THE ROLE OF
ACC DEAMINASE IN PLANT GROWTH PROMOTION

by

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Abstract

We have proposed that plant growth-promoting bacteria, that possess the enzyme ACC (1-aminocyclopropane-1-carboxylic acid) deaminase, can reduce the amount of ethylene produced by the plant and promote root elongation. Ethylene is required by many plants for seed germination but high levels of ethylene produced by fast growing roots can inhibit root elongation. In this study, levels of ACC, the immediate precursor of ethylene, were measured in canola seedlings, and in canola seed exudates and extracts. We show that ACC is exuded by the seeds and that, in the presence of the plant growth-promoting bacterium, *Enterobacter cloacae* CAL3, ACC levels in both exudates and extracts are lowered.

Enterobacter cloacae CAL3, the plant growth-promoting bacterium used in this study, produces low levels of ACC deaminase activity and is capable of synthesizing high levels of IAA (indole acetic acid). An attempt was made to isolate the ACC deaminase gene from this bacterium. Four clones, based on their growth on minimal medium containing ACC as the sole source of nitrogen, were selected from the *Enterobacter cloacae* CAL3 clone bank for DNA sequence analysis. None of the derived amino acid sequences of the DNA inserts were homologous with known ACC deaminase genes but were homologous with genes encoding enzymes or functional proteins that might enhance the growth of the bacterium or the uptake of ACC.

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So many people have been involved in the completion of this thesis. I am grateful to those who have helped me with my research, particularly Dr. Saleh Shah—without his help, the completion of this thesis would have been delayed even further. I thank my colleagues—Jiping Li, who bestowed on me her vast lab skills and good humour in generous measures; Cheryl Patten, whose deftness at balancing academic and family life, was an inspiration; Wenbo Ma, who shared invaluable advice about computers and DNA sequencing, and the other members of Dr. Glick's lab, who were more than lab-mates—friends, comrades, and kindred spirits. My supervisor, Dr. Bernard Glick, deserves special recognition: he offered me an opportunity that I could not refuse and nudged me towards its completion.

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Dedication

For my dad, Gordon Penrose,

who believed that science should be fun.

Table of Contents

List Of Tables

List of Figures

"And I keep hearing from the cellar bin

The rumbling sound

Of load on load of apples coming in.

For I have had too much

Of apple-picking: I am overtired of the great harvest I myself desired.

There were ten thousand thousand fruit to touch,

Cherish in hand, lift down, and not let fall."

After Apple-Picking, Robert Frost

1. Introduction

1.1. Plant growth-promoting bacteria

Throughout the ages, man has striven to produce agricultural crops in ever-greater abundance and variety. In this regard, farmers have often focused their efforts on soil improvement in order to maximize yields. The easiest method of improving soil fertility is by adding chemical fertilizers. Today, more than 100 million tonnes of nitrogen fertilizers and more than 90 million tonnes of potash and phosphate fertilizers are used annually worldwide to increase the yield of crop plants (Glick et al., 1999b). Despite the success in using these compounds to improve crop yields, they can, under certain circumstances, pollute the environment and may contribute to a number of human and animal health problems.

The potential negative environmental impacts of large-scale use of chemical fertilizers together with their increased cost has prompted a public outcry for alternatives to replace chemical fertilizers. Bacterial inoculants capable of facilitating plant growth have been considered reasonable substitutes but, in the past, suffered from a lack of consistency when used under field conditions. However, it is plausible that in the future, the use of bacterial inoculants will become part of everyday agricultural practice; we have

the tools of modern molecular biology to enable a better understanding of the mechanisms utilized by these organisms, and to improve their efficacy in agriculture.

Bacteria that provide some benefit to plants are likely to be found in the soil. In addition to bacteria, soil contains a large number of different types of microorganisms including fungi, actinomycetes, protozoa and algae (Paul and Clark, 1989). Of these, bacteria are by far the most common; they can grow rapidly and have the ability to utilize a wide range of different substances as either carbon or nitrogen sources. While many soil bacteria are bound to the surface of soil particles and are found in soil aggregates, many more interact specifically with the roots of plants. In fact, the concentration of bacteria that is found around the roots of plants, i.e., in the rhizosphere, is generally much greater than the bacterial density, or concentration, that is found in the rest of the soil (Lynch, 1990). The high concentration of bacteria around the roots of plants presumably occurs because of the presence of high levels of nutrients (especially small molecules such as amino acids, sugars and organic acids) that are exuded from the roots of most plants, and can then be used to support bacterial growth and metabolism (Whipps, 1990).

The interaction between bacteria and plant roots may be beneficial, harmful or neutral for the plant, and sometimes the effect of a particular bacterium may vary as the soil conditions change

(Lynch, 1990). For example, a bacterium that facilitates growth by providing plants with fixed nitrogen, which is usually present in only limited amounts in the soil, is unlikely to provide any benefit to plants when large amounts of chemical nitrogen fertilizer are added to the soil.

While there are some soil bacteria that are pathogenic to plants, most of the more agronomically important plant disease-causing soil microorganisms are fungi, including the genera *Fusarium, Pythium* and *Rhizoctonia*. Most of the research in the area of microbial phytopathogenesis is directed toward ameliorating the negative impact that these organisms have on plants. However, an understanding of the mechanism(s) of microbial phytopathogenesis may indirectly contribute to a better understanding of some of the mechanisms that are used by plant growth-promoting bacteria since phytopathogens and plant growth-promoting bacteria utilize many of the same mechanisms.

The bacteria that provide some benefit 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 of plants (Kloepper et al., 1988; van Peer and Schippers, 1989; Frommel et al., 1991). The symbiotic bacteria, especially Rhizobia, have been studied extensively

and, although the world market for these microorganisms is relatively small at the present time, they have been developed as a "biological" means of increasing crop yields (Vance, 1983; Bohlool, 1990; Paau, 1991; Sharma et al., 1993). Beneficial free-living soil bacteria are generally referred to as plant growth-promoting rhizobacteria or PGPR and are found in association with the roots of many different plants (Kloepper et al., 1989; Table 1). 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 identical metabolic capabilities. Thus, for example, some *Pseudomonas putida* strains actively promote plant growth while others have no measurable effect on plants.

Plant growth-promoting bacteria can have an impact on plant growth and development in two different ways: indirectly or directly (Table 2). The indirect promotion of plant growth occurs when these bacteria decrease or prevent some of the deleterious effects of a phytopathogenic organism (usually a fungus) by any one or more of several different mechanisms. On the other hand, the direct promotion of plant growth by plant growth-promoting bacteria generally entails providing the plant with a compound that is synthesized by the bacterium or facilitating the uptake of nutrients from the environment.

Table 1. Some free-living soil bacteria that can function as plant growth-promoting bacteria. Not all strains of the listed organisms can act as plant growth-promoting bacteria. (Glick et al., 1999b.)

Azorhizobium caulinodans	*Citrobacter freundii*
Azospirillum amazonense	*Curtobacterium flaccumfaciens*
Azospirillum halopraeferens	*Enterobacter agglomerans*
Azospirillum irakense	*Enterobacter cloacae*
Azospirillum lipoferum	*Erwinia herbicola*
Azospirillum brasilense	*Flavomonas oryzihabitans*
Azotobacter chroococcum	*Hydrogenophaga pseudoflava*
Bacillus cereus	*Klebsiella planticola*
Bacillus coagulans	*Kluyvera ascorbata*
Bacillus laterosporus	*Kluyvera cryocrescens*
Bacillus licheniformis	*Phyllobacterium rubiacearum*
Bacillus macerans	*Pseudomonas aeruginosa*
Bacillus megaterium	*Pseudomonas aureofaciens*
Bacillus mycoides	*Pseudomonas corrugata*
Bacillus pasteurii	*Pseudomonas fluorescens*
Bacillus polymyxa	*Pseudomonas marginalis*
Bacillus pumilus	*Pseudomonas putida*
Bacillus sphaericus	*Pseudomonas rubrilineans*
Bacillus subtilis	*Rathayibacter rathayi*
Burkholderia cepacia	*Serratia marcescens*
Burkholderia gladioli	*Stenotrophomonas sp.*
Burkholderia graminis	*Streptomyces griseoviridis*
Burkholderia vietnamiensis	

Table 2. Some mechanisms used by plant growth-promoting bacteria to stimulate plant growth

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Indirect Mechanisms

- Antibiotic production
- Depletion of iron from the rhizosphere
- Induced systemic resistance
- Synthesis of antifungal metabolites
- Production of fungal cell wall lysing enzymes
- Competition for sites on the root

Direct Mechanisms

- Solubilization of phosphorus
- Nitrogen fixation
- Sequestering iron by siderophores
- Production of phytohormones (auxins, cytokinins, gibberellins)
- Lowering ethylene concentration

A perusal of the scientific literature indicates that most of the interest in plant growth-promoting bacteria relates to the ability of some of these organisms to function (indirectly) as biocontrol agents (Glick et al., 1999b). On the other hand, the bacteria that act by directly stimulating plant growth have received much less attention. This probably is a reflection of the generally held view that in the field it is easier to reproducibly demonstrate the efficacy of biocontrol plant growth-promoting bacteria rather than bacteria that act by directly stimulating plant growth. Technical problems notwithstanding, the use of bacteria that promote plant growth directly represents an enormous potential opportunity for both agriculture and horticulture.

1.1.1. Mechanisms of direct growth promotion

There are several ways in which different plant growth-promoting bacteria have been reported to directly facilitate the proliferation of their plant hosts. Plant growth-promoting bacteria may fix atmospheric nitrogen and supply it to plants; they may synthesize siderophores which can solubilize and sequester iron from the soil and provide it to plant cells; they may synthesize several different phytohormones, including auxins and cytokinins, which can act to enhance various stages of plant growth; they may have mechanisms for the solubilization of minerals such as phosphorus

which then become more readily available for plant growth; and they may synthesize some less well characterized low molecular mass compounds or enzymes that can modulate plant growth and development (Brown, 1974; Kloepper et al., 1986; Davison, 1988; Kloepper et al., 1989; Lambert and Joos, 1989; Patten and Glick, 1996). From the numerous studies concerning the mechanisms that are used by plant growth-promoting bacteria, it has become apparent that a particular bacterium may affect plant growth and development using any one, or more, of these mechanisms. Moreover, since many plant growth-promoting bacteria possess several traits that enable them to facilitate plant growth, a bacterium may utilize different traits at various times during the life cycle of the plant. For example, following seed germination a plant growth-promoting bacterium may lower the plant's ethylene concentration thereby decreasing the ethylene inhibition of seedling root length. Once the seedling has depleted the resources that are contained within the seed, a plant growth-promoting bacterium may help to provide the plant with a sufficient amount of iron and phosphorus from the soil. Through early plant development, plant growth promoting bacteria may stimulate cell division by providing appropriate levels of auxins, cytokinins or gibberellins and help plants to tolerate a variety of environmental stresses, such as flooding and drought, by lowering

the potentially detrimental level of stress ethylene that can form as a response to these stresses.

Since one of the major benefits that Rhizobia provide to plants is fixed nitrogen in exchange for fixed carbon (photosynthate), it was naively thought that diazotrophic plant growth-promoting bacteria might also function in this way. However, not all plant growth-promoting bacteria are diazotrophic. In addition, many of the plant growth-promoting bacteria that are diazotrophic fix only limited amounts of nitrogen, and not nearly enough to provide for their own as well as the host plant's nitrogen requirements (Hong et al., 1991a). Other diazotrophic plant growth-promoting bacteria can fix high levels of nitrogen; however, most researchers believe that even these organisms provide the plant with only small amounts of fixed nitrogen. Thus, for the majority of plant growth-promoting bacteria nitrogen fixation is only a minor component of the benefit that the bacterium provides to the plant, although under certain circumstances, some diazotrophs may provide their plant hosts with a significant portion of the fixed nitrogen that they require.

It is likely that the impact of all of the mechanisms, by which the bacterium provides a compound or nutrient such as fixed nitrogen, phosphorus or iron to the plant, varies considerably depending upon the soil composition. For example, the growth of cactus plants that are cultivated in extremely nutrient-poor sandy

soils is dramatically enhanced when the plants are inoculated with *Azospirillum* (Y. Bashan, unpublished observation). On the other hand, numerous researchers have observed that plant growth-promoting bacteria have little or no measurable effect on plant growth when the plants are cultivated in nutrient-rich soil and grown under optimal conditions.

It is generally assumed that bacterial stimulation of plant growth requires the binding of the bacterium to the plant root. While this is undoubtedly true for most plant growth-promoting bacteria effects, binding may not always be required (Reddy and Rahe, 1989; Hong et al., 1991b). For example, in one study of onion in the field, significant growth promotion was observed even though only very low levels of root colonization by plant growth-promoting bacteria were detected (Reddy and Rahe, 1989). In addition, electron microscopy studies of the roots of young canola seedlings grown in growth pouches, under gnotobiotic conditions in the presence of the plant growth-promoting bacterium *Pseudomonas putida* GR12-2, indicated that bacterial adherence to the seed coat alone was sufficient to enhance root elongation during the first few days of seedling development (Hong et al., 1991b). In the latter case, root colonization may play another, and later, role in the plant growth-promoting activity of this bacterium (Glick et al., 1997). That is, root colonization

may become more important once the developing seedling has exhausted the nutrients that are available in the seed.

The mechanism that has been most often invoked to explain the various effects of plant growth-promoting bacteria on plants is the production of phytohormones, and most of the attention has focused on the role of the phytohormone auxin (Brown, 1974; Tien et al., 1979; Patten and Glick, 1996). Since plants as well as plant growth-promoting bacteria can synthesize auxin, it is important when assessing the consequences of treating a plant with a plant growth-promoting bacterium to distinguish between the bacterial stimulation of plant auxin synthesis on the one hand, and auxin that is synthesized by the plant growth-promoting bacterium, on the other (Gaudin et al., 1994). Thus, the growth of plants treated with an auxin-secreting plant growth-promoting bacterium can be affected by the amount of auxin that the bacterium produces such that the observed response may vary from one species of plant to another depending upon the existing hormonal levels within the treated plant.

A relatively recent discovery implicates the plant hormone ethylene in a mechanism of plant growth promotion. It has been found that a number of plant growth-promoting bacteria contain the enzyme 1-aminocyclopropane-1-carboxylate (ACC) deaminase (Jacobson et al., 1994; Shah et al., 1997; Glick et al., 1998; Shah et al.,

1998; Belimov et al., submitted for publication) and that this enzyme can cleave the plant ethylene precursor ACC, and thereby lower the level of ethylene in a developing or stressed plant.

1.2. Ethylene

For centuries farmers from around the world have used ethylene to manipulate crop plants. In the fourth century B.C., Theophrastus, a student of Aristotle's and author of the first known botanical treatise, recorded that the Egyptian fig "will not fully mature unless it is cut and anointed with oil" (McGee, 1984). It was reported that the Chinese enclosed mature pears in a room with burning incense to ripen them more quickly (Abeles et al., 1992). In Russia during the 1860s, young cucumber plants were fumigated with wood smoke to increase the formation of fruitful flowers and produce an earlier harvest. Citrus growers used kerosene stoves in packing sheds and railway cars in California in the early 1900s not only to prevent frost damage to citrus fruit but also to enhance colour development in the fruit. In all of these instances farmers encouraged the ripening of their harvests by exposing them to biologically effective levels of ethylene (Abeles et al., 1992).

Ethylene was not recognized as the causative agent of these practices until the Russian scientist, Dimitri Neljubov, began his work with etiolated pea seedlings in greenhouses in St. Petersburg

late in the nineteenth century (Abeles et al., 1992). He noticed that the seedlings grew horizontally inside the greenhouse but in the normal vertical manner outside the building. The air inside the greenhouse was contaminated with "illuminating gas," the main source of light during the nineteenth century produced by the partial combustion of coal. Neljubov discovered that ethylene, one of a number of unsaturated gases released during the burning of coal (and other organic fuels), could accumulate and reach physiologically effective levels in an enclosed space. For the first time, ethylene was identified as a biologically active compound (Abeles et al., 1992).

Since Neljubov's discovery, a great deal has been learned about ethylene. It is one of the simplest organic molecules (CH_2=CH_2) with biological activity, and can, because of its volatile nature, function as an efficient plant growth regulator even at very low concentrations (Abeles et al., 1992). Ethylene is important for normal development in plants as well as for their response to stress (Deikman, 1997). Many aspects of growth including growth of vegetative tissues such as roots, stems and petioles, and all the stages of development are affected by ethylene (Deikman, 1997). Flower senescence, leaf and petal abscission, and fruit ripening are apparently co-ordinated by ethylene and its biosynthesis is induced during these developmental phases (Abeles et al., 1992). A variety of other processes appear to involve ethylene: rhizobia nodulation of legumes, root development of plant

cuttings, and plant response to heavy metals, ozone, pathogens and flooding (Glick et al., 1999a).

Although ethylene is important for the normal development of higher plants including most aquatic and semi-aquatic plants, there are a number of exceptions in which this hormone does not seem essential. The growing shoots of over wintering tubers of a common aquatic weed, *Potamogeton pectinatus* (Devlin and Karczmarczyk, 1975) lack ethylene but are vigorous and morphologically normal (Summers et al., 1996). Normal vegetative growth has been observed in plant tissues grown under conditions in which ethylene concentrations are greatly reduced by hypobaric pressure (Reid, 1987) and in plant tissues treated with high doses of silver, an inhibitor of ethylene action (Cameron and Reid, 1983). Some mutant lines of *Arabidopsis thaliana* and tomato that are specifically insensitive to ethylene appear to develop normally and the development of these plants is not seriously affected by lowering their ethylene biosynthesis levels (Smalle and Van Der Straeten, 1997). However, tobacco plants transformed with a gene from *Arabidopsis thaliana* that confers ethylene insensitivity became sensitive to normally non-pathogenic soil-borne fungi. In this case, it appears that the particular pathogen determines whether ethylene is involved in plant resistance against microorganisms (Knoester et al., 1998).

The production of ethylene is regulated by a large number of environmental and internal factors (e.g., temperature, light, gravity, nutrition, and hormones). The term "stress ethylene" was coined by Abeles (1973) to describe the acceleration of ethylene biosynthesis associated with biological and environmental stresses, and pathogen attack (Morgan and Drew, 1997). 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 (Bestwick and Ferro, 1998) can be both the cause of some of the symptoms of stress (e.g., onset of epinastic curvature and formation of arenchyma), and the inducer of responses which will enhance survival of the plant under adverse conditions (e.g., production of antibiotic enzymes and phytoalexins).

During much of plant growth and development, ethylene production is low, but during senescence and ripening large quantities are synthesized (Bestwick and Ferro, 1998). Ethylene-induced senescence results in large losses of fruit and vegetables annually (Huxtable et al., 1998) (e.g., from "pit-splitting" and "pod shatter"). The phenomenon of "pit splitting" in peach fruit results in premature ripening, fruit drop and flesh wooliness (Mizutani et al., 1998a); seed is lost from fully mature pods during "pod shatter" in *Brassica napus L.* (oilseed rape/canola) (Child et al., 1998). Certain post-harvest disorders of fruits and vegetables are caused by ethylene:

for example, lettuce and cucumbers cannot be stored in the same space as ethylene-producing stock such as tomatoes. The shelf life of many cut flowers is limited because they are extremely sensitive to ethylene. The production of ethylene in fruits and vegetables following cuts or bruises may be sufficient to hamper storage effectiveness (Wilkinson et al., 1997). Substantial fruit loss may occur during transportation and storage due to the autocatalytic production of ethylene in which a small amount of ethylene initiates an avalanche of ethylene production in adjacent plants or plant tissues (Bestwick and Ferro, 1998).

In agriculture it is important to control ethylene levels, often by lowering them in order to prevent economic losses. Current strategies include the use of controlled atmosphere storage, development of chemical inhibitors of ethylene biosynthesis and action, isolation of mutant plant lines with altered ethylene biosynthesis sensitivity and/or response, molecular cloning and characterization of genes encoding enzymes of the ethylene biosynthetic pathway for the construction of transgenic plants with impaired ethylene biosynthesis, and most recently, the investigation of some plant growth-promoting bacteria that, in addition to their ability to enhance plant growth, also appear capable of lowering ethylene levels within the plant (Hall et al., 1996).

