containing bacterium down-regulated a gene involved in an ethylene-induced plant stress response and up-regulated genes involved in plant growth. This data is consistent with the model [13] of how deaminase-containing plant growth-promoting bacteria lower plant ethylene levels and limit stress-caused damage to plants.

In preliminary experiments, commercially available *Arabidopsis thaliana* 60-mer oligonucleotide microarrays have been used to assess the effect of ACC deaminase activity (from a root expressed transgene) on gene expression in canola roots and shoots (Stearns and Glick, unpublished results). The data suggest that lowering plant ACC levels (and hence presumably ethylene levels as well) by the transgene results in an increase in the transcription of genes involved in the biosynthesis and functioning of IAA. Since IAA can promote plant cell elongation and proliferation, in these plants growth promotion attributed to transgene expression may be a consequence of both the lowered level of ethylene and the increased level of IAA.

## 6. Conclusion

With the demonstration that soil bacteria expressing the enzyme ACC deaminase, or the transgene transformed into plants are able to provide a significant measure of protection to a wide range of plants from many different biotic and abiotic stresses, many researchers have become interested in using the activity of this enzyme as an adjunct to agricultural and horticultural practice. And, given the plethora of biological responses to increased amounts of ethylene, the ability to enzymatically attenuate those levels is an important and powerful tool for both basic and applied plant scientists. However, in the long run, for this technology to be most useful in an applied setting, it is necessary for the relationship between plants and ACC deaminase-containing bacteria to be thoroughly understood at a basic level. Thus, the various ACC deaminase genes, both microbial and plant, and their detailed modes of regulation need to be thoroughly characterized, the physiological impacts of these genes need to be well understood in a variety of plants and plant tissues, and the genes whose expression is altered (either up or down) needs to be elaborated in detail including an understanding as to how the proteins encoded by these genes contribute to plant growth and development. The magnitude of the task at hand notwithstanding, there is every reason to believe that gaining a greater understanding of these processes will enhance and facilitate efforts to wean our society off of its dependence on a wide range of agricultural chemicals.

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