Chemicals have been used to control ethylene levels in plants. The application of compounds such as rhizobitoxin, an amino acid secreted by some microorganisms, and its synthetic analogue, aminoethoxyvinylglycine (AVG), can inhibit ethylene biosynthesis; silver thiosulfate, can inhibit ethylene action, and 2-chloroethylphosphoric acid (ethephon), regarded in agriculture as "liquid ethylene," can release ethylene (Abeles et al., 1992). Sisler and Serek (1997) discovered that cyclopropenes can block ethylene perception and are potentially useful for extending the vaselife of cut flowers and the display life of potted plants. All of these chemicals are potentially harmful to the environment: AVG and silver thiosulfate are highly toxic in food, and silver thiosulfate causes blackspotting in flowers (Bestwick and Ferro, 1998). Recently, tropolone compounds were isolated from wood by Mizutani et al. (1998b). These compounds, which can inhibit the growth of wood-rotting fungi, were shown to inhibit the biosynthesis of ethylene in excised peach pits.

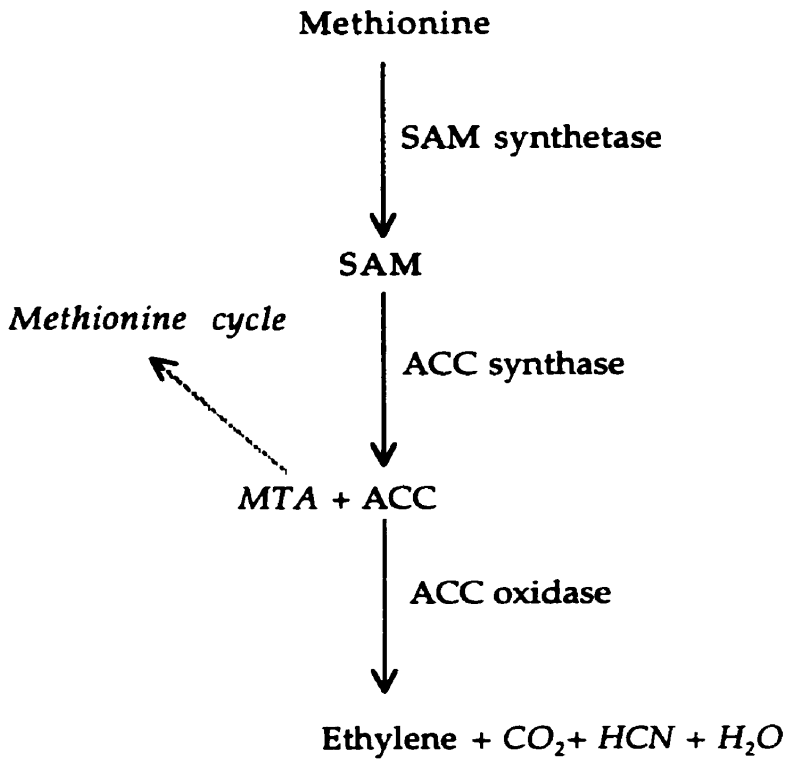
1.2.1. Ethylene biosynthetic pathway

In higher plants ethylene is produced from L-methionine via the intermediates, S-adenosyl-L-methionine (SAM) and 1-aminocyclo-propane-1-carboxylic acid (ACC) (Yang and Hoffman, 1984; Fig. 1). The enzymes involved in this metabolic sequence are

SAM synthetase, which catalyzes the conversion of methionine to SAM (Giovanelli et al., 1980); ACC synthase, which is responsible for the hydrolysis of SAM to ACC and 5'-methylthioadenosine (MTA) (Kende, 1989); and ACC oxidase which metabolizes ACC to ethylene, carbon dioxide, and cyanide (John, 1991).

Ethylene can be metabolized to ethylene glycol or ethylene oxide (Sanders et al., 1989), but it is not essential to enzymatically remove this hormone because it is a simple gas that can readily diffuse from plant tissues (Kende and Zeevaart, 1997). However, the volatility of ethylene makes it difficult to measure. Head space analysis by flame-ionization gas chromatography is the most frequently used method of measurement. Recently, a laser-driven photoacoustic detector has been developed to measure hormone released by intact plants with a claimed sensitivity of at least 200 times that of flame-ionization gas chromatography (Summers et al., 1996).

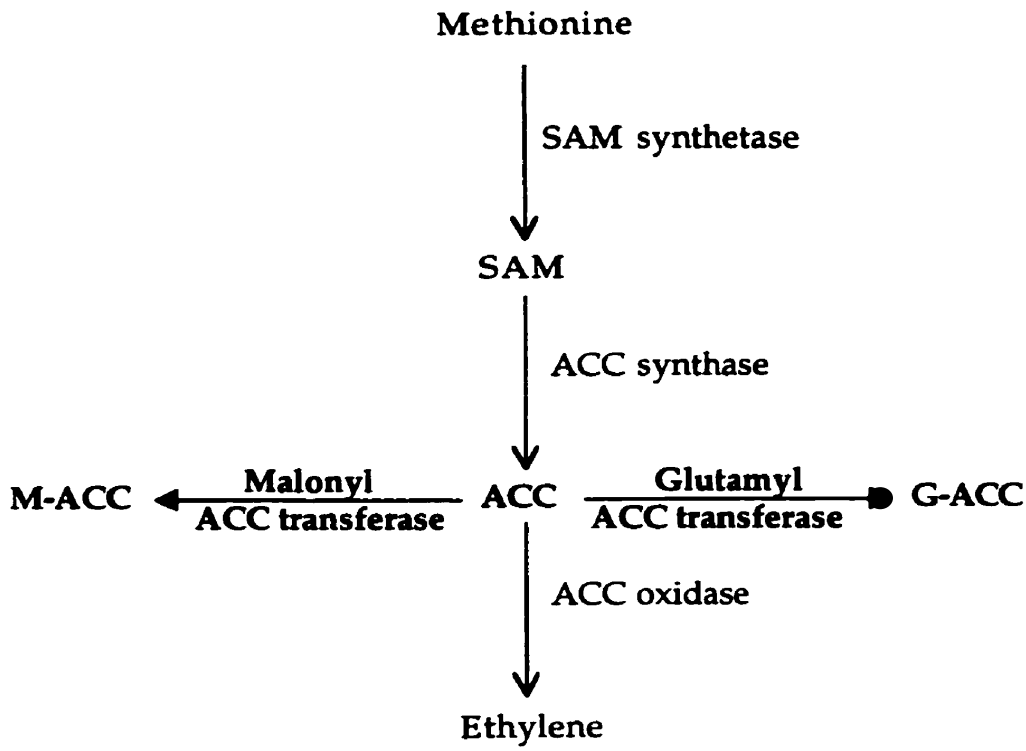
Fig. 1. The formation of ethylene in higher plants. The biosynthesis of ethylene from methionine is shown in plain type; the other products of the reactions are shown in italics as is the route to the methionine cycle. Abbreviations: SAM, S-adenosyl-L-methionine; ACC, 1-aminocyclopropane-1-carboxylic acid; MTA, 5'-methyl-thioadenosine; CO_2, carbon dioxide; HCN, hydrogen cyanide.


Methionine
SAM synthetase
SAM
Methionine cycle
ACC synthase
MTA + ACC
ACC oxidase
Ethylene + CO_2+ HCN + H_2O

Hydrogen cyanide, produced during ethylene biosynthesis in amounts stoichiometrically equivalent to ethylene (Yang and Hoffman, 1984), is the main source of endogenous hydrogen cyanide for many plants (Manning, 1988; Goudy et al., 1989). Cyanide is a phytotoxic agent capable of inhibiting enzymes involved in major metabolic processes (Solomanson, 1982). Although most plant tissues possess β-cyanoalanine synthase, the key enzyme for detoxifying cyanide in higher plants (Miller and Conn, 1980), the cyanide produced during ethylene biosynthesis is not necessarily removed from the plant cells (Grossmann, 1996). Cyanide may have a regulatory function in plant metabolism (Grossmann, 1996), particularly as a positive regulator of ethylene synthesis; cyanide has been implicated in the stimulation of ethylene synthesis in climacteric tissue, possibly at the ACC oxidase step (Pirrung and Brauman, 1987) and in the catalytic activation and inactivation of the enzyme, ACC oxidase (Grossmann, 1996).

Many plant growth substances including auxins, cytokinins, giberellins and abscicic acid, can exist in conjugated forms (Kende and Zeevaart, 1997). ACC, the immediate precursor of ethylene, can also occur in conjugated forms, i.e., 1-(malonylamino)cyclopropane-1-carboxylic acid (M-ACC) (Amrhein et al., 1981) and 1-(γ-L-glutamylamino)cyclopropane-1-carboxylic acid (G-ACC) (Martin et al., 1995) (Fig. 2).

Fig. 2. Synthesis of ACC conjugates. The conjugation reactions of ACC are shown in boldface type. Abbreviations: SAM, S-adenosyl-L-methionine; ACC, 1-aminocyclo-propane-1-carboxylic acid; M-ACC, 1-(malonylamino)-cyclopropane-1-carboxylic acid; G-ACC, 1-(γ-L-glutamylamino)cyclopropane-1-carboxylic acid.


Methionine
SAM synthetase
SAM
ACC synthase
M-ACC
Malonyl
ACC transferase
ACC
Glutamyl
ACC transferase
G-ACC
ACC oxidase
Ethylene

The malonylation of ACC with malonyl CoA is catalyzed by the enzyme ACC N-malonyltransferase (Guo et al., 1992); although the enzyme responsible for catalyzing the formation of G-ACC has not been identified, γ-L-glutamyltranspeptidase is a likely candidate (Fluhr and Mattoo, 1996). The role of these ACC-conjugates is uncertain but they may contribute to the regulation of ACC levels and rate of ethylene formation because ACC sequestered as ACC-conjugates cannot accumulate and subsequently be converted to ethylene (Fluhr and Mattoo, 1996). Although the malonylation of ACC is thought to be irreversible under physiological conditions (Kionka and Amrhein, 1984), it appears that under certain stress conditions, M-ACC can serve as a source of ACC (Jiao et al., 1986; Hanley et al., 1989).

In experiments following his discovery of some of the effects of ethylene in 1901, Neljubov observed a distinct pattern in the horizontal growth of etiolated pea seedlings in the presence of ethylene. This growth pattern, seen in most dicotyledenous seedlings exposed to ethylene, is named the "triple response" because it describes a trio of unique growth changes including inhibition of normal root geotropic response, inhibition of root and hypocotyl elongation, and exaggerated apical curvature (Abeles et al., 1992). The "triple response" has been very useful in the isolation of mutants in the ethylene biosynthetic pathway of *Arabidopsis thaliana* (Guzman

and Ecker, 1990). Mutant seedlings have been selected by their loss of capacity for the triple response or by their constitutive induction of the response in the absence of ethylene (Fluhr and Mattoo, 1996).

Genetic mutations have been isolated in the ethylene biosynthetic pathways of plants other than *Arabidopsis thaliana* at later stages of development: delayed fruit ripening was used to select the tomato mutants identified as *Nr*, never ripe; *rin*, ripening inhibitor, and *nor*, non-ripening (Fluhr and Mattoo, 1996); extended vase life was used to identify at least two mutations that rendered carnation flowers insensitive to ethylene (Larsen et al., 1995; Brandt and Woodson, 1992).

Several auxin mutants are included amongst the ethylene mutant lines because many plant processes are controlled by ethylene in close conjunction with auxin: auxin can increase the rate of ethylene biosynthesis (Smalle and Van Der Straeten, 1997); ethylene can affect auxin transport positively (Musgrave and Walters, 1973) or negatively (Beyer and Morgan, 1970; Suttle, 1988); and ethylene may influence auxin perception (Bertell et al., 1990; Liu and Reid, 1992). The ethylene-related mutants have been very useful for studying the role of ethylene and auxin during germination, etiolated seedling growth, and root and shoot growth, when it is particularly difficult to separate the effects of the two hormones (Smalle and Van Der Straeten, 1997). Ethylene biosynthesis was not induced by cytokinin

in recently identified mutants of *Arabidopsis thaliana* (Vogel et al., 1998).

1.2.2. Key enzymes of the ethylene biosynthetic pathway

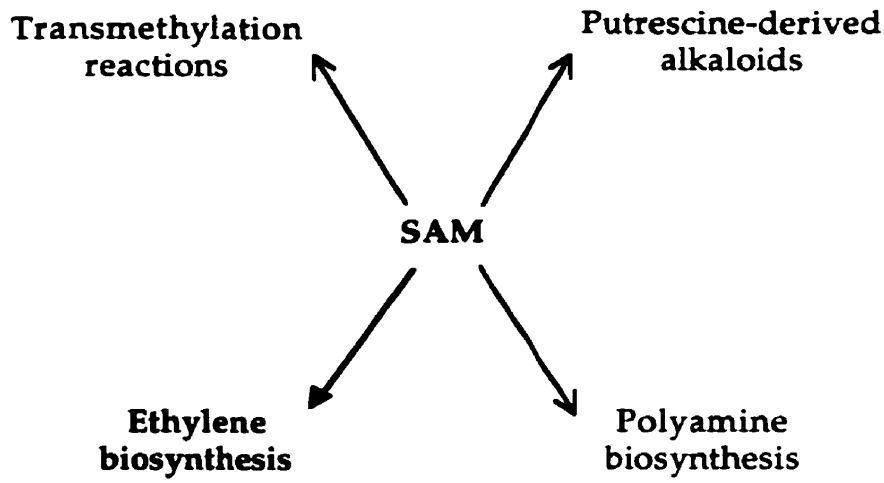
The tight regulation of ethylene synthesis in plants depends largely on the key enzymes of its biosynthetic pathway: SAM synthetase, ACC synthase and ACC oxidase. The genes encoding these enzymes and their controlling elements have been characterized, modified and reintroduced into plants in both their sense and antisense versions (Fluhr and Mattoo, 1996). These enzymes are all encoded by genes belonging to multigene families (Peleman et al., 1989; Larsen and Woodson, 1991; Kende and Zeevaart, 1997) whose transcripts, at least in the case of ACC synthase and ACC oxidase, and quite possibly SAM synthetase, are differentially regulated (Fluhr and Mattoo, 1996). Although ACC synthase and ACC oxidase are generally considered to be the enzymes that catalyze the rate-limiting steps (Kende and Zeevaart, 1997), the activity of SAM synthetase may also function in this capacity (Peleman et al., 1989).

1.2.2.1. SAM synthetase

During ethylene biosynthesis SAM is replenished by the methionine cycle at the expense of ATP (Yang and Hoffman, 1984). Plants, unlike other eukaryotes, can synthesize methionine and have the capacity to maintain methionine, SAM and threonine fluxes even during adverse conditions (Ravanel et al., 1998). Methionine, which exists at low levels in plants, is regenerated from 5'-methylthioadenosine, by a salvage pathway, which recycles the methylthio group of 5'-methylthioadenosine (Yang and Hoffman, 1984). The presence of this salvage pathway in most plant tissues (Miyazaki and Yang, 1987) ensures maximum availability of methionine, and therefore SAM, to the plant (Fluhr and Mattoo, 1996).

SAM synthetase provides SAM for a number of diverse metabolic reactions (Fluhr and Mattoo, 1996; Fig. 3). In addition to ethylene biosynthesis, SAM is involved in numerous transmethylation reactions in lipids, nucleic acids, proteins and carbohydrates (Tabor and Tabor, 1984), serves as a substrate for polyamine biosynthesis (Even-Chen et al., 1982), is involved in some stress responses (Morgan and Drew, 1997), and has been implicated in plant-pathogen interactions (Ravanel et al., 1998). Although a small amount of SAM, an abundant metabolite in most organisms (Boerjan et al., 1995), is diverted to ethylene synthesis (Yu and Yang,

Fig. 3: Pivotal role of SAM in nitrogen metabolism. The biosynthesis of ethylene from SAM is shown in boldface type. Abbreviation: SAM, S-adenosyl-L-methionine. Adapted from Tiburcio et al., 1997.


Transmethylation reactions
Putrescine-derived alkaloids
SAM
Ethylene biosynthesis
Polyamine biosynthesis

1979), SAM levels may regulate ethylene production (Fluhr and Mattoo, 1996).

SAM synthetase, an important "housekeeping enzyme", is ubiquitous, exists in multiple molecular forms, and in most plants, is inhibited by its own product (Peleman et al., 1989; Larsen and Woodson, 1991). SAM synthetase genes from different plants appear to be highly homologous at the amino acid level but at the nucleotide level show some diversity in the regions of these genes surrounding the open reading frame (ORF) (Espartero et al., 1994).

Transcripts of SAM synthetase genes accumulate differentially in different plant organs, and during senescence and various types of stress (Woodson et al., 1992; Kawalleck et al., 1992; Gowri et al., 1991; Espartero, et al., 1994), and enzyme activity appears to correspond to its transcript levels in plant organs (Peleman et al., 1989). Recently the expression of an organ-specific SAM synthetase has been correlated with changes in rates of ethylene synthesis during drought stress (Mayne et al., 1996). This evidence supports a role for SAM synthetase in the regulation of stress ethylene responses (Morgan and Drew, 1997).

1.2.2.2. ACC synthase

ACC synthase catalyzes the conversion of SAM to ACC and 5'-methylthioadenosine (Yang and Hoffman, 1984). The synthesis of

this enzyme is stimulated by signals from the plant hormones auxin, cytokinin and ethylene, and by environmental stresses including those of a physical, chemical or biological nature (Ecker, 1995). Ethylene production is induced by the *de novo* synthesis of ACC synthase which is elicited by this multitude of internal cues and external inducers (Kende, 1989).

ACC synthase is difficult to purify because it is very labile and its concentration in plant tissue is extremely low (Kim and Yang, 1992) even in tissues with increased levels such as tomato pericarp (Bleecker et al., 1988). The most frequently used assay of enzyme activity is a procedure in which the ACC, produced by ACC synthase from added substrate (SAM) in the presence of the cofactor, pyridoxal phosphate, is chemically converted to ethylene and then measured by gas chromatography (Lizada and Yang, 1979; Boller et al., 1979). Although rapid and relatively simple, this method has some difficulties associated with it because ACC is measured indirectly (Chauvaux et al., 1997). A range of methods have been developed to measure ACC directly and all involve the derivatization of ACC prior to its measurement (Lanneluc-Sanson et al., 1986; Bushey et al., 1987; Hall et al., 1989 & 1993; Chauvaux et al., 1997; Penrose et al., submitted for publication).

ACC synthase is a cytosolic enzyme that was initially identified in homogenates of ripening tomato pericarp tissue, has an

approximate K_m for SAM of approximately 13 μM (Boller et al., 1979; Yu et al., 1979), and was first purified from wounded, lithium-treated tomato pericarp (Bleecker et al., 1986). Mehta et al. (1988) noted the presence of at least three isoenzymes in the tissue of ripening tomato fruit, each isoenzyme with a distinct pI. In addition to tomato pericarp (Bleecker et al., 1986; Privalle and Graham, 1987; Satoh and Yang, 1988; Van der Straeten et al., 1989), ACC synthase has been purified from tissues of several other plants (Table 3).

The activity of ACC synthase requires the presence of the coenzyme, pyridoxal phosphate (Yang and Hoffman, 1984), which is loosely bound to the enzyme (Mattoo and Suttle, 1991) by a lysine residue (Yip et al., 1990). This lysine residue is located in the active site of the enzyme purified from apple tissue (Yip et al., 1990) and appears to be conserved amongst ACC synthases (Zarembinski and Theologis, 1994).

Table 3: Purified ACC synthases

Plant source	Reference
tomato pericarp	Bleecker et al., 1986, 1988
	Privalle & Graham, 1987
	Satoh and Yang, 1988
	Van Der Straeten et al., 1989, 1990
	Edelman & Kende, 1990
	Rottmann et al., 1991
squash mesocarp	Imaseki et al., 1988
	Nakajima and Imaseki, 1986
	Nakajima et al., 1990
	Nakagawa et al., 1988
zucchini	Sato et al., 1991
	Sato & Theologis, 1989
apple fruit	Dong et al., 1991a; Yip et al., 1991
mung bean hypocotyl	Tsai et al., 1988

ACC synthase has a rigid specificity for the stereochemistry of SAM (Khani-Oskouee et al., 1984) and is active only with its (S,S)-diastereomer, the form of SAM that occurs almost exclusively in nature (Satoh and Yang, 1989a). Two reactions are catalyzed by ACC synthase: the formation of ACC via α,γ-elimination and the formation of the highly reactive intermediate vinylglycine via β,γ-elimination. It is likely that the short half-life of ACC synthase results from the β,γ-elimination reaction of SAM to form vinylglycine (Satoh and Yang, 1989b) in which the covalent binding of vinylglycine to the active site of ACC synthase irreversibly inhibits the activity of the enzyme (Satoh and Yang, 1989a & b). Not long after ACC synthase was purified from tomato fruit (Bleeker et al., 1986) the cDNA for an ACC synthase gene was cloned from zucchini fruit (Sato and Theologis, 1989), and the nucleotide sequence and its derived amino acid sequence were determined (Sato et al., 1991). ACC synthase cDNAs and genomic sequences have now been cloned from numerous plant species (Table 4).

Nucleotide sequencing of the ACC synthase cDNA clones and their derived amino acid sequences have provided most of the information from which the structure of ACC synthase can be predicted (Kende, 1993). The presence of multiple ACC synthase genes that encode proteins of varying molecular mass and isoelectric point (pI) is consistent with the number of isoforms of the enzyme.

When these isoforms, identified on the basis of their predicted amino acid sequences, are classified according to pI, they fall into four categories: acidic, near neutral, neutral and alkaline. Each of these groups has highly conserved regions or residues that are distinct from the other groups (Fluhr and Mattoo, 1996).

The highest level of homology among the ACC synthase genes is found in the region corresponding to the interior portion of the ACC synthase polypeptides and the most divergence is seen in the carboxyl terminus, a hypervariable, positively charged region (Zarembinski and Theologis, 1994). The carboxyl terminus contains domains that influence structural and catalytic aspects of the enzyme, including the one responsible for substrate-based inhibition of ACC synthase by SAM and others that affect dimerization and some kinetic parameters of the enzyme (Li and Mattoo, 1994). Analysis and characterization of the apple ACC synthase crystal structure have revealed the dimeric nature of the enzyme (Hohenester et al., 1994; White et al., 1994; Li et al., 1997; Tarum and Theologis, 1998). It appears that ACC synthase from tomato fruit functions as a dimer and has an intersubunit or shared subunit with its active site (Tarum and Theologis, 1998).

Table 4: Cloned ACC synthases

Plant Source	Reference
apple	Dong et al., 1991b
	Lay-Yee and Knighton, 1995
Arabidopsis thaliana	Liang, et al., 1992
	Van Der Straeten et al., 1992
broccoli	Pogsun et al., 1995
carnation	Park et al., 1992; Fluhr and Mattoo, 1996
melon	Miki et al., 1995
mung bean	Kim et al., 1992; Botella et al., 1992a, b; 1993
mustard	Wen et al., 1993
orchid	O'Neill et al., 1993
petunia	Fluhr and Mattoo, 1996
rice	Zarembinski and Theologis, 1993
soybean	Liu et al., 1993
tobacco	Bailey et al., 1992
tomato	Van Der Straeten et al., 1990; Olson et al., 1991
	Rottmann et al., 1991; Yip et al., 1992
	Lincoln et al., 1993; Mattoo et al., 1993
wheat	Subramanian et al., 1996
winter squash	Nakajima et al., 1990; Nakagawa et al., 1991
zucchini	Huang et al., 1991

Biochemical and sequence comparisons have shown that ACC synthases are similar to, and may be evolutionarily related to aminotransferases, prevalent pyridoxal phosphate-dependent enzymes, which often function as dimers. The active site dodecapeptide of ACC synthases shows some sequence similarity with the same region in several aminotransferases and, of the 12 amino acids conserved among aminotransferases, all but one have been found in the ACC synthases (Rottmann et al., 1991; Theologis, 1992).

Five distinct highly conserved regions have been identified amongst the ACC synthase genes (Fluhr and Mattoo, 1996). Of these five regions, the most notable is around the active site (Kende, 1993). Active site residues have been tentatively identified in apple ACC synthase: Lys-273, the base that catalyzes C^{α} proton abstraction; Tyr-223, a residue involved in the orientation of pyridoxal phosphate; Arg-407, the principle ligand for the substrate α-carboxylate group (White et al., 1994) and Tyr-85 (Li et al., 1997).

Members of the multigene family of ACC synthases are differentially expressed in response to developmental, environmental and hormonal factors (Kende and Zeevaart, 1997). Increases in the synthesis of ACC synthase have been observed during fruit ripening, flower fading, and in response to exogenous signals such as wounding, mechanical strain, auxin, cytokinin and

ethylene (Kende, 1993). Transcripts of ACC synthase genes are differentially regulated (Fluhr and Mattoo, 1996). An investigation of the differential expression of tomato ACC synthases revealed that transcripts of one isoform increased during fruit ripening, those of another increased in response to wounding and transcripts of a third isoform increased when treated with auxin (Kende and Zeevaart, 1997).

Induction of the genes in this family appears to occur in a regulated manner. ACC synthase genes expressed in response to a particular stimulus, such as auxin, share more similarity with ACC synthase genes induced by that stimulus in another species than to other ACC synthase genes in the same plant species (Liang et al., 1992; Trebitsh et al., 1997). Differential expression of ACC synthase genes has been seen in a number of tissues in a variety of plant species including tomato: four genes are differentially regulated during ripening, by wounding and by auxin treatment in tomato fruits, cell cultures and hypocotyls (Yip et al., 1992).

ACC synthase is primarily regulated at the level of transcription, although there is some evidence that regulation may also be post-transcriptional (Chappell et al., 1984; Felix et al., 1991). In this regard, fungal elicitors induced ACC synthase activity in the presence of RNA synthesis inhibitors. ACC synthase gene expression has also been induced by protein synthesis inhibitors such as

cyclohexamide (Zarembinski and Theologis, 1993; Liang et al., 1992; Zarembinski and Theologis, 1994; Liu et al., 1993). Induction by cyclohexamide suggests that ACC synthase gene expression may be controlled by a labile repressor (or repressors) (Liang et al., 1992; Theologis et al., 1985) and/or by a rapidly turning over RNAse capable of degrading labile enzyme transcripts (Franco et al., 1990).

1.2.2.3. ACC oxidase

At the time Yang and Hoffman (1984) outlined the ethylene biosynthetic pathway, they described an enzyme responsible for the conversion of ACC to ethylene and named it ethylene-forming enzyme. After years of trying to isolate this enzyme, it was finally purified in a case of "reversed biochemistry": the gene was cloned prior to the purification of the enzyme (Kende, 1993). By examining the deduced amino acid sequence of the putative gene for ethylene-forming enzyme (Hamilton et al., 1990), researchers discovered similarities between it and the sequences of genes for flavanone 3-hydroxylase and other iron and ascorbate-dependent dioxygenases (Ververidis and John, 1991).

When extracted and assayed under conditions known to preserve flavanone 3-hydroxylase activity, ethylene-forming enyzme activity was fully recovered and the cofactors, iron and ascorbate, were identified (Ververidis and John, 1991). Though it was known

that carbon dioxide enhanced the activity of ACC oxidase in a number of plant systems (Yang and Hoffman, 1984), Dong et al. (1992a) demonstrated that *in vitro* ACC oxidase activity in apple fruit was entirely dependent on carbon dioxide and established the equation (shown below) describing the conversion of ACC to ethylene by ethylene-forming enzyme (Yang and Dong, 1993). Because of the oxidative nature of this reaction, ethylene-forming enzyme was renamed ACC oxidase (Fluhr and Mattoo, 1996).

$$ACC + O_2 + \text{ascorbate} \xrightarrow{Fe^{2+},\ CO_2} C_2H_4 + CO_2 + HCN + \text{dehydroascorbate} + 2H_2O$$

During the purification of ACC oxidase from apple fruit, Dong et al. (1992a) noted that for full enzyme activity, the presence of carbon dioxide was required in crude extracts; subsequently, carbon dioxide was deemed an essential activator of ACC oxidase (Fernández-Maculet et al., 1993). Carbon dioxide not only stimulates the rate of reaction but also increases the affinity of ACC oxidase towards ACC (Fernández-Maculet et al., 1993; Ponteleit and Dilley, 1993; Smith and John, 1993; Finlayson and Reid, 1994), towards oxygen (Smith and John, 1993) and towards ascorbate (Finlayson and Reid, 1994).

The assay of ACC oxidase activity is based upon the ability of the enzyme to convert exogenous ACC to ethylene (Yang and

Hoffman, 1984) which is subsequently measured by gas chromatography (Ververadis and John, 1991: Dong et al., 1992a).

ACC oxidase was first purified from melon fruit (Smith et al., 1992); the enzyme had a molecular weight of approximately 41 kDa, an apparent K_m of 60 μM for ACC, and a pH optimum of approximately 7.5 (Smith et al., 1992). The enzyme has subsequently been purified and characterized from a variety of plant tissues (Table 5).

There are a number of unanswered questions concerning which parts of the cell produce ethylene and the location of ACC oxidase in plant tissue. Enzyme purified from melon fruit was discovered in the soluble fraction (Smith et al., 1992) whereas the apple fruit enzyme was associated with the pellet fraction (Dong et al., 1992a). ACC oxidase is located intracellularly and can function in this site, but there is also evidence of the enzyme in the apoplasm in ripening fruit and other plant tissues, although the mechanism by which it arrives at this site is unclear (John, 1997).

Table 5: Purified and partially purified ACC oxidases

Plant Source	Reference
apple fruit	Kuai and Dilley, 1992; Dong et al., 1992
	Dupille et al., 1993; Pirrung et al., 1993
	Fernández-Maculet and Yang, 1992
avocado fruit	McGarvey and Christoffersen, 1992
banana fruit	Moya-Leon and John, 1995
carnation petal	Nijenhuis-de Vries et al., 1994
cherimoya fruit	Escribano et al., 1996
citrus peel	Dupille and Zacarius, 1996
melon fruit	Smith et al., 1992
pear fruit	Vioque and Castellano, 1994
winter squash	Hyodo et al., 1993

Membrane perturbants influence both ethylene production and the activity of ACC oxidase (Mattoo and White, 1991); the *in vivo* ACC oxidase activity, unlike the *in vitro* activity, requires the presence of the plasma membrane (Lieberman 1979; Yang and Hoffman, 1984; Ververidis and John, 1991). John (1997) proposed that a charge on the plasma membrane maintains ascorbate in the reduced state required by the ACC oxidase in the apoplast; *in vitro* activity does not require a membrane because exogenous ascorbate is supplied to the reaction.

Inhibitors of ACC oxidase from melon fruit include Co^{2+}, sulfhydryl modifiying reagents, free radical scavengers, free radical quenchers, and EDTA which acts by chelating free iron in the reaction medium. The most potent inhibitor is 1,2-dihydroxynaphthalene, presumably also due to its iron binding capacity (Smith et al., 1992). The enzyme isolated from avocado fruit is competitively inhibited by 2-aminoisobutyric acid (McGarvey and Christoffersen, 1992).

ACC oxidase is a member of the 2-oxoacid-dependent dioxygenases, a family of enzymes characterized by requirements for Fe (II) and ascorbate, and loose substrate specificity (John, 1997). Conservation of histidine residues amongst ACC oxidases and other dioxygenases of this family suggests that critical histidine residues may be located at the active site of ACC oxidase (Christoffersen et al., 1993).

ACC is an α-dialkyl amino acid with two enantiotopic β-methylene groups in a cyclopropane ring structure (Honma and Shimomura, 1978). Previous to its purification, Hoffman and Yang (1982) observed that ACC oxidase showed a stereospecificity for the (1R, 2S) isomer of ACC, a distinctive trait that was used frequently to confirm identity of the enzyme (Ververidis and John, 1991; Hamilton et al., 1990; Dong et al., 1992a; McGarvey and Christoffersen, 1992).

Differential screening was used to identify the first ACC oxidase gene (Slater et al., 1985). pTOM13, a clone isolated from a cDNA library prepared from ripening tomato fruit, was found to encode a 35 kDa protein whose expression was correlated with enhanced ethylene synthesis (Slater et al., 1985; Smith et al., 1986). The protein encoded by pTOM13 was identified as ACC oxidase by a combination of antisense experiments (Hamilton et al., 1990) and *in vivo* expression in yeast (Hamilton et al., 1991; Wilson et al., 1993) and *Xenopus* oocytes (Spanu et al., 1991). ACC oxidase cDNAs have since been cloned from numerous plants (Table 6).

Table 6: Cloned ACC oxidases

Plant Source	Reference
Apple	Dong et al., 1992b; Ross et al., 1992
Arabidopsis thaliana	Gómez-Lim et al., 1993
Broccoli	Pogsun et al., 1995
Carnation	Wang and Woodson, 1991
Cucumis melo L.	Balague et al., 1993
Kiwi	MacDiarmid and Gardner, 1993
Mung bean	Kim and Yang, 1994
Mustard	Pua et al., 1992
Orchid	Nadeau et al., 1993; Nadeau and O'Neill, 1995
Peach	Callahan et al., 1992
Pea	Peck et al., 1993
Pelargonium	Wang et al., 1994
Petunia	Wang and Woodson, 1992; Tang et al., 1993
Tomato	Köck et al., 1991; Holdsworth et al., 1987
	Deikman and Fischer, 1988
Winter squash	Hyodo et al., 1993

Nucleotide sequencing has shown that a number of these cloned genes share a high degree of homology with the DNA carried within pTOM13 (Wang and Woodson, 1991; Dong et al., 1992b; Ross et al., 1992). Analyses of nucleotide and derived amino acid sequences of ACC oxidase genes have shown that a multigene family encodes the enzyme. To date, it has been shown that multiple genes encode ACC oxidase in petunia, tomato and mung bean; all of the gene members are expressed as functional ACC oxidases (Fluhr and Mattoo, 1996).

The proteins encoded by these genes are highly homologous and the genes share identical numbers and positions of introns (Fluhr and Mattoo, 1996). A comparison of the nucleotide sequences of the ACC oxidase genes reveals sequence conservation in the internal coding regions but not in the C-terminal regions that are divergent (Kim and Yang, 1994). There is significant homology in a 40 bp sequence in the 5'-non-coding region that includes the transcription initiation site and TATA box but not in the remainder of the 5'-non-coding region (Tang et al., 1993).

ACC oxidase is constitutively present in most plant tissues at a low level of activity (Ecker, 1995). Enzyme activity is induced by ACC during fruit ripening, flower senescence and mechanical wounding, and in response to elicitors, novel fungal proteins and tobacco mosaic virus (McGarvey and Christoffersen, 1992; Wang and Woodson, 1992;

Fluhr and Mattoo, 1996). Increases in levels of ACC oxidase activity have also been observed in some plants in response to internal and external factors, such as auxin and ethylene, that are known to influence ethylene formation (Kende, 1993). Treatment of tissues with ethylene has been found to stimulate the development of ACC oxidase activity in a variety of vegetative and reproductive tissues through a positive feedback loop: the induction of ACC synthase by ethylene leads to the production of ACC which is converted by the low constitutive level of ACC oxidase activity to ethylene (Kende, 1993). The increased level of enzyme activity is associated with an accumulation of ACC oxidase transcripts, a fact that implies transcriptional regulation of this enzyme (Fluhr and Mattoo, 1996).

Increases in both transcript and activity levels of ACC oxidase have also been induced by auxin in pea seedlings (Peck and Kende, 1995). In some circumstances, the accumulation of ACC oxidase transcripts occurs earlier than those of ACC synthase. The apparently coordinated induction of transcripts from these two enzymes causes an intense autocatalytic ethylene production. Short chain oligogalacturonic acids derived from plant cell wall pectin were also shown to be active both in eliciting ACC oxidase expression and in the production of ethylene (Simpson et al., 1998).

The genes characterized in the tomato and petunia multigene families appear to be expressed in a regulated manner. Distinct

patterns of expression of three ACC oxidase genes have been observed in various organs and at different stages of development in tomato (Kende and Zeevaart, 1997). The patterns of expression differ somewhat for the ACC oxidase genes from petunia and tomato: more of the petunia genes are organ specific than those of tomato (Fluhr and Mattoo, 1996).

1.3. Transgenic plants

Much of the molecular work being done with the enzymes responsible for ethylene biosynthesis has culminated in the construction of transgenic plants with impaired ethylene biosynthesis. The reduced synthesis of ethylene in these plants does not appear to cause any major vegetative phenotypic abnormalities other than delayed leaf senescence (Picton et al., 1993; Grier et al., unpublished observations). The fruit produced by these transgenic plants show significant delays in ripening and the mature fruit remains firm longer than non-transgenic control plants (Good et al., 1994). It seems possible that ethylene is not required for many developmental processes although survival during periods of stress may depend on ethylene (Smalle and Van Der Straeten, 1997).

Tomato plants that express antisense ACC synthase RNA under the control of constitutive or fruit specific promoters displayed greatly reduced ethylene production and detached fruit that never

soften or ripen (Oeller et al., 1991). A similar effect was observed in transgenic fruit with antisense ACC oxidase (Hamilton et al., 1990; Picton et al., 1993) that neither softened nor developed a red colour (Hobson and Murray, 1995).

Transgenic tomato plants carrying ACC deaminase, a microbial enzyme that hydrolyzes ACC to form α-ketobutyrate and ammonia, synthesize very little ethylene and exhibit a prolonged ripening phase—ethylene synthesis was reduced in leaves by more than 95% and in fruit by 90% (Klee et al., 1991). The prolonged ripening phase only occurs when ethylene is reduced by 95% but not when it is decreased by approximately 70%. Moreover, in some cases, seed germination appeared to be delayed (Grichko et al., submitted). ACC deaminase has also been expressed in transgenic petunia, tobacco and *Arabidopsis* plants; ethylene synthesis was greatly reduced in all tissues where it was expressed (Yang and Hiatt, 1991; Klee and Kishore, 1992; Romano et al., 1993). Reports of the use of this bacterial enzyme to reduce ethylene levels in plants provided the impetus for studies examining the possible role of ACC deaminase in the promotion of plant growth by certain soil bacteria. For example, the assessment of the plant growth-promoting capabilities of the bacterium *Pseudomonas putida* GR12-2 (Lifshitz et al., 1986) was based on enhanced seedling root length following imbibition of the seed in the presence of the bacterium (Glick et al., 1994a; 1994b).

It is well known that the production of ethylene is important during the early phase of plant growth. Ethylene is required by many plant species for seed germination, although the levels needed may vary greatly (Smalle and Van Der Straeten, 1997), and the rate of ethylene production increases during germination and seedling growth (Abeles et al., 1992). Although a low level of ethylene appears to enhance both root initiation and outgrowth, and a small amount of ethylene present in well-ventilated roots promotes root extension, the high levels of ethylene produced by fast growing roots, which can either raise the internal ethylene concentration or accumulate around the roots, regularly lead to inhibition of root elongation (Mattoo and Suttle, 1991; Ma et al., 1998). It is possible that the longer roots seen by Glick et al. (1994a) from seeds treated with the plant growth-promoting bacterium, *Pseudomonas putida* GR12-2, were a result of a reduced ethylene concentration, either in or around the roots, following germination, and that the lower level of ethylene was due to the presence of the enzyme ACC deaminase in the bacterium (Jacobson et al., 1994).

1.4. ACC deaminase

ACC was first isolated in 1957 from cider apple and perry pear fruit (Burroughs 1957), and from ripe cow berries (Vähätalo and Virtanen, 1957) and has until recently, only been found in plant

tissue. However, Cytotrienin A, an antiobiotic produced by the soil bacterium, *Streptomyces* sp. RK95-74, contains a unique ACC unit as part of its structure (Zhang et al., 1998). In 1957, there were also reports of two microbial enzymes involved in the metabolism of α-dialkyl-α-amino acids but both enzymes were found to be inactive with ACC as a substrate. It seemed, from these early reports, that any microorganism capable of utilizing ACC would metabolize it by a unique reaction (Honma and Shimomura, 1978). In 1978, an enzyme capable of degrading ACC was isolated from *Pseudomonas* sp. strain ACP, and from the yeast, *Hansenula saturnus* (Honma and Shimomura, 1978; Minami et al., 1998). Since then, ACC deaminase has been detected in the fungus, *Penicillium citrinum*, (Honma, 1993) and in a number of other bacterial strains (Klee and Kishore, 1992; Jacobson et al., 1994, Glick et al., 1995; Campbell and Thomson, 1996; Table 7), all of which originated from the soil either as soil sample isolates or as microbes typically found in the soil. Many of these microorganisms were identified by their ability to grow on minimal media containing ACC as its sole nitrogen source (Honma and Shimomura, 1978; Klee et al., 1991; Honma, 1993; Jacobson et al., 1994; Glick et al., 1995; Campbell and Thomson, 1996; Burd et al., 1998; Belimov et al., submitted for publication).

Table 7. Soil microorganisms that have shown ACC deaminase activity

n.d. indicates not determined.

Genus and Species	Strain	Source	K_m for ACC (mmol)	Reference
Pseudomonas	ACP	soil isolate	1.5	Honma and Shimomura, 1978
Hansenula saturnus		soil isolate	2.6	Honma and Shimomura, 1978; Minami et al. 1998
Penicillium citrinum		soil isolate	4.6	Honma, 1993
Pseudomonas chloroaphis	6G5	Drahos collection	9.0	Klee and Kishore, 1992 Drahos et al. 1988
Pseudomonas	3F2	Drahos collection	5.8	Klee and Kishore, 1992; Drahos et al. 1988
Pseudomonas		Drahos collection		Klee and Kishore, 1992 Drahos et al. 1988
n.d.	388	soil isolate St. Charles, USA	8.6	Klee and Kishore, 1992
n.d.	391	soil isolate Malaysia	17.4	Klee and Kishore, 1992
n.d.	392	soil isolate Peru	7.1	Klee and Kishore, 1992
n.d.	393	soil isolate St. Charles, USA	5.9	Klee and Kishore, 1992
n.d.	401	soil isolate St. Charles, USA	7.8	Klee and Kishore, 1992
n.d.	T44	soil isolate Tanzania	11.8	Klee and Kishore, 1992
n.d.	PR1	soil isolate Puerto Rico	4.1	Klee and Kishore, 1992
Pseudomonas putida	GR12-2	soil isolate, grass Canadian Arctic	n.d.	Jacobson et al. 1994 Lifshitz et al. 1986

Pseudomonas putida	UW1	soil isolate, bean Waterloo, Canada	n.d.	Glick et al. 1995 Shah et al. 1997
Enterobacter cloacae	UW2	soil isolate, clover Waterloo, Canada	n.d.	Glick et al. 1995 Shah et al. 1997
Pseudomonas putida		soil isolate, corn Waterloo, Canada	n.d.	Glick et al. 1995 Shah et al. 1997
Enterobacter cloacae	UW4	soil isolate, reeds Waterloo, Canada	n.d.	Glick et al. 1995 Shah et al. 1997
Pseudomonas fluorescens	CAL1	soil isolate, oats San Benito, USA	n.d.	Glick et al. 1995 Shah et al. 1997
Enterobacter cloacae	CAL2	soil isolate, tomato King City, USA	n.d.	Glick et al. 1995 Shah et al. 1997
Enterobacter cloacae	CAL3	soil isolate, cotton Fresco, California USA	n.d.	Glick et al. 1995 Shah et al. 1997
Kluyvera ascorbata	SUD 165	metal contaminated soil isolate, Sudbury, Ont., Canada	n.d.	Burd et al. 1998
Pseudomonas	Am1	peat-boggy soil isolate mustard and pea St. Petersburg, Russia	n.d.	Belimov et al. submitted for publication
Pseudomonas	Am2	peat-boggy soil isolate mustard and pea St. Petersburg, Russia	n.d	Belimov et al. submitted for publication
Pseudomonas	Am3	peat-boggy soil isolate mustard and pea St. Petersburg, Russia	n.d	Belimov et al. submitted for publication
Pseudomonas	Bm1	derno-podzolic soil isolate mustard and pea St. Petersburg, Russia	n.d	Belimov et al. submitted for publication
n.d.	Bm2	derno-podzolic soil isolate mustard and pea St. Petersburg, Russia	n.d	Belimov et al. submitted for publication
Pseudomonas	Bm3	derno-podzolic soil isolate mustard and pea St. Petersburg, Russia	n.d	Belimov et al. submitted for publication
Pseudomonas	Cm1	loamy soil isolate mustard and pea St. Petersburg, Russia	n.d	Belimov et al. submitted for publication

Pseudomonas	Cm2	loamy soil isolate mustard and pea St. Petersburg, Russia	n.d	Belimov et al. submitted for publication
Pseudomonas	Cm3	loamy soil isolate mustard and pea St. Petersburg, Russia	n.d.	Belimov et al. submitted for publication
Pseudomonas	Cm4	loamy soil isolate mustard and pea St. Petersburg, Russia	n.d.	Belimov et al. submitted for publication
Pseudomonas	Dp1	sandy soil isolate pea St. Petersburg, Russia	n.d.	Belimov et al. submitted for publication
Pseudomonas	Dp2	sandy soil isolate pea St. Petersburg, Russia	n.d.	Belimov et al. submitted for publication
Pseudomonas	Dp3	sandy soil isolate pea St. Petersburg, Russia	n.d.	Belimov et al. submitted for publication
Pseudomonas	Dp4	sandy soil isolate pea St. Petersburg, Russia	n.d.	Belimov et al. submitted for publication
Pseudomonas	Dc1	sandy soil isolate carex St. Petersburg, Russia	n.d.	Belimov et al. submitted for publication
Pseudomonas	Dc2	sandy soil isolate carex St. Petersburg, Russia	n.d.	Belimov et al. submitted for publication
n.d.	Ep1	sewage sludge soil isolate pea Vladimir, Russia	n.d.	Belimov et al. submitted for publication
Pseudomonas	Ep2	sewage sludge soil isolate pea Vladimir, Russia	n.d.	Belimov et al. submitted for publication
Pseudomonas	Ep3	sewage sludge soil isolate pea Vladimir, Russia	n.d.	Belimov et al. submitted for publication
Pseudomonas	Ep4	sewage sludge soil isolate pea Vladimir, Russia	n.d.	Belimov et al. submitted for publication
Pseudomonas	Fp1	post-glacial clay soil isolate, pea Ultuna, Sweden	n.d.	Belimov et al. submitted for publication
n.d.	Fp2	post-glacial clay soil isolate, pea Ultuna, Sweden	n.d.	Belimov et al. submitted for publication

Three ACC-degrading microorganisms were isolated from a collection of 597 bacterial strains (Drahos et al., 1988) comprised mostly of fluorescent *Pseudomonas* species and a few common soil microorganisms (Klee et al., 1991). Seven other novel organisms were found in soil samples collected from disparate locations on four continents (Klee et al., 1991). *Pseudomonas putida* GR12-2, was isolated from the rhizosphere of grass in the Canadian High Arctic (Lifshitz et al., 1986) and was found to have plant growth-promoting capabilities (Hong et al., 1991b). Another seven strains, all of which had growth-promoting capabilities, were isolated from soil samples gathered in Waterloo, Ontario, Canada and California, U.S.A (Glick et al., 1995). One strain was isolated from nickel contaminated soil in Sudbury, Ontario, Canada (Burd et al., 1998). From a collection of 103 soil microorganisms isolated from various regions in South Africa, 81 displayed ACC degrading abilities; of these isolates, the 7 bacterial strains which displayed the most vigorous growth on ACC were identified as strains of *Pseudomonas* (Campbell and Thomson, 1996). Twenty-two ACC deaminase-containing strains were isolated from the rhizosphere of pea (*Pisum sativum L.*), saperda mustard (*Brassica juncea L.*) and carex (*Carex cespitosa L.*) plants grown in one of five different soils or long standing sewage sludge. Most of these isolates, tentatively identified as strains of *Pseudomonas*, showed significant

plant growth-promoting capabilities (Belimov et al., submitted for publication).

Enzymatic activity has been assayed by monitoring the production of either ammonia or α-ketobutyrate, the products of ACC hydrolysis (Honma and Shimomura, 1978). While ACC deaminase has been found only in microorganisms, there are no microorganisms that synthesize ethylene via ACC (Fukuda et al., 1993).

ACC deaminase has been purified to homogeneity from *Pseudomonas* sp. strain ACP (Honma and Shimomura, 1978) and partially purified from *Pseudomonas* sp. strain 6G5 (Klee et al., 1991) and *Pseudomonas putida* GR12-2 (Jacobson et al., 1994); enzyme activity is localized exclusively in the cytoplasm (Jacobson et al., 1994). The molecular mass and form is similar for the ACC deaminase purified from all three sources. The enzyme is a trimer (Honma, 1985); the size of the holoenzyme is approximately 104 to 105 kDa (Honma and Shimomura, 1978; Honma, 1985; Jacobson et al., 1994) and the subunit mass is approximately 36,500 daltons (Honma and Shimomura, 1978; Jacobson et al., 1994). Similar subunit sizes were predicted from nucleotide sequences of cloned ACC deaminase genes from *Pseudomonas* strains ACP (Sheehy et al., 1991) and 6G5 (Klee et al., 1991), and *Enterobacter cloacae* UW4 (Shah et al., 1997).

K_m values for the binding of ACC by ACC deaminase have been estimated for enzyme extracts of 12 microorganisms at pH 8.5. These values ranged from 1.5 to 17.4 mmol (Honma and Shimomura, 1978; Klee and Kishore, 1992; Honma, 1993) indicating that the enzyme does not have a particularly high affinity for ACC (Glick et al., 1998a).

ACC deaminase activity has been induced in both *Pseudomonas* sp. strain ACP and *Pseudomonas putida* GR12-2 by ACC, at levels as low as 100 nM, (Honma and Shimomura, 1978; Jacobson et al., 1994); both bacterial strains were grown on a rich medium and then switched to a minimal medium containing ACC as its sole nitrogen source. The rate of induction, similar for the enzyme from the two bacterial sources, was relatively slow: complete induction required eight to ten hours. Enzyme activity increased only approximately 10-fold over the basal level of activity even when the concentration of ACC increased up to 10,000-fold. The level of ACC deaminase activity observed when *Pseudomonas putida* GR12-2 was grown on minimal medium plus ammonium sulfate represented a basal level of activity which never exceeded 5% of the total activity measured in extracts grown on minimal medium containing ACC (Jacobson et al., 1994). Other amino acids including L-alanine, DL-alanine and DL-valine can induce enzyme activity to a small extent; α-aminoisobutyric acid can induce enzyme activity to

almost the same level as ACC with *Pseudomonas* sp. strain ACP (Honma, 1983) but not with strain UW4 (Li and Glick, unpublished results).

The activity of ACC deaminase was optimum at a temperature of 30°C for enzyme from *Pseudomonas putida* GR12-2 (Jacobson et al., 1994), and at pH 8.5 for enzyme from both *Pseudomonas* sp. strain ACP and *Pseudomonas putida* GR12-2 (Honma and Shimomura, 1978; Jacobson et al., 1994). The pH profile of GR12-2 ACC deaminase activity displays values of approximately 7.7 and 9.2. These pK_a values indicate that histidine and cysteine residues may be involved in the catalytic functioning of the enzyme (Jacobson et al., 1994).

1.4.1. Biochemical mechanism

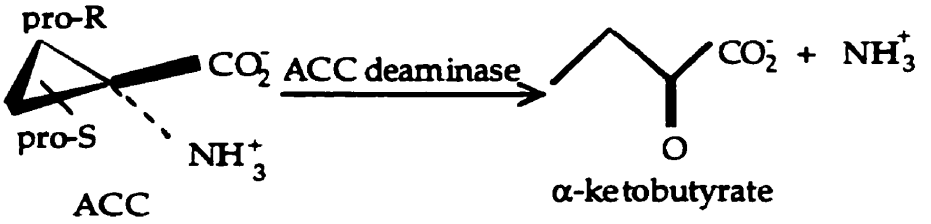
Pyridoxal phosphate is a tightly bound cofactor of ACC deaminase in the amount of approximately three moles of enzyme-bound pyridoxal phosphate per mole of enzyme or one mole per subunit (Honma, 1985). The aldimine/ketimine complex formed between pyridoxal phosphate and the substrate has a characteristic absorption spectrum which shows a maximum absorbance at 418 nm and may be used to monitor the formation and disappearance of intermediate enzyme-substrate complexes (Walsh et al., 1981).

ACC deaminase catalyzes a cleavage of ACC that includes cyclopropane ring fragmentation, and deamination of ACC to form

α-ketobutyrate and ammonia (Murakami et al., 1997). Cyclopropane ring cleavage occurs in the bond between the α-carbon and the pro-S methylene carbon of ACC (Honma et al., 1979; Liu et al., 1984; Fig. 4) but the reaction mechanism is not fully understood (Murakami et al., 1997).

Other amino acids such as alanine and serine, and ACC-related compounds including 2-alkyl-ACC and vinyl-ACC can also function as substrates for ACC deaminase purified from *Pseudomonas* sp. strain ACP but the enzyme shows an unusual specificity for D-amino acids and is inactive with any of the L-amino acids tested or their derivatives (Honma, 1983; Walsh et al., 1981). ACC deaminase is competitively inhibited by L-isomers of amino acids including alanine, α-aminobutyric acid and homoserine although the strongest inhibition is seen with L-alanine and L-serine (Honma et al., 1979) and the affinity of the enzyme for these competitive inhibitors is highest at pH 8.5 (Honma, 1985).

Fig. 4. Reaction catalyzed by ACC deaminase. The site of cyclopropane ring cleavage occurs in the bond between the α-carbon and the pro-S methylene carbon of ACC. Abbreviations: ACC, 1-aminocyclopropane-1-carboxylic acid.


pro-R
CO_2^-
ACC deaminase
CO_2^- + NH_3^+
pro-S
NH_3^+
O
ACC
α-ketobutyrate

Two key amino acid residues have been located in the enzyme from *Pseudomonas* sp. strain ACP: a reactive thiol group at cysteine 162 and the pyridoxal phosphate binding site at lysine 51 (Honma et al., 1993). In addition to formation of an internal aldimine with pyridoxal phosphate, lysine 51 serves specifically in the formation of holoenzyme and in catalysis (Murakami et al., 1997). The amino acid sequence surrounding this pyridoxal phosphate binding residue of ACC deaminase from *Pseudomonas* sp. strain ACP is similar to that of the tryptophan synthase β-subunit (Honma, 1993), an enzyme which catalyzes both the synthesis of tryptophan from L-serine and indole, and the deamination of L-serine (Hyde et al., 1988).

1.4.2. Gene isolation and characterization

The entire amino acid sequence of ACC deaminase purified from *Pseudomonas* sp. strain ACP protein was determined, and the information from the amino acid sequence was used to clone the ACC deaminase gene from this strain—a genomic library was screened with a PCR generated fragment of the ACC deaminase gene (Sheehy et al., 1991). Sequence analysis of the cloned gene revealed an open reading frame of 1014 bp encoding a polypeptide with a predicted molecular mass (Sheehy et al., 1991) similar to the subunit mass of the purified enzyme (Honma, 1985). The deduced amino acid sequence of the open reading frame, 338 residues in length, was

identical to the chemically determined sequence of the purified protein (Sheehy et al., 1991).

Genes encoding ACC deaminase have subsequently been cloned from a number of different soil bacteria including *Pseudomonas* sp. strains 6G5 and 3F2 (Klee et al., 1991; Klee and Kishore, 1992), *Pseudomonas* sp. strain 17 (Campbell and Thomson, 1996), yeast, *Hansenula saturnus* (Minami et al., 1998) and *Enterobacter cloacae* strains CAL2 and UW4 (Glick et al., 1995; Shah et al., 1998). The latter two strains were originally designated *Pseudomonas* sp. strain CAL2 and UW4 but further characterization revealed that both of these strains were actually *Enterobacter cloacae* (Shah et al., 1997). The selection of the genes isolated from *Pseudomonas* sp. strains 6G5 and 3F2 was based on the activity of the enzyme in *Escherichia coli*. A cosmid library prepared from DNA of either strain, was introduced into *Escherichia coli* MM294 and screened for the ability to grow on media containing ACC as a sole source of nitrogen (Klee et al., 1991; Klee and Kishore, 1992).

Enterobacter cloacae strains CAL2 and UW4 are two of seven different ACC deaminase-containing plant growth-promoting bacteria (CAL#1-3 and UW#1-4) (Glick et al., 1995) subjected to PCR with primers designed from the most conserved regions of genes from *Pseudomonas* sp. strains ACP and 6G5 (Shah et al., 1998). Of these seven strains only CAL2 and UW4 gave the expected PCR

product and were proven to be amplified from ACC deaminase genes ACP and 6G5 (Klee et al., 1991; Sheehy et al., 1991; Shah et al., 1998). Similarly, PCR amplification of DNA extracts from *Pseduomonas* sp. strains P15 and P17, also using primers designed from the most conserved regions of genes from strains ACP and 6G5, revealed that only P17 had an ACC deaminase gene similar to those of ACP and 6G5 (Campbell and Thomson, 1996).

The ACC deaminase genes cloned from *Pseudomonas* sp. strains 6G5, and F17, and *Enterobacter cloacae* strains UW4 and CAL2 all have an ORF of 1014 nucleotides that encode a protein containing 338 amino acids with a calculated molecular weight of approximately 36.8 kDa. (Klee et al., 1991; Campbell and Thomson, 1996; Shah et al., 1998). The genes from these strains are highly homologous to each other: at the nucleotide level 6G5, F17, UW4 and CAL2 are 85% to 95% identical to each other (Campbell and Thomson, 1996; Shah et al., 1998) and most of the dissimilarities are in the wobble position (Shah et al., 1998). However, the DNA sequences from strains UW4 and CAL2 show only 74% to 75% homology with the sequence of the ACC deaminase gene from *Pseudomonas* sp. strain ACP (Sheehy et al., 1991; Shah et al., 1998).

At the amino acid level the sequences from strains 6G5, 3F2, F17, UW4 and CAL2 are approximately 96-99% identical. Almost all of the amino acid differences are due to conservative replacements

(e.g., valine to isoleucine). However, when compared to strain ACP, the amino acid sequences from these strains show only 81 to 82% identity (Shah et al., 1998). It appears that sequences for ACC deaminase fall into two different groups: one set includes strains 6G5, 3F2, F17, UW4, and CAL2 whose sequences are very similar to one another, and the other group is comprised of ACP which is most dissimilar to all of the other sequences (Campbell and Thomson, 1996; Shah et al., 1998).

A putative Shine-Delgarno consensus ribosome binding sequence, AAGGA, is present upstream from the ATG start codon at position -13 in the ACC deaminase genes from CAL2 and UW4 (Shah et al., 1998). This sequence is identical in 6G5, F17, CAL2, and UW4, although UW4 lacks 35 nucleotides in the 5' terminal upstream sequence. An alignment of the 5' terminal upstream sequences shows high homology in strains CAL2, ACP and 6G5 up to -130 bp from the ATG start codon and up to -93 bp in the case of strain UW4 (Shah et al., 1998). Beyond this point, the homology is considerably lower except for one small stretch of eight nucleotides located at position -175. Sequence data indicates that strain UW4 contains a DNA region similar to that of the anaerobic transcription regulator, FNR, (fumarate and nitrate regulator) at positions -39 to -49 (Grichko and Glick, submitted for publication). Moreover, the ACC deaminase gene promoter in strain UW4 is under the transcriptional control of a

nearby gene that has a DNA sequence similar to a leucine-responsive regulatory protein and (the putative leucine-responsive regulatory protein) is transcriptionally regulated by ACC (Grichko and Glick, submitted for publication).

There appears to be little homology in the 3′ terminal region of the ACC deaminase genes from the strains in which it has been characterized (Klee et al., 1991; Campbell and Thomson, 1996; Shah et al., 1998). However, two of these strains have repeated sequences in the 3′ non-coding region that may act as transcription termination sites: the gene from ACP contains multiple direct repeats and an inverted repeat potentially capable of forming a stem-loop structure (Sheehy et al., 1991) and UW4 also contains an inverted repeat from which it is possible to form a hairpin-like structure (Shah et al., 1998).

Results from Southern hybridizations confirmed that, of the nine ACC-degrading strains detected in soil isolates from Ontario, Canada; California, USA; and South Africa, only three, CAL2, UW4 and P17, contained ACC deaminase genes homologous to those of ACP and 6G5 (Shah et al., 1998; Campbell and Thomson, 1996). The other six strains produced only weakly hybridizing bands or no signal at all, despite the fact that they had all demonstrated strong ACC deaminase activity (Jacobson et al., 1994; Campbell and Thomson, 1996; Shah et al., 1997). These results suggest that there may be

different types of ACC deaminase genes in various strains (Shah et al., 1998).

1.4.3. Evolution

The isolation and at least partial characterization of six different ACC deaminase genes has been reported. Five of these six genes are highly homologous to one another while the sixth gene, the gene encoding the enzyme from the strain used as a source for all of the biochemical studies performed by Honma and his co-workers, *Pseudomonas* sp. strain ACP, is somewhat different from the others (Klee et al., 1991; Shah et al., 1998; Campbell and Thomson, 1996). In addition, five of the seven strains isolated by Glick et al. (1995) and one of the two strains isolated by Campbell and Thomson (1996), all of which have ACC deaminase activity, were not able to bind the probes used to isolate the ACC deaminase genes from strains *Enterobacter cloacae* UW4 and CAL2, and *Pseudomonas* sp. P17, respectively. These findings are in accordance with the notion that more than one type of ACC deaminase gene may exist. If this is the case, then ACC deaminase genes may have arisen by convergent evolution from duplication and modification of genes encoding various pyridoxal phosphate-requiring deaminases or amino-transferases. This explanation 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 also limited.

1.4.4. Expression of ACC deaminase in bacteria and plants

The ACC deaminase genes cloned from *Pseudomonas* sp. ACP and *Enterobacter cloacae* UW4 have been expressed in *Escherichia coli* cells (Sheehy et al., 1991; Shah et al., 1998). Active enzyme was found in extracts of *Escherichia coli* cells transformed with the ACC deaminase gene cloned from ACP; in this case induction by ACC was not required for expression of this gene (Sheehy et al., 1991). Moreover, despite high levels of activity expressed in transformed *Escherichia coli*, transformants would not grow on minimal medium containing ACC as the sole nitrogen source (Sheehy et al., 1991). The reason for this is unclear; however, Sheehy et al. (1991) suggest that it may reflect the inability of this particular *Escherichia coli* strain to take up ACC. On the other hand, *Escherichia coli* cells transformed with the ACC deaminase gene from UW4 demonstrated a higher level of activity when grown on minimal medium with ACC as the sole source of nitrogen than when grown on either minimal medium plus ammonium sulfate or complete medium. This result, which indicates that this gene is being expressed from its own promoter, was confirmed by an analysis of the DNA sequences upstream from the ACC deaminase gene (Shah et al., 1998).

A broad host range plasmid (pRK415) containing the ACC deaminase gene from *Enterobacter cloacae* UW4 was introduced into two non-plant growth-promoting *Pseudomonas* strains, *Pseudomonas putida* ATCC 17399 and *Pseudomonas fluorescens* ATCC 17400, by conjugational transfer. The resulting strains acquired the ability to grow on minimal media using ACC as the sole source of nitrogen, and to promote the elongation of canola roots (Shah et al., 1998).

Some of the cloned ACC deaminase genes have been expressed in plant tissues (Klee and Kishore, 1992; Yang and Hiatt, 1991; Robison et al., submitted for publication). ACC deaminase activity was detected in tobacco and petunia tissue transformed with the gene from strain ACP (Yang and Hiatt, 1991), in petunia, tobacco, *Arabidopsis*, and tomato plants transformed with the gene from strain 6G5 (Klee and Kishore, 1992), and in tomato plants transformed with the gene from strain UW4 (Robison et al., submitted for publication). The synthesis of ethylene was greatly reduced in all tissues of phenotypically normal transgenic plants where ACC deaminase was expressed under the control of the constitutive promoter from cauliflower mosaic virus (Klee and Kishore, 1992; Grichko et al., submitted for publication). Changes were observed during development of plants expressing ACC deaminase activity: senescence of tobacco flowers was slowed down considerably (Yang

and Hiatt, 1991); the initiation of ripening of detached tomato fruit was delayed significantly (Klee, 1993); and the duration of the tomato ripening process was increased substantially (Klee and Kishore, 1992). Transgenic tomato plants challenged with the pathogens, *Xanthomonas campestris* pv. *vesicatoria* and *Verticillium dahliae* Kleb showed significant reductions in the necrotic lesions of bacterial spot disease and verticillium wilt respectively (Lund et al., 1998; Robison et al., submitted for publication).

1.4.5. The role of ACC Deaminase in the functioning of plant growth-promoting bacteria

Each of the seven plant growth-promoting rhizobacteria isolated by Glick et al. (1995) from different soil samples in two geographically disparate locations was able to utilize ACC as a nitrogen source, contain ACC deaminase activity and enhance seedling root elongation of ethylene sensitive plants under gnotobiotic conditions (Glick et al., 1995; Hall et al., 1996; Shah et al., 1997). The increase in the length of the roots of young (five to seven day old) seedlings of canola, tomato, lettuce, wheat, oats and barley following treatment of the seeds with wild-type *Pseudomonas putida* GR12-2 was similar to the response of these plants when their seeds were treated with the ethylene inhibitor, AVG (Hall et al., 1996).

The plant growth-promoting bacterium, *Pseudomonas putida* GR12-2, which contains the enzyme ACC deaminase, stimulates root elongation (Lifshitz et al., 1987; Glick et al., 1994a; 1994b) and significantly reduces the level of ACC in emerging roots and shoots (Penrose et al., submitted for publication). Three separate mutants of *Pseudomonas putida* GR12-2, deficient in ACC deaminase activity, have lost the ability to promote canola root elongation under gnotobiotic conditions (Glick et al., 1994a; 1994b).

An IAA overproducing mutant of *Pseudomonas putida* GR12-2 was found to be inhibitory to root elongation (Xie et al., 1996). It is likely that the increased level of IAA secreted by this mutant is taken up by the plant and interacts with the enzyme ACC synthase. Thus, the "excess" bacterial IAA stimulates the synthesis of ACC which is in turn converted to ethylene (Yang and Hoffman, 1984; Kende, 1993). In fact, the inhibitory effect of high exogenous IAA levels on root length has been known for quite some time and is generally attributed to the stimulation of ethylene synthesis by IAA (Patten and Glick, 1996).

Glick et al. (1998) proposed a model by which plant growth-promoting bacteria can lower plant ethylene levels and in turn stimulate plant growth. In this model (Fig. 5) the plant growth-promoting bacteria bind to the surface of either the seed or root of a developing plant; in response to tryptophan and other small

molecules in the seed or root exudates (Whipps, 1990; Bayliss et al., 1993; 1997), the plant growth-promoting bacteria synthesize and secrete IAA (Hong et al., 1991b; Fallik et al., 1994), some of which may be taken up by the plant. This IAA, together with endogenous plant IAA, can stimulate plant cell proliferation, plant cell elongation or induce the synthesis of ACC synthase which is able to convert SAM to ACC (Kende, 1993).

It is postulated that much of the ACC produced by this latter reaction may be exuded from seeds or plant roots along with other small molecules normally present in seed or root exudates (Bayliss et al., 1993; 1997). The ACC in the exudates may be taken up by the bacteria and subsequently hydrolyzed by the enzyme, ACC deaminase, to ammonia and α-ketobutyrate. The uptake and cleavage of ACC by plant growth-promoting bacteria decreases the amount of ACC outside the plant. Increasing amounts of ACC are exuded by the plant in order to maintain the equilibrium between internal and external ACC levels. As a result, the presence of the bacteria induces the plant to synthesize more ACC than it would otherwise need and as well, stimulates the exudation of ACC from the plant.

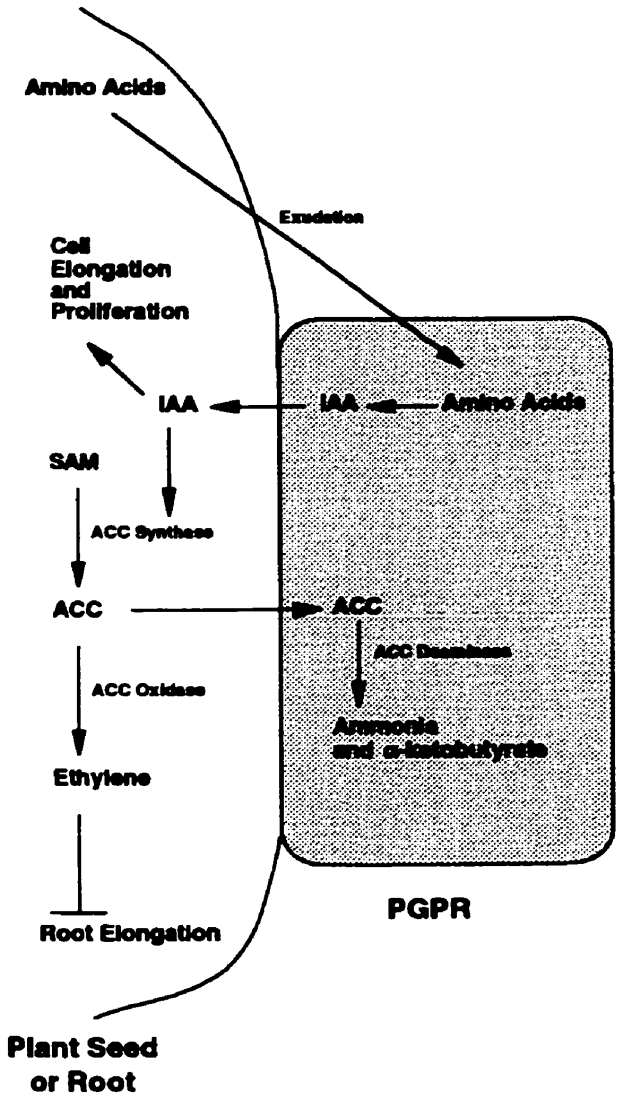
Thus, plant growth-promoting bacteria are supplied with a unique source of nitrogen in the form of ACC that enables them to proliferate under conditions in which other soil bacteria may not flourish. As a result of lowering the ACC level within the plant,

either the endogenous level or the IAA-stimulated level, the amount of ethylene in the plant is also reduced.

It is proposed that plant growth-promoting bacteria that possess the enzyme ACC deaminase and are bound to seeds or roots of seedlings, can reduce the amount of plant ethylene and the extent of its inhibition on root elongation. Thus, these plants should have longer roots and possibly longer shoots as well, inasmuch as stem elongation, except in flooding resistant plants, is also inhibited by ethylene (Abeles et al., 1992).

If the model that was previously proposed to explain the role of ACC deaminase in plant growth promotion (Jacobson et al., 1994; Glick et al., 1995; Hall et al., 1996; Glick et al., 1998) has any validity, a mutation in a pre-existing gene that generated the first ACC deaminase gene was fortuitous (Shah et al., 1998). Such a mutation could allow a soil bacterium to utilize a unique nutrient source, i.e., ACC, which, under conditions of low nitrogen availability, would allow that bacterium to proliferate. Soil bacteria that have ACC deaminase activity should then have a selective advantage over other soil bacteria in situations in which the main bacterial nutrients are from exudates of plants (Shah et al., 1998). It should be borne in mind that soil bacteria may acquire ACC deaminase genes by mechanisms other than fortuitous mutation—transfer of such a gene from another soil bacterium is another possible mechanism.

Fig. 5. A representation of the role of ACC deaminase in the promotion of plant root elongation. Abbreviations: IAA, indole-3-acetic acid; SAM, S-adenosylmethionine; ACC, 1-aminocyclopropane-1-carboxylic acid; PGPR, plant growth-promoting bacteria; ⊥ indicates the inhibition of root elongation.


Amino Acids
Exudation
Cell
Elongation
and
Proliferation
IAA
IAA
Amino Acids
SAM
ACC Synthase
ACC
ACC
ACC Deaminase
ACC Oxidase
Ammonia
and α-ketobutyrate
Ethylene
Root Elongation
PGPR
Plant Seed
or Root

The regulation of ethylene production in plants, especially to prevent increased ethylene production and accumulation, may reduce many of the inhibitory effects of this hormone (Klee et al., 1991; Jacobson et al., 1994; Sheehy et al., 1991). Many agricultural and horticultural crops are particularly sensitive to ethylene levels which regulate fruit ripening and control the deleterious effects of senescence in vegetables and flowers (Li et al., 1996; Sisler and Serek, 1997). The bacterial enzyme, ACC deaminase, is potentially a valuable tool for controlling the levels, and hence the effects, of ethylene in plants. ACC deaminase has already been used to substantially reduce ethylene levels in transgenic tomato plants which have exhibited a prolonged ripening phase (Klee and Kishore, 1992; Yang and Hiatt, 1991), and to lower stress ethylene levels following infection by bacterial and fungal pathogens (Lund et al., 1998; Robison et al., submitted for publication).

Strains of plant growth-promoting bacteria that contain ACC deaminase are known to reduce ACC, and hence ethylene, levels in canola seedlings (Penrose et al., submitted for publication), promote root elongation in a variety of plants (Hall et al., 1996), decrease the deleterious effects of flooding on tomato plants (Grichko and Glick, submitted for publication), and prolong the shelf-life of ethylene sensitive cut flowers (Nayani et al., 1998). There has also been a considerable decrease in the levels of stress ethylene in plants grown

in the presence of ACC deaminase-containing plant growth-promoting bacteria. Canola seedlings grown in the presence of high levels of nickel produced much less ethylene when the seeds were inoculated with a nickel resistant plant growth-promoting strain that also contained ACC deaminase. It is likely that the plant stress ethylene induced by the nickel was reduced by the activity of the bacterial ACC deaminase (Burd et al., 1998). Moreover, biocontrol strains of bacteria carrying ACC deaminase genes were able to more effectively protect several plants against various phytopathogens; cucumber plants were protected against *Pythium* damping-off and potato slices, exposed to *Erwinia caratovora* in a small sealed bag, were protected against *Erwinia* soft rot. It appears that the damage caused by the pathogen was reduced by the activity of ACC deaminase that lowered the level of stress ethylene produced by the plant (Wang et al., submitted for publication).

2. Materials and Methods

2.1. Bacterial strains and plasmids

2.1.1. Bacterial strains: description and culture conditions

The bacterial strains and plasmids used in this study are shown in Table 8 and the growth conditions are summarized in Table 9. *Escherichia coli* DH5α (Hanahan, 1983) was used as the host cell for the construction and maintenance of recombinant plasmids in this study. It is a recombinant-deficient suppressing strain in which the φ80*lacZ*ΔM15 allows α-complementation with the amino terminus of β-galactosidase encoded in the pUC18 vector (Sambrook et al., 1989). This *Escherichia coli* strain was grown aerobically in Lennox L Broth Base (LB) (Gibco BRL Life Technologies, Paisley, Scotland) or nutrient broth (NB) (Difco Laboratories, Detroit, MI) at 37°C. When required, the medium was supplemented with the appropriate antibiotic at these final concentrations: ampicillin (Roche Molecular Biochemicals), 100 $\mu g \cdot mL^{-1}$ or 950 $\mu g \cdot plate^{-1}$; tetracycline (Roche Molecular Biochemicals), 15 $\mu g \cdot mL^{-1}$ or 625 $\mu g \cdot plate^{-1}$.

Several strains of bacteria were identified and isolated from soil samples based on their ability to utilize 1-aminocyclopropane-1-carboxylic acid (ACC) as the sole source of nitrogen (Glick et al., 1995). Originally, some of these strains, including *Enterobacter cloacae* CAL3, were designated as *Pseudomonas* sp., but were later classified as

Enterobacter cloacae following fatty acid analysis (Shah et al., 1997). *Enterobacter cloacae* CAL3, as well as *Pseudomonas putida* GR12-2 (Lifshitz et al., 1986) (kindly provided by Dr. Gerry Brown, Agrium Inc. Sasksatoon, Canada), were used in this study. All of these strains were initially isolated from soil around plant roots: *Enterobacter cloacae* CAL3 from cotton in Fresno, California, USA, and *Pseudomonas putida* GR12-2 from grass in the Canadian High Arctic, Canada. Based on their ability to promote canola seedling root elongation under gnotobiotic conditions, each of these strains is considered to be a plant growth-promoting bacterium (Glick et al., 1995). *Pseudomonas putida* ATCC 17399 was obtained from the American Type Culture Collection. It is a psychrophilic bacterium that does not demonstrate plant growth-promoting ability and was used as a negative control in this study. The *Pseudomonas* and *Enterobacter* strains were grown aerobically in tryptic soybean broth (TSB) (Difco Laboratories, Detroit, MI) at 25°C with the exception of *Enterobacter cloacae* CAL3 that was grown at 30°C.

2.1.2. Culture conditions for the induction of ACC deaminase activity

In experiments that required the induction of ACC deaminase activity, bacteria were cultured in minimal media with ACC as the sole source of nitrogen. *Escherichia coli* was grown in M9 minimal medium (Miller, 1972) (grams·L^{-1}): 6.0 grams KH_2PO_4, 3.0 grams Na_2HPO_4, 1.5 grams NaCl, pH 7.4 with the following elements added asceptically just

before use (per litre) 493 mg $MgSO_4 \cdot 7H_2O$, 1.47 mg $CaCl_2 \cdot 2H_2O$, 2.0 grams glucose, and 337 mg thamine HCl. The *Enterobacter* and *Pseudomonas* strains were cultivated in DF salts medium (Dworkin and Foster, 1958) (grams$\cdot L^{-1}$): 4.0 grams KH_2PO_4, 6.0 grams Na_2HPO_4, 0.2 grams $MgSO_4 \cdot 7H_2O$, 2.0 grams glucose, 2.0 grams gluconic acid and 2.0 grams citric acid with trace elements (per litre): 1 mg $FeSO_4 \cdot 7H_2O$, 10 µg H_3BO_3, 11.2 µg $MnSO_4 \cdot H_2O$, 124.6 µg $ZnSO_4 \cdot 7H_2O$, 78.2 µg $CuSO_4 \cdot 5H_2O$, and 10 µg MoO_3, pH 7.2. When required, the medium was supplemented with the appropriate antibiotic at these final concentrations: ampicillin, 100 µg$\cdot mL^{-1}$; tetracycline, 15 µg$\cdot mL^{-1}$ (Roche Molecular Biochemicals). A 0.5 M solution of ACC (Calbiochem-Novobiochem Corp., La Jolla, CA) which is heat labile, was prepared under sterile conditions, aliquoted and frozen at –20°C; just prior to inoculation or induction, the frozen ACC solution was added to a final concentration of 3.0 mM.

Bacterial cells were grown to mid or late-log phase in 15 mL of rich medium, divided between two culture tubes. Each tube contained 7.5 mL of rich medium and the required antibiotic, and was inoculated with 5 µL of the appropriate strain that had been stored in glycerol at –20° but not thawed more than four times. The cultures were grown overnight in a shaking water bath at 200 rpm and the accumulated biomass was harvested by centrifugation of the contents of the combined tubes at 8000 x g for 10 minutes at 4°C in a Sorvall RC5B/C

centrifuge using an SS34 rotor. The supernatant was removed and the cells were washed with 5 mL of the appropriate minimal medium. Following an additional centrifugation for 10 minutes at 8000 x g in a SS34 rotor in a Sorvall RC5B/C centrifuge at 4°C, the cells were suspended in 7.5 mL of minimal medium plus 3 mM ACC and antibiotic, if necessary, in fresh culture tubes. The bacterial cells were returned to the shaking water bath at 200 rpm for 24 hours to induce the activity of ACC deaminase. The *Pseudomonas* strains were incubated at 25°C and *Escherichia coli* and *Enterobacter cloacae* CAL3 were incubated at 30°C. The bacteria were harvested by centrifugation at 8000 x g for 10 minutes at 4°C in an SS34 rotor of a Sorvall RC5B/C centrifuge. The supernatant was removed, the cells washed in the buffer or solution appropriate for their intended use and the pelleted cells stored for further use.

Bacteria were also plated on minimal media, either M9 or DF salts, with ACC as the sole nitrogen source and their growth was monitored for 3 days at 25°C (*Pseudomonas* strains) or 30°C (*Enterobacter cloacae* CAL3 and *Escherichia coli*). The plates were prepared with 1.8% Bacto-Agar (Difco Laboratories, Detroit, MI), which has a very low nitrogen content, and then spread with the appropriate antibiotic (ampicillin, 950 $\mu g \cdot plate^{-1}$; tetracycline, 625 $\mu g \cdot plate^{-1}$); ACC (30 μmoles) was spread on the plates just before they were used.

Table 8. Bacterial strains and plasmids

Strains or plasmids	Description	Source or reference
Strains		
Enterobacter cloacae CAL3	Wild-type strain isolated from soil; cotton field; Fresno, USA; has PGPR activity	Glick et al., 1995 Shah et al., 1998
Pseudomonas putida GR12-2	Wild-type strain isolated from soil; grass field; Canadian High Arctic; has PGPR activity	Lifshitz et al., 1986
Pseudomonas putida ATCC 17399	Psychrophile; has no PGPR activity	ATCC
Escherichia coli DH5α	F⁻φ80d*lacZ*ΔM15,*lacZ* Δ(YA-*arg*F)U169 *rec*A1 *end*A1 *hsd*R17 (r_K^-, m_K^+) *sup*E44λ↓*thi*-1 *gyr*A *rel*A1	Hanahan, 1983
Vectors		
pUC18	High copy number, *E. coli* cloning vector; multiple cloning site; gene for lacZ'; linearized with *Bam*HI, dephosphorylated with bacterial alkaline phosphatase (BAP); Amp^r	Yanisch-Perron et al., 1985; Amersham-Pharmacia
pRK415	Low copy number, broad host range vector; multiple cloning site; gene for *lacZ*αIncP; Tet^r	Keen et al., 1988
Plasmids		
pRKACC	3 kb fragment encoding ACC deaminase from UW4 gene cloned into pRK415; Tet^r	Shah et al., 1998
pUC319	2 kb fragment from CAL3 cloned into pUC18; Amp^r	Shah, unpublished
PUC320	2 kb fragment from CAL3 cloned into pUC18; Amp^r	Shah, unpublished
PUC324	0.2 kb fragment from CAL3 cloned into pUC18; Amp^r	Shah, unpublished
PUC327	6.5 kb fragment from CAL3 cloned into pUC18; Amp^r	Shah, unpublished

Abbreviations: Amp^r, ampicillin resistance; Tet^r, tetracycline resistance

Table 9. Growth conditions of bacterial strains

Bacterial strain	Culture collection number	Standard culture conditions	Growth conditions for induction of ACC deaminase
Enterobacter cloacae CAL3	155	TSB, 30°C	DF + 3 mM ACC, 30°C
Pseudomonas putida GR12-2	37	TSB, 25°C	DF + 3 mM ACC, 25°C
Pseudomonas putida pRK415	353	TSB, Tet, 25°C	DF + 3 mM ACC, Tet, 25°C
Pseudomonas putida pRKACC	331	TSB, Tet, 25°C	DF + 3 mM ACC, Tet, 25°C
Escherichia coli pRK415	79	Nutrient Broth, Tet, 37°C	M9 + 3mM ACC, Tet, 30°C
Escherichia coli pRKACC	312	Nutrient Broth, Tet, 37°C	M9 + 3mM ACC, Tet, 30°C
Escherichia coli pUC18/320	320	Nutrient Broth, Amp, 37°C	M9 + 3mM ACC, Amp, 30°C
Escherichia coli pUC18/321	321	Nutrient Broth, Amp, 37°C	M9 + 3mM ACC, Amp, 30°C
Escherichia coli pUC18/324	324	Nutrient Broth, Amp, 37°C	M9 + 3mM ACC, Amp, 30°C
Escherichia coli pUC18/327	327	Nutrient Broth, Amp, 37°C	M9 + 3mM ACC, Amp, 30°C

2.2. Gnotobiotic root elongation assay

The gnotobiotic root elongation assay was used as a method of assessing the effect of various conditions on the growth of canola seedlings. The protocol employed in this study was a modification of the procedure developed by Lifshitz et al. (1987) and was used to measure the elongation of canola roots from seeds treated with different strains of bacteria or chemical ethylene inhibitors. Some strains were grown in rich media only; others were transferred to minimal media for the induction of ACC deaminase activity. All bacterial cells were grown to late log phase in 15 mL of rich media, divided between two culture tubes: TSB was used for *Pseudomonas* and *Enterobacter* strains and nutrient broth for *Escherichia coli*. Each tube contained 7.5 mL of rich medium and the required antibiotic, and was inoculated with 5 µL of the appropriate strain. The cultures were grown overnight in a shaking water bath at 200 rpm; the contents of the two culture tubes that contained the same strain were combined and centrifuged at 8000 x g for 10 minutes at 4°C in a Sorvall RC5B/C centrifuge using an SS34 rotor. The supernatant was removed, the pellet suspended in 5 mL of sterile 0.03 M $MgSO_4$ and then centrifuged in an SS34 rotor in a Sorvall RC5B/C centrifuge at 8000 x g for 10 minutes at 4°C. The above step was repeated twice to ensure that the pellet was washed thoroughly. Following centrifugation after the final wash, the cells were suspended in 0.5 mL of sterile 0.03 M $MgSO_4$ and then placed on ice. A 0.5 mL

sample was removed from the cell suspension and diluted 8 to 10 times in 0.03 M $MgSO_4$; the absorbance of the sample was measured at 600 nm. This measurement was used to adjust the absorbance at 600 nm, of the bacterial suspension, to 0.15 with sterile 0.03 M $MgSO_4$. A solution of 10^{-4}M AVG, (L-α-(2-amino-ethoxyvinyl) glycine hydro-chloride (Sigma Chemicals), a chemical ethylene inhibitor, was dissolved in sterile 0.03M $MgSO_4$, aliquoted and stored at –20°C.

Seed-pack growth pouches (Northrup King Co., Minneapolis, MN) were prepared for gnotobiotic assay of canola root elongation. Following the addition of 12 mL of distilled water to each growth pouch, the growth pouches were wrapped in aluminum foil in groups of 10, and autoclaved at 121°C for 15 minutes.

Canola seeds (*Brassica campestris* cv. Reward, kindly provided by Dr. G. Brown, Agrium, Inc.) were disinfected immediately before use. The seeds (approximately 0.2 gram/treatment) were soaked in 70% ethanol for one minute in glass petri dishes (60 X 15 mm); the ethanol was removed and replaced with 1% sodium hypochlorite (household bleach). After 10 minutes the bleach solution was suctioned off and the seeds were thoroughly rinsed with sterile distilled water: at least 5 times, sterile distilled water was added to the dish of seeds, swirled and removed by suction. Each dish was incubated at room temperature for one hour with the appropriate treatment: sterile 0.03 M $MgSO_4$ (used as a negative control), bacterial suspensions in sterile 0.03 M $MgSO_4$

(absorbance of 0.15 at 600 nm), 10^{-4} M AVG (used as a positive control). Following incubation with each treatment, the seeds were placed in growth pouches with sterilized forceps: six seeds were set in each growth pouch and ten pouches were used for each treatment. The pouches were grouped together according to treatment and placed upright in a rack (Northrup King Co., Minneapolis, MN) such that the pouches were not touching. Two empty pouches were placed at the ends of each rack. Racks were placed in a clean plastic bin containing sterile distilled water, to a depth of approximately 3 cm, and covered loosely with clear plastic wrap to prevent dehydration. Pouches were incubated in a growth chamber (Conviron CMP 3244) which was maintained at 20 ± 1°C with a cycle beginning with 12 hours of dark followed by 12 hours of light (18 $\mu mol \cdot m^2 \cdot sec^2$). Each rack was positioned such that the centre of the row of pouches was 8 inches below and 5 inches lateral to the light source. The primary root and shoot lengths were measured on the fifth day of growth and the data were analyzed. Seeds that failed to germinate two days after they were sown were marked and the roots that subsequently developed from these seeds were not measured.

2.3. Collection of canola seed tissue and exudate during germination

Canola seeds (*Brassica campestris* cv. Reward) were exposed to various treatments and afterward, incubated in the dark for up to 50

hours. At specific time points during the incubation, samples of the germinating seeds were harvested and the seed exudate was collected from them. At each time point, seed tissue and exudate were gathered from duplicate samples of 200 seeds.

The seeds were disinfected immediately before use. Two hundred seeds (0.4 ± 0.008 grams) were weighed into an aluminum weigh boat and soaked in 5 mL of 10% hydrogen peroxide (BDH Inc., Toronto, Ont.) at room temperature. After two minutes, the hydrogen peroxide solution was removed by suction and the seeds were rinsed with sterile distilled water at least four times, during which time water was added to the dish of seeds, swirled and removed by suction. Each dish was incubated at room temperature for one hour with 5 mL of the appropriate treatment: 0.03M $MgSO_4$, (used as a negative control), *Enterobacter cloacae* CAL3 in 0.03M $MgSO_4$ (absorbance of 0.15 at 600 nm), *Pseudomonas putida* pRK415 in 0.03M $MgSO_4$ (absorbance of 0.15 at 600 nm). Following incubation, the solution used for seed treatment (0.03M $MgSO_4$ or bacterial suspension) was removed from the seeds and they were rinsed twice with sterile distilled water. After the water was removed by suction, the seeds were transferred to a 100 µm nylon sterile cell strainer (Becton Dickinson Labware, Franklin Lakes, NJ) set into a sterile disposable polypropylene petri plate (60 x 15 mm). One mL of autoclaved distilled water was added to each petri dish. Three petri plates, each containing seeds that received a different treatment, were

clustered together in a circular plastic container (15.5 x 4.5 cm) (Tupperware). The containers were covered loosely and incubated in the dark at 20° ± 1°C up to 66 hours in a growth chamber (Conviron CMP 3244). After 20 hours of incubation, one mL of sterile water was added to the remaining petri dishes and following 44 hours of incubation another mL of water was added to the samples.

At these specified times: 1, 2, 3.5, 5, 7, 15, 20, 24, 44, 50 hours after treatment, three petri plates, each containing seeds that received a different treatment, were removed from the growth chamber. The cell sieve was removed from each petri plate and the seeds transferred by sterile forceps to autoclaved screw cap 1.5-mL microfuge tubes (VWR Scientific Products). The tubes were immediately placed in liquid nitrogen and the frozen seeds stored at –80°C.

Two samples of canola seeds that had not been incubated at 20°C were also frozen in liquid nitrogen and stored at –80°C; they consisted of 200 seeds, disinfected but not treated, and 200 seeds, disinfected and treated with sterile 0.03 M $MgSO_4$ for one hour.

After the germinating seedlings had been gathered from the strainers at each time point, the seedling exudate was removed from the petri plate (and any clinging to the cell strainer) with a one-mL sterile disposable syringe (Becton Dickinson Labware) and #20 gauge needle (Becton Dickinson Labware). The exudate was filtered through a 0.2 µm sterile syringe filter (Gelman Sciences, Ann Arbour, MI), pre-wetted

with sterile distilled water. The filtrate was collected into 1.5 mL glass vials (12 x 32 mm) capped with silicon septa (75/10) and polypropylene open top lids (all from Chromatographic Specialties Inc.) and immediately frozen at –80°C.

2.4. Protein Extraction

2.4.1. Preparation of bacterial crude extracts

Bacterial crude extracts were prepared according to the following procedure. Cell pellets were suspended in 5 mL of 0.1 M Tris-HCl, pH 7.6, centrifuged at 8000 x g at 4°C for 10 minutes in an SS34 rotor in a Sorvall RC5B/C centrifuge. The supernatant was discarded; the pellet was suspended in one mL of same buffer and transferred to a 1.5-mL microfuge tube. The contents of the 1.5-mL microfuge tube were centrifuged at 16000 x g for 5 minutes in a Brinkmann microfuge and the supernatant removed with a fine-tip transfer pipet (Fisher Scientific). The pellet was either frozen at –20°C (to be turned into a crude extract at a later time) or suspended in 600 µL of 0.1 M Tris-HCl pH 8.5. Thirty µL of toluene (Caledon Laboratories, Georgetown, Ont.) were added to the cell suspension and vortexed at the highest setting for 30 seconds. At this point, a 100-µL aliquot of the "toluenized cells" was set aside and stored at 4°C for protein assay at a later time. The remaining toluenized cell suspension was used immediately for assay of ACC deaminase activity.

2.4.2. Preparation of crude plant extracts

A modification of the protocol described by Siefert et al. (1994) was used to make crude extracts of canola (*Brassica campestris* cv. Reward) tissue from seeds (disinfected only, or disinfected and treated with 0.03 M $MgSO_4$), seedlings (grown up to 66 hours in the dark) and 4.5-day old plants grown for the gnotobiotic root elongation assay. Roots and shoots excised from the approximately 60 seedlings grown for the root elongation assay, were set in aluminum weigh boats, immediately frozen in liquid nitrogen and stored at –80°C. All of the glassware used in the preparation of crude plant extracts i.e., mortars and pestles, solution bottles, centrifuge tubes, pipets, pasteur pipets, and glass vials and silicon septa, was heated overnight at 275°C and cooled to room temperature just before being used. Each of the frozen tissue samples was ground in a pre-chilled mortar and pestle, suspended in 2.5 mL of 0.1 M sodium acetate pH 5.5 and kept on ice for 15 minutes. The contents of the mortar were scraped into a 15-mL glass centrifuge tube and the mortar and pestle were rinsed with 0.5 mL of the same buffer. The ground tissue suspension together with the rinses, were centrifuged in an SS34 rotor at 17,500 x g in a Sorvall R5C/B centrifuge for 15 minutes at 4°C to remove cell debris. The supernatant was collected and clarified by centrifugation in a Beckman L8-70 ultracentrifuge at 100,000 x g in a 70.1 Ti rotor for one hour at 4°C and then, if necessary, by an additional centrifugation at 100,000 x g for 15 minutes. The clarified

supernatant was collected and distributed into one-mL aliquots, some of which were stored at –80°C in glass vials for ACC determination by HPLC (high performance liquid chromatography), and the remainder stored in 1.5 mL microfuge tubes at 4°C for protein determination.

2.5. Determination of protein content

2.5.1. Protein concentration assay

The protein concentrations in all bacterial and plant extracts were measured according to the protocol of the "Protein Assay", based on the method of Bradford (1976), purchased from Bio-Rad Laboratories. The concentrated dye reagent (Coomassie Brilliant Blue G-250) was diluted with distilled water in a 1:4 ratio and then filtered through Whatman #1 filter paper to remove particles. Protein concentrations of samples were determined by using BSA (bovine serum albumin) (Bio-Rad Laboratories) as the standard. A standard concentration curve was produced from a series of known BSA concentrations, prepared within a linear range from 0.1 to 0.8 $mg \cdot mL^{-1}$, each in a volume of 100 µL. Each point in the series was transferred to a glass test tube (100 X 13 mm) and assayed in triplicate. After 5 mL of the diluted dye reagent was added to each test tube, it was vortexed and incubated at room temperature between 5 and 20 minutes. The absorbance of each sample was measured at 595 nm in a Novaspec II spectrophotometer (Pharmacia Biospec Tech) kindly made available by Dr. Barbara Moffatt. A standard

curve was obtained by fitting the best straight line through the absorbance data plotted against concentration (Appendix I).

2.5.2. Protein concentration assay of crude bacterial extracts

The 100-µL aliquots of toluenized cell suspensions, which had been set aside and stored at 4°C during the preparation of crude bacterial cell extracts, were each mixed with 100 µL of 0.1 N NaOH and incubated for 10 minutes at 100°C. After the mixtures had cooled, between 20 and 50 µL of each sample were transferred to a clean glass test tube (100 x 13 mm); the volume was adjusted to 100 µL with 0.1 M Tris-HCl pH 8.5, and 5 mL of the diluted dye reagent was added to the tube. The contents of the tube were vortexed and incubated for 5 to 20 minutes at room temperature. The absorbance of the samples was measured at 595 nm in a Novaspec II spectrophotometer.

2.5.3. Protein concentration assay of crude plant extracts

Aliquots of the crude plant extracts, set aside and stored at 4°C, were each transferred to clean glass test tubes (100 x 13 mm) and the volume was adjusted to 100 µl with 0.1 M sodium acetate pH 5.5. Varying amounts of the different extracts were transferred to the tubes, depending on the concentration of the extract: routinely, 30 µL of seed extract, 70 µL of shoot extract, and 100 µL of root extract were used. Sufficient buffer to bring the volume up to 100 µL was added. All of the

samples of crude plant extracts were assayed in triplicate. After 5 mL of the diluted dye reagent were added to each test tube, it was vortexed and incubated at room temperature between 5 and 20 minutes. The absorbance of each sample was measured at 595 nm in a Novaspec II spectrophotometer.

2.6. Measurement of ACC deaminase activity

2.6.1. Assay of ACC deaminase activity

The assay of ACC deaminase activity in the crude extract is a modification of the method of Honma and Shimomura (1978) which measures the amount of α-ketobutyrate produced by the enzyme. The number of μmoles of α-ketobutyrate produced by ACC deaminase is determined by comparing the absorbance at 540 nm of a sample to a standard curve of α-ketobutryate ranging between 0.1 and 1.0 μmoles. A stock solution of 100 mM α-ketobutryate (Sigma Chemical Co.) was prepared in 0.1 M Tris-HCl pH 8.5 and stored at 4°C. Just prior to use, the stock solution was diluted with the same buffer to make a 10 mM solution from which a standard concentration curve was generated. Each in a series of known α-ketobutryate concentrations was prepared in a volume of 200 μL and transferred to a glass test tube (100 x 13 mm); each point in the series was assayed in duplicate. Three hundred μL of the 2,4-dinitrophenylhydrazine reagent (0.2% 2,4-dinitrophenylhydrazine in 2 N HCl) (Sigma Chemical Co.) was added to each glass tube

and the contents vortexed and incubated at 30°C for 30 minutes during which time the α-ketobutyrate was derivatized as a phenylhydrazone. The colour of the phenylhydazone was developed by the addition of 2 mL of 2 N NaOH; after mixing, the absorbance of the mixture was measured at 540 nm in a Novaspec II spectrophotometer.

2.6.2. Assay of ACC deaminase activity in crude bacterial extracts

Bacterial crude extracts were assayed for ACC deaminase activity. All sample measurements were carried out in duplicate. Two hundred µL of the toluenized cells were placed in a fresh 1.5-mL microfuge tube; 20 µL of 0.5 M ACC were added to the suspension, briefly vortexed, and then incubated at 30°C for 15 minutes. Following the addition of one mL of 0.56 N HCl, the mixture was vortexed and centrifuged for 5 minutes at 16000 x g in a Brinkmann microfuge at room temperature. One mL of the supernatant was vortexed together with 800 µL of 0.56 N HCl in a clean glass tube (100 x 13 mm). Thereupon, 300 µL of the 2,4-dinitrophenylhydrazine reagent (0.2% 2,4-dinitrophenylhydrazine in 2 N HCl) were added to the glass tube, the contents vortexed and then incubated at 30°C for 30 minutes. Following the addition and mixing of 2 mL of 2 N NaOH, the absorbance of the mixture was measured at 540 nm in a Novaspec II spectrophotometer.

The absorbance of the assay reagents including the substrate, ACC, and the bacterial extract were taken into account. After the

indicated incubations, the absorbance at 540 nm of the assay reagents in the presence of ACC was used as a reference for the spectrophotometric readings; it was subtracted from the absorbance of the bacterial extract plus the assay reagents in the presence of ACC. The contribution of the extract, i.e., the absorbance at 540 nm of extract and the assay reagents without ACC, was determined and subtracted from the absorbance value calculated above. This value was used to calculate the amount of α-ketobutyrate generated.

2.7. Measurement of ACC by HPLC

2.7.1 Chemicals

The Waters AccQ•Fluor™ Reagent Kit, AccQ•Tag eluent A concentrate (a pre-mixed concentrated acetate-phosphate buffer) and the amino acid hydrolysate standard, a mixture of 17 hydrolysate amino acids (tryptophan, glutamine, and asparagine not included) each at a concentration of 2.5 mM with the exception of cysteine which is 1.25 mM, were supplied by Waters Chromatography, Inc. The Waters AccQ•Fluor™ Reagent Kit contained the chemicals for derivatization: AccQ•Fluor reagent powder (6-aminoquinolyl-N-hydroxysuccinimidyl carbamate) (AQC), AccQ•Fluor reagent borate buffer and AccQ•Fluor reagent diluent (acetonitrile). ACC, β- and γ-aminobutyric acid were purchased from Calbiochem-Novabiochem Corp. (La Jolla, CA), HPLC grade acetonitrile from Caledon Laboratories (Georgetown, Ont.

Canada), α-aminobutyric acid from Fisher Chemicals, and L-α-(2-amino-ethoxyvinyl) glycine hydrochloride (AVG) from Sigma Chemical Corp. Individual amino acids: L-cysteine, L-isoleucine, L-leucine, L-lysine, L-methionine, L-proline, L-tyrosine and L-valine, were puchased from Sigma Chemical Corp. All water used was purified by a Milli-Q® Water System (Millipore Co. Bedford, MA), autoclaved and then filtered through a 0.45 μm HA filter (Millipore Co. Bedford, MA).

2.7.2. Treatment of glassware

All glassware used in this procedure was washed and flushed at least 6 times with tap water, twice with deionized water and twice more with distilled water. Just prior to use, the cleansed glassware was wrapped in aluminum foil, heated overnight at 275°C and cooled to room temperature. Solutions and samples were stored in heat-treated bottles and vials (including septa and lids).

2.7.3. Preparation of standard solutions

Stock 2.5-mM solutions of ACC, α-aminobutyric acid, β-aminobutyric acid, γ-aminobutyric acid, cysteine, isoleucine, leucine, lysine, methionine, proline, tyrosine, valine, and a mixture of 17 hydrolysate amino acids were prepared in 25 mL of 0.1 N HCl in a 25-mL volumetric flask. These solutions were diluted with sterile distilled water to yield a concentration of 0.1 mM. The 2.5-mM and 0.1-mM stock

solutions were divided into 0.5-mL aliquots, frozen at –20°C, thawed once when needed and then discarded. With the exception of ACC and cysteine, the 0.1 mM solutions were further diluted with sterile distilled water to generate concentrations between 5 and 25 pmol·20 μL^{-1} injection. Dilutions of the 0.1-mM solutions of ACC and cysteine yielded between 1 and 25 pmol ACC·20 μL^{-1} injection, and between 5 and 50 pmol cysteine·20 μL^{-1} injection. Standard mixtures of ACC and α-aminobutyric, and of ACC, α-, β-, and γ aminobutyric acids were prepared in sterile distilled water to yield 12.5 pmol·20 μL^{-1} injection. The amino acid hydrolysate standard was diluted such that each 20-μL injection included 25 pmol of each of the 17 amino acids with the exception of cysteine which contained 12.5 pmol. Aliquots of the standard solutions were frozen at –20°C and when required, thawed once and used. Injections were made of fresh ACC solutions, or of those stored at 4°C or –20°C for varying lengths of time.

2.7.4. Derivatization procedure

Standard solutions of ACC, α-aminobutyric acid, β-aminobutyric acid, γ-aminobutyric acid, cysteine, isoleucine, leucine, lysine, methionine, proline, tyrosine, valine, ACC and α-aminobutyric acid; ACC, α-, β- and γ- aminobutyric acids; the amino acid hydrolysates and plant extracts were coupled with ACQ according to the directions in the Waters AccQ•Fluor™ Reagent Kit Instruction Manual. The AccQ•Fluor

derivatization reagent, once reconstituted, was stable for one week. The derivatization reagent was reconstituted by adding one mL of acetonitrile (vial 2B) to the AccQ•Fluor reagent powder, vortexing for 10 seconds, and heating on top of a 55°C heating block for no more than 10 minutes to dissolve the powder. The concentration of the reconstituted AccQ•Fluor reagent is approximately 10 mM in acetonitrile; amino acid derivatization is optimal when the reconstituted AccQ•Fluor reagent is in excess and the pH is between pH 8.2 to 10. The derivatization reactions were carried out in duplicate in 6 x 55 mm glass sample tubes (Waters Chromatography, Inc.). Ten µL of standard or sample solution were placed in each tube; 70 µL of AccQ•Fluor borate buffer were added to it and the mixture was immediately vortexed for several seconds. Following the addition of 20 µL of reconstituted AccQ•Fluor; the mixture was briefly vortexed again, allowed to stand at room temperature for one minute and then heated at 55°C for two minutes in a heating block. Once cooled to room temperature (5–10 minutes) the solution was either injected immediately or sometime during the next week. According to the manufacturer's directions, amino acids derivatized by this procedure are quite stable and can be stored at room temperature for at least one week.

2.7.5. HPLC determination of ACC content

The Acc•Tag Column, a high-efficiency 4 μm Nova-Pak™ C_{18} column specifically certified for use with the AccQ•Tag Method (Waters Chromatography, Inc.) was used to separate the amino acid derivatives produced by the AccQ•Fluor derivatization reaction and a Hewlett Packard column heater was used to maintain the column temperature at 37°C. Amino acid derivatives were detected and measured by using a Hewlett Packard HPLC system which consisted of a 1050 Series Quaternary Pump and a 104a Programmable Fluorescence Detector. A PC computer system (DTK 3300 386/33) was used to run the supporting computer software, *i.e.* Hewlett Packard's ChemStation (DOS Series).

The solvent system included eluent A, a diluted solution of Waters AccQ•Tag acetate-phosphate buffer concentrate prepared daily, (50 mL concentrate diluted with 500 mL 18 Megohm Milli-Q water), eluent B, HPLC-grade acetonitrile, and eluent C, 18 Megohm Milli-Q water. The solvents were continuously sparged with helium and the solvent lines were purged for at least 60 seconds prior to use to remove any air bubbles present. The AccQ•Tag column was conditioned with 60% eluent B/40% eluent C at a flow rate of 1 mL·minute^{-1} for 30 minutes and then equilibrated with 100% eluent A for 10 minutes at a flow rate of 1 mL·minute^{-1} before injection of the first sample. The gradient recommended by Waters Chromatography, Inc. for separation of the AccQ•Tag-labelled amino acids in Table 10 was modified to

enhance resolution of the ACC peak. The modified gradient is presented in Table 11.

The Hewlett Packard 104a Programmable Fluorescence Detector was set up according to the Waters AccQ•Tag Amino Acid Analysis Method™ and was turned on at least 40 minutes prior to sample injection. The settings were as follows: excitation wavelength, 250 nm; emission wavelength, 395 nm; response time, 4; pmt gain, 15, and lamp setting, 3-5W/220Hz.

Once the column was conditioned and equilibrated, and the detector was warmed up, a standard solution, containing 12.5 pmol of ACC and α-aminobutyric acid or 12.5 pmol of ACC, α-, β-, and γ-aminobutyric acid, was injected. Following the injection of standard solutions, samples were injected and analyzed; the run time for each sample was 23 minutes and included washing and re-equilibrating the column following the separation of the derivatized amino acids. Duplicates of each standard and sample were derivatized and injected. The needle port was rinsed with eluent A prior to each injection in order to reduce contamination from previously injected samples, and at least every two or three days, eluent A was injected and the blank gradient examined for the presence of any other contaminants. The injection volume of all samples including blanks, standards and plant extracts was 20 μL. Plant tissue extracts were diluted just prior to derivatization. The quantity of sample hydrolyzed and derivatized in 20 μL was

estimated to be 0.1 to 1.0 μg (4 to 40 pmoles) of protein, based on a protein average molecular weight of 25,000 daltons.

Table 10. Gradient Table for Waters AccQ•Tag System

Time (min)	Flow Rate (min/min)	%A	%B	%C
0	1.0	100.0	0	0
0.5	1.0	99.0	1.0	0
18.0	1.0	95.0	5.0	0
19.0	1.0	91.0	9.0	0
29.5	1.0	83.0	17.0	0
*33.0	1.0	0	60.0	40
36.0	1.0	100.0	0	0
45.0	1.0	100.0	0	0

Abbreviations: A, Waters AccQ•Tag acetate-phosphate buffer concentrate (50 mL diluted with 500 mL 18 Megohm Milli-Q water); B, HPLC–grade acetonitrile; C, 18 Megohm Milli-Q water.

Note: *From this point in the gradient, the column is being washed and conditioned for the next sample.

Table 11. Gradient table for Waters AccQ•Tag system modified for ACC elution

Time (min)	Flow Rate (min/min)	%A	%B	%C
0	1.0	100.0	0	0
0.5	1.0	99.0	1.0	0
3.0	1.0	91.0	9.0	0
13.0	1.0	88.0	12.0	0
14.0	1.0	83.0	17.0	0
*16.0	1.0	0	60	40
18.0	1.0	100.0	0	0
23.0	1.0	100.0	0	0

Abbreviations: A, Waters AccQ•Tag acetate-phosphate buffer concentrate (50 mL diluted with 500 mL 18 Megohm Milli-Q water); B, HPLC–grade acetonitrile; C, 18 Megohm Milli-Q water.

Note: *From this point in the gradient, the column is being washed and conditioned for the next sample.

2.8. Isolation of plasmid DNA by alkaline lysis miniprep

A modification of the alkaline lysis miniprep procedure (Sambrook et al., 1989) was used to isolate plasmid DNA. An overnight culture inoculated with a single bacterial colony in 2 mL of rich media (LB for *Escherichia coli*) supplemented with the appropriate antibiotic, was distributed among 1.5-mL microfuge tubes and centrifuged at 16000 x g in a Brinkmann microfuge for one minute, to pellet the cells. The supernatant was discarded and the pellet gently suspended in 200 µL of Solution I (50 mM glucose, 25 mM Tris-HCl, pH 8.0; 10 mM EDTA, pH 8.0). After the addition of 200 µL of freshly made Solution II (0.2 N NaOH, 1% SDS) the suspension was shaken vigorously and left to sit at room temperature for 5 minutes, during which time the cell membranes were ruptured; the chromosomal DNA was denatured but the plasmid DNA was left intact. This was followed by the addition of 200 µL of ice-cold Solution III (3.0 M potassium acetate pH 4.8) to the lysed cells; the tube was inverted several times and then kept on ice for 5 minutes. The lysed cell suspension was neutralized by the low pH of Solution III that caused the SDS-protein complexes to precipitate and the chromosomal DNA strands to renature and become entangled in one another. Following centrifugation in a Brinkmann microfuge at 16000 x g for 5 minutes, the pellet was discarded and 600 µL of supernatant collected. The supernatant, which contained plasmid DNA, was treated with RNAse (final concentration of 50 µg/µL) (Roche Molecular

Biochemicals) for 30 minutes at 37°C to digest the RNA. Protein was then removed by extraction with an equal volume of phenol: chloroform: isoamyl alcohol (25:24:1); the supernatant was shaken vigorously for one to two minutes and centrifuged at 16000 x g in a Brinkmann microfuge for 5 minutes. The upper aqueous phase was collected in a 1.5-mL microfuge tube and an equal volume of isopropanol added to it. Following 10 inversions of the tube, the plasmid DNA was allowed to precipitate at room temperature for 30 to 60 minutes. The precipitated DNA was collected in the pellet following centrifugation at 16000 x g in a Brinkmann microfuge for 10 minutes. The supernatant was removed and the pellet washed by shaking it with 500 µL of ice cold 70% ethanol. Following centrifugation at 16000 x g for 3 minutes at 4°C, the ethanol was removed and the pellet dried under vacuum for 5 minutes. The pellet was resuspended in 50 – 100 µL TE buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA, pH 8.0), aliquoted and stored at 4°C and –20°C.

2.9. Isolation of total genomic DNA

Chromosomal DNA was isolated from *Enterobacter cloacae* CAL3 by a modification from the procedure of Meade et al. (1982). A 10-mL culture of the bacterium was grown in TSB medium overnight at 30°C with shaking at 200 rpm; the cells were distributed among 1.5-mL microfuge tubes and centrifuged at 16000 x g in a Brinkmann microfuge

for 2 minutes. The harvested cells were washed with 1.5 mL TES (10 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 8.0; 50 mM NaCl,) and then suspended in 500 µL of TE buffer. The bacterial cells were lysed by incubation with 50 µL of a freshly prepared lysozyme solution (2 $mg \cdot mL^{-1}$ in TE) at 30°C for 20 minutes. This was followed by the addition of 50 µL of sarkosyl/pronase (10% sarkosyl in TE containing 5% pronase) (sarkosyl and pronase both purchased from Sigma Chemicals) and a further incubation at 37°C for one hour. During this incubation, the progression of lysis was determined by an increase in viscosity of the suspension. When lysis was evident, 70 µL of 3.0 M sodium acetate, pH 5.2, were gently mixed with the lysate which was then extracted with a series of solvents: once with 250 µL of phenol, twice with phenol: chloroform (1:1) and once with 250 µL chloroform: isoamyl alcohol (24:1). For each extraction, the tubes were inverted gently for approximately 10 minutes, centrifuged at 12000 x g for 10 minutes in a Brinkmann microfuge and the upper aqueous phase collected in a clean 1.5-mL microfuge tube. The supernatant from two tubes was pooled to give an approximate volume of 700 µL; the DNA was precipitated with an equal volume of isopropanol, and was evident as a coiled thread. The DNA was removed by spooling it on a yellow pipet tip, washed twice with ice-cold 70% ethanol and centrifuged at 16000 x g in a Brinkmann microfuge for 3 minutes at 4°C. After the

second wash, the ethanol supernatant was removed; the pellet was dried under vacuum for 5 minutes and dissolved in 100 µL 0.1 M TE buffer.

2.10. Manipulation of DNA

2.10.1. Restriction endonuclease digestion

For a single enzyme digestion, the DNA sample was incubated with the specific restriction enzyme and its buffer according to the manufacturer's instructions. When a double enzyme digestion was required, the restriction buffer was selected in accordance with the table in the Roche Molecular Biochemicals Catalogue. Each restriction enzyme digest contained 1 – 5 µL of DNA (100 – 1000 ng), 2 µL of the appropriate 10X buffer (1X), 1 µL of restriction enzyme (0.125 – 2 units) and sufficient sterile distilled water to bring the volume up to 20 µL. The digestion mixture was vortexed and incubated between 0.5 and 18 hours at 37°C. After the digestion was completed, the enzyme(s) were inactivated by heating for 20 minutes at 65°C.

2.10.2. Dephosphorylation of plasmid DNA

Before a plasmid vector was used in a cloning experiment, it was treated with alkaline phosphatase (because this enzyme catalyzes the removal of 5'-phosphate residues from DNA vector fragments and thus prevents self-ligation of the vector). Initially, the plasmid was digested with the appropriate restriction enzyme and then diluted to 50 µL with

its corresponding buffer. Following inactivation of the restriction enzyme, the vector was treated with 0.1 unit of alkaline phosphatase (Roche Molecular Biochemicals) and incubated at 37°C for 30 minutes. The alkaline phosphatase was then inactivated by heating at 85°C for 15 minutes and the dephosphorylated vector was ready to be used for ligation. Alternatively, the vector *Bam*HI/BAP, a commercial preparation of pUC18 digested to completion with the restriction endonuclease *Bam*HI and then treated with alkaline phosphatase, was purchased from Amersham Pharmacia Biotech, Quebec, Canada.

2.10.3. DNA ligation

DNA fragments were ligated into plasmid vectors by using T4 DNA ligase according to the manufacturer's instructions. If the DNA was used directly following restriction digestion, the restriction enzymes were inactivated first. The cohesive-end ligation reactions used in this study contained a total of 20 µL and were composed of 2 units of T4 DNA ligase (MBI Fermentas, Vilnius, Lithuania), 1X ligation buffer (66 mM-HCl; 5 mM $MgCl_2$; 5 mM dithiothreitol; 1 mM ATP, pH 7.5) and insert DNA and plasmid vector DNA in a molar ratio of approximately 3:1. The ligation mixture was incubated at 16°C overnight. Following ligation, the T4 DNA ligase was inactivated by heating at 65°C for 10 minutes; it has been suggested that by inactivating the enzyme, the

number of transformants may be increased by up to two orders of magnitude (Michelsen, 1995).

2.11. Introduction of foreign DNA into bacterial cells

Plasmids created for the construction of the *Enterobacter cloacae* CAL3 genomic DNA libraries were transformed into (and later maintained in) *Escherichia coli* DH5α competent cells. Preparation of the competent cells was based on the procedure of Maniatis et al. (1982). Thirty mL of LB medium were inoculated with 300 µL of an overnight culture of the desired bacterial strain. The flask was incubated at 37°C with shaking at 200 rpm for approximately 3.5 hours or until the culture reached an optical density of 0.4 at 600 nm. The cells were chilled on ice for 2 hours and harvested by centrifugation in an SS34 rotor at 3000 x g for 10 minutes in the Sorvall RC5B/C centrifuge at 4°C. The cells were gently suspended in 1 – 2 mL of freshly prepared, ice-cold Trituration buffer (Promega): 100 mM $CaCl_2$, 70 mM $MgCl_2$, and 40 mM sodium acetate, pH 5.5. The volume of the cells was adjusted to 25 mL with the Trituration buffer and the suspension allowed to stand on ice for 45 minutes. The diluted cells were collected by centrifugation at 1800 x g for 10 minutes at 4°C and then gently suspended in 2 mL of ice-cold Trituration buffer. At this point, the cells were aliquoted and frozen. The competent cells were gently mixed by swirling with 80% glycerol to a final concentration of 15% ($v \cdot v^{-1}$), apportioned into 200-µL aliquots in

1.5 mL microfuge tubes, quick-frozen in liquid nitrogen and stored at –80°C.

Prior to transformation, the required number of aliquots of competent cells was thawed on ice. Between 5 and 10 µL of ligation product or isolated plasmid DNA (about 500 ng of DNA) were added to each aliquot of competent cells, gently mixed and then incubated for 30 – 60 minutes on ice. The mixture was exposed to heat shock for 45 – 90 seconds in a 42°C water bath and then placed on ice for 2 minutes. Following this, 800 µL of SOC medium (0.1% tryptone [$w \cdot v^{-1}$], 0.05% yeast extract [$w \cdot v^{-1}$], 0.05% NaCl [$w \cdot v^{-1}$], 10 mM $MgSO_4$, and 10 mM$MgCl_2$, pH 7.0, and 20 mM sterile glucose which was added after the medium was autoclaved) were added to the transformed competent cells and they were warmed to 37°C for 5 minutes. The recovered cells were shaken for one hour at 37°C and then plated on selective medium containing the appropriate antibiotic.

2.12. Construction of the genomic DNA libraries

Total genomic DNA isolated from *Enterobacter cloacae* CAL3 was partially digested with the restriction endonuclease *Sau*3AI under conditions that produced DNA fragments between 3 and 10 kb in size. Three digestions of the genomic DNA, 1 µL of DNA/digestion, were carried out for 30 minutes at 37°C, with three concentrations of *Sau*3AI (1.25, 0.625 and 0.3125 units/µL) (MBI Fermentas). Partially digested

DNA fragments from the digestions with 1.25 and 0.625 units/μL of *Sau*3AI were ligated into the vector *Bam*HI/BAP, a commercial preparation of pUC18, and the ligation reaction mixtures, containing different genomic DNA fragments, were transformed into *Escherichia coli* DH5α cells. Transformants were plated on LB medium containing 100 $\mu g \cdot mL^{-1}$ ampicillin; these plates were spread with 40 μL of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galacto-pyranoside) (MBI Fermentas, Vilnius, Lithuania) from a 20 $mg \cdot mL^{-1}$ stock prepared in dimethyl formamide, and 4 μL of IPTG (isopropylthio-β-D-galactoside) (Roche Molecular Biochemicals) from a 200 $mg \cdot mL^{-1}$ stock, 30 minutes before use. Approximately 10 – 20% of the transformants were white colonies, which represents transformants containing genomic DNA inserts.

2.13. Screening of the genomic libraries

Following transformation, the *Enterobacter cloacae* CAL3 genomic DNA library was recovered in 50 mL in LB media plus ampicillin at 30°C overnight. The cells were washed in M9 minimal media and incubated with the same media plus 3 mM ACC as the sole nitrogen source at 37°C for 48 hours. The bacteria were plated on M9-ACC plates containing 3 mM ACC as the only nitrogen source (10 plates). About 30 clones grew on the M9-ACC plates; they were streaked twice on M9-ACC plates and then cultured in M9-ACC liquid. A portion of each of these liquid cultures was plated on LB media plus ampicillin in order to

produce single colonies. Approximately 150 single colonies were then transferred to M9-ACC plates to select those colonies that could utilize ACC as a source of nitrogen; only 12 clones grew on the M9-ACC plates. The plasmid from each of these clones was isolated and digested with restriction enzymes to confirm the presence of an insert.

Preparation and initial screening of the *Enterobacter cloacae* CAL3 clone bank was done in collaboration with Dr. Saleh Shah.

2.14. Agarose gel electrophoresis

Plasmid DNA, uncut or restriction endonuclease-digested, was separated on a 0.8% agarose gel prepared in 1X TAE (Tris-Acetate EDTA buffer) according to Sambrook et al. (1989). The working solution of 1X TAE (0.04 M Tris-acetate, 0.001 M EDTA, pH 8.0) was prepared from 50X stock, which contained (per litre): 242 grams Tris base, 57.1 mL glacial acetic acid, and 100 mL of 0.5 M EDTA, pH 8.0. Prior to solidification, ethidium bromide (5 $\mu g \cdot ml^{-1}$) (Biorad Laboratories) was added to the agarose solution. Immediately before samples were loaded on the gel, uncut plasmid DNA, suspended in 10 µL of TE, was incubated with 0.5 mL RNAse (final concentration of 2.5 $mg \cdot mL^{-1}$) at 37°C for 15 minutes. Two µL of 6X loading buffer (0.25% bromophenol blue, 40% [$w \cdot v^{-1}$] glycerol) were added to 10 µL of DNA and the entire 12 µL were loaded per well. GeneRuler™, a one kb DNA ladder, (MBI Fermentas, Vilnius, Lithuania) was placed in the outermost well of the

gel (5 $\mu L \cdot well^{-1}$) and electrophoresis was carried out at 100 volts for 1.25 hour. The gel was rinsed in deionized water and then visualized on an ImageMaster® VDS (Pharmacia Biotech); the image was documented by photography.

2.15. DNA sequence analysis

DNA sequencing was done at the MOBIX Central Facility, McMaster University, Hamilton, Ontario. Several clones from the *Enterobacter cloacae* CAL3 clone bank were sent for sequencing. Plasmid DNA was isolated by the alkaline lysis miniprep method from these clones (#319, 320, 324 and 327), and was prepared for sequencing according to the instructions issued by MOBIX.

3. Results

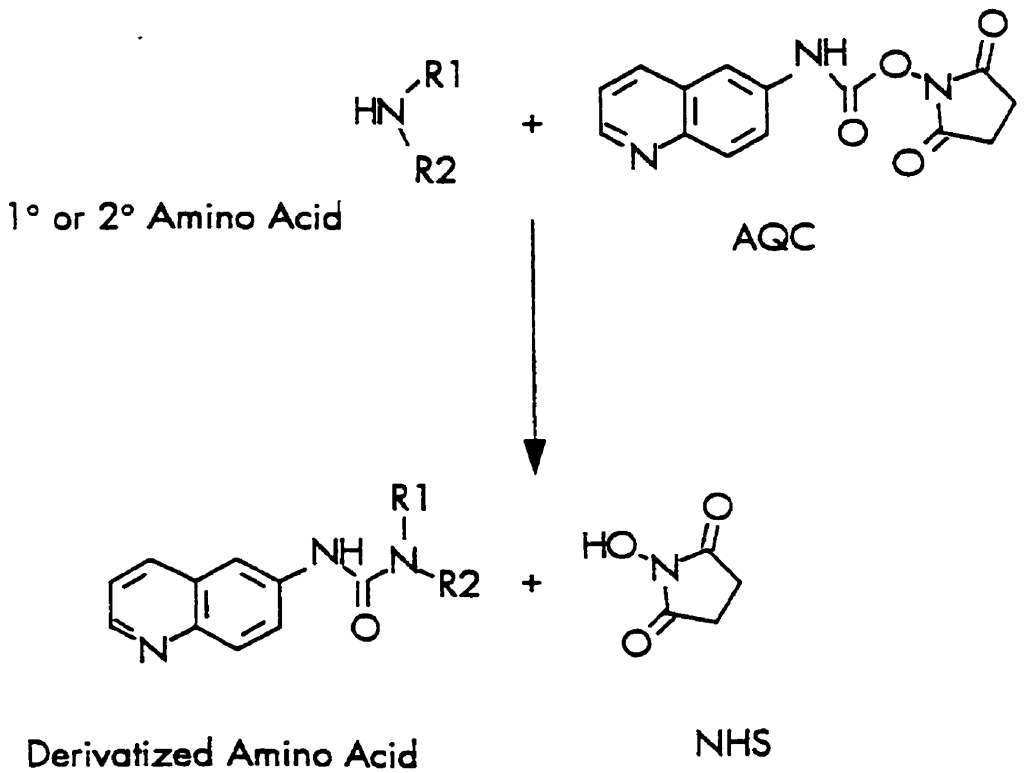
3.1. Measurement of ACC by HPLC

In order to be able to test the model described in Chapter 1, a way of measuring ACC in plant tissue was needed. Since all of the available methods for ACC quantification had problems and limitations associated with their use, the Waters AccQ•Tag Method™, designed to measure amino acids, was adapted for ACC analysis. This procedure is simple and relatively sensitive. ACC, which is an amino acid, was derivatized with the Waters AccQ•Fluor reagent; the ACC derivatives separated by reversed phase high performance liquid chromatography, HPLC, and quantitated by fluorescence.

3.1.1. Derivatization of ACC

A procedure, based on the Waters AccQ•Tag Method™ of amino acid analysis, was used for the quantification of ACC in plant tissues, and seed exudates and extracts. The Waters AccQ•Fluor reagent, AQC, (6-amino-quinolyl-N-hydroxysuccinimidyl carbamate) is a member of a new class of amine-derivatizing compound that reacts with primary and secondary amino acids to yield stable ureas that fluoresce strongly at 395 nm. ACC was coupled with the Waters AccQ•Fluor reagent (Fig. 6); the ACC derivatives were separated on an AccQ•Tag column, a high efficiency C18 column, by means of reversed phase HPLC, and

Fig. 6. Derivatization of an amino acid. The reaction between an amino acid and Waters AccQ•Fluor reagent, AQC, yields a stable urea and NHS. Abbreviations: AQC, 6-aminoquinolyl-N-hydroxysuccinimidyl carbamate; NHS, N-hydroxysuccinimide


R1
HN
R2
+
NH
O
O
N
O
O
N
1° or 2° Amino Acid
AQC
R1
NH
N
R2
O
N
+
HO
N
O
O
Derivatized Amino Acid
NHS

quantified by fluorescence (Fig. 7a). Other AccQ•Tag-labelled amino acids including α-, β-, γ- aminobutyric acid, tyrosine, valine and methionine were also separated on the AccQ•Tag column by this procedure (7b). In a slower reaction, excess reagent hydrolyzes to produce AMQ (6-aminoquinoline), NHS (N-hydroxysuccininimde) and carbon dioxide. The destruction of excess reagent is complete within a minute. AMQ, the major hydrolysis product fluoresces weakly at 395 nm and produces a peak that is easily resolved chromatographically on a C18 column at approximately 5.9-6.0 minutes (Fig. 7a). The other byproducts, NHS and carbon dioxide, do not appear to interfere with the amino acid analysis.

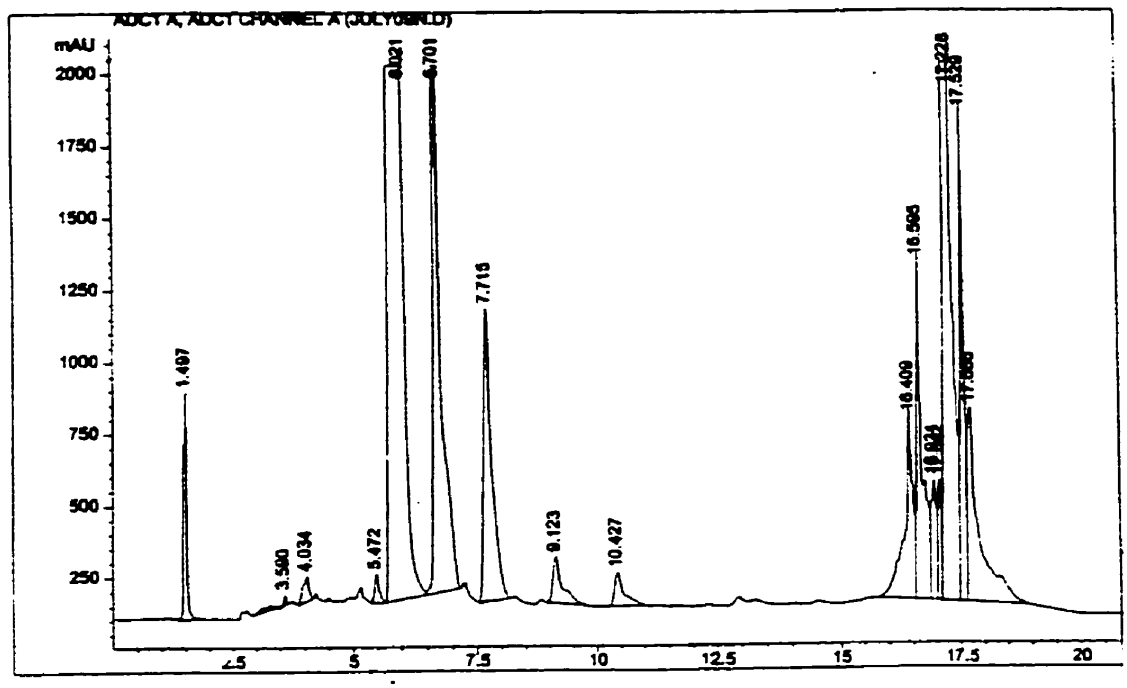
3.1.2. Separation of ACC

The separation of ACC from other AccQ•Tag-labelled amino acids (Fig. 8) was enhanced by modification of the gradient recommended by Waters Chromatography, Inc. (Fig. 9). Amino acids that normally eluted in the first 21 minutes of the Water's gradient, were compressed into 6.5 minutes, and amino acids including ACC, that eluted in the next 5 minutes of the Water's gradient, were instead spread out over 7 minutes. The length of time for elution of the remaining amino acids, and column washing and conditioning, was decreased from 19 to 9 minutes. Normally, the column was being washed and

Fig. 7a. Chromatogram of a standard solution of ACC. Fifteen pmoles of AccQ•Tag-labelled ACC were injected into an AccQ•Tag column, detected and measured by using a modified Waters gradient with a Hewlett Packard HPLC system (1050 Series Quaternary Pump and a Programmable Fluoresence Detector), and analyzed with Hewlett Packard's ChemStation computer software. Peaks occur at 6.021 minutes, AMQ; 6.701 minutes, NH_3; 7.715 minutes, ACC; 16.409 – 17.686 minutes, elements stripped from column.

Fig. 7b. Chromatogram of a standard solution of ACC and amino acids. Twenty-five pmoles of AccQ•Tag-labelled amino acids were measured as in Fig. 7a. Peaks occur at 7.672 minutes, ACC; 8.235 minutes, α-aminobutyric acid (ABA); 8.673 minutes, β-ABA; 9.259 minutes, γ-ABA; 12.612 minutes, tyrosine; 14.657 minutes, valine; 15.470 minutes, methionine.

a.


mAU
2000
1750
1500
1250
1000
750
500
250
1.497
3.590
4.034
5.472
7.715
9.123
10.427
16.409
16.595
2.5
5
7.5
10
12.5
15
17.5
20

b.

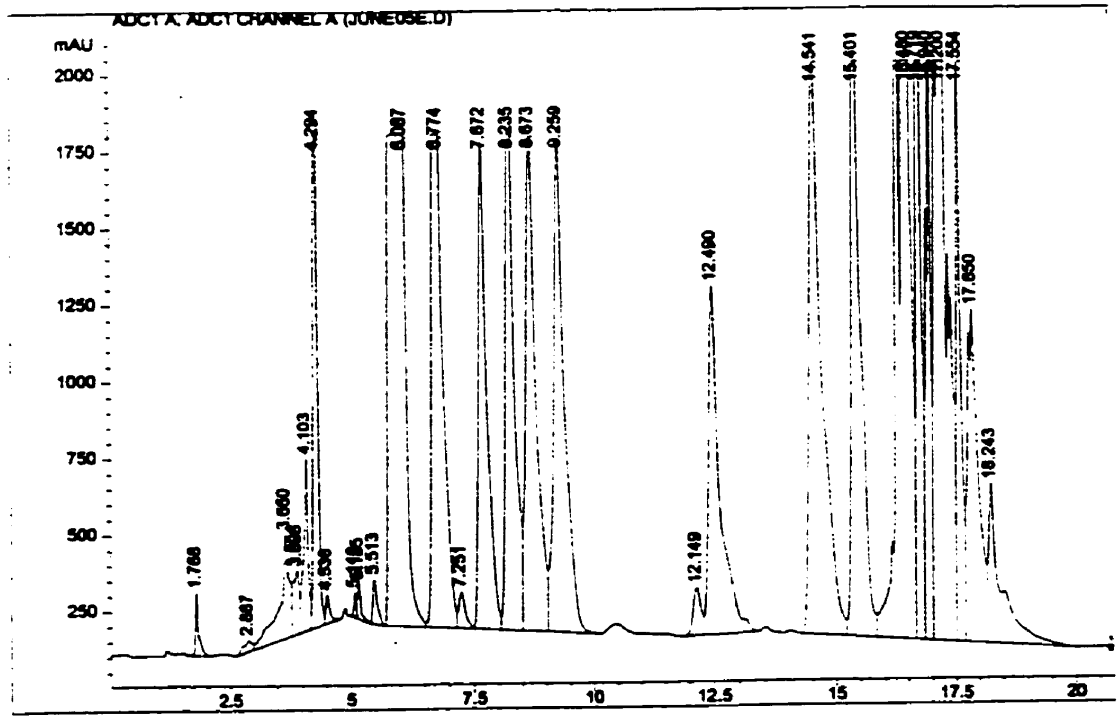
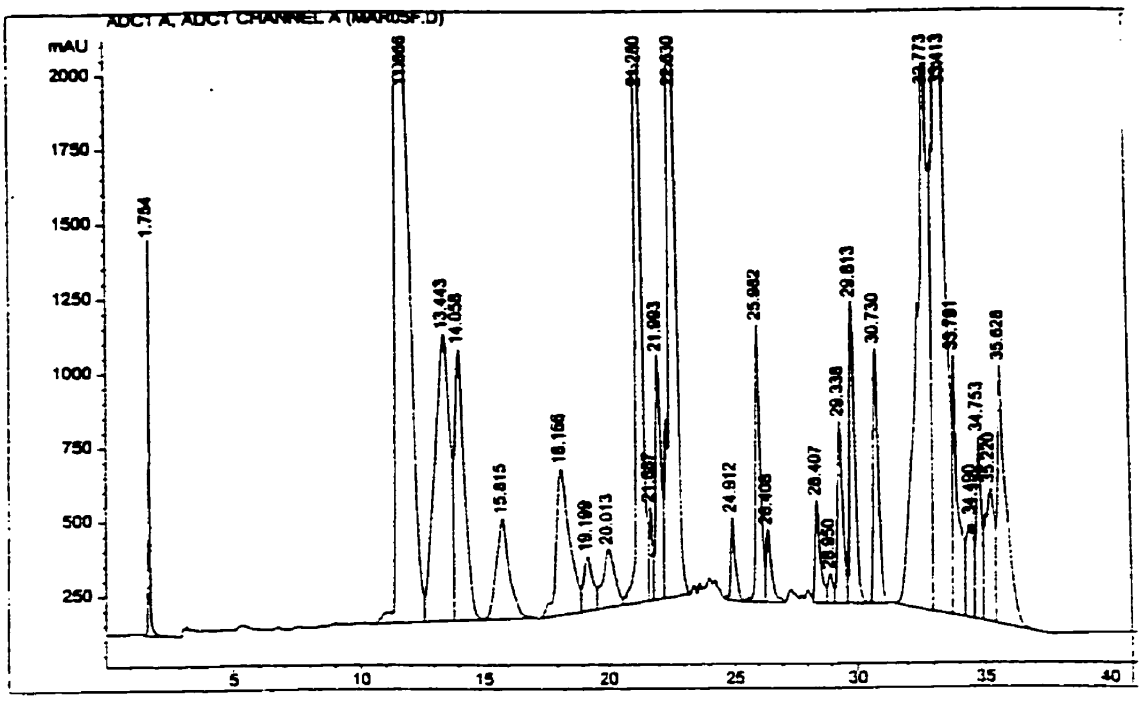

mAU
2000
1750
1500
1250
1000
750
500
250
1.766
2.887
4.294
5.513
6.087
6.774
7.672
8.235
8.673
9.259
12.149
12.490
14.541
15.401
17.554
17.850
18.243
2.5
5
7.5
10
12.5
15
17.5
20

Fig. 8a. Chromatogram of amino acids present in an extract of tissue harvested from canola seeds one hour after incubation at 20°C; prior to incubation, the seeds had been treated with *Enterobacter cloacae* CAL3 for one hour and then rinsed with sterile water. The extract was diluted 1:25 and the AccQ•Tag-labelled amino acids were separated by using Water's recommended gradient.

Fig. 8b. Chromatogram of amino acids present in the same canola seed extract as Fig. 8a with the addition of 10 pmoles of ACC. The ACC peak eluted at 22.182 minutes.

a.


ADCT A, ADCT CHANNEL A (MAR05F.D)
mAU
2000
1750
1500
1250
1000
750
500
250
1.784
13.443
14.058
15.815
18.166
19.199
20.013
21.280
21.983
22.630
24.912
25.982
28.407
28.813
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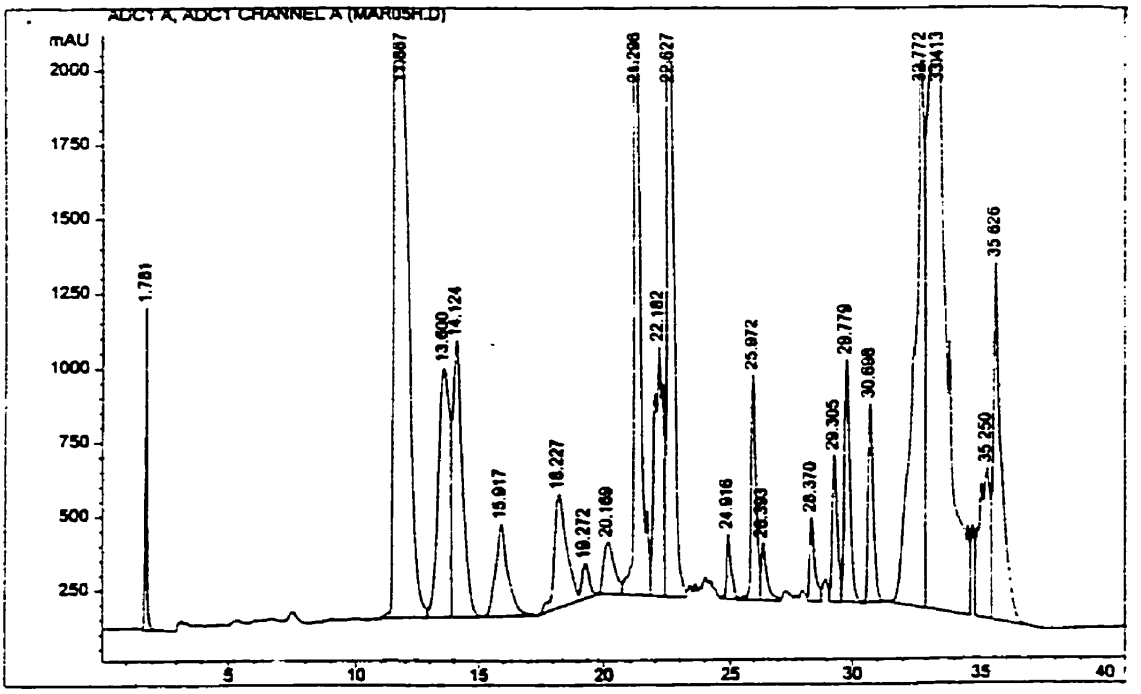
34.753
35.628
5
10
15
20
25
30
35
40

b.


ADCT A, ADCT CHANNEL A (MAR05H.D)
mAU
2000
1750
1500
1250
1000
750
500
250
1.781
13.600
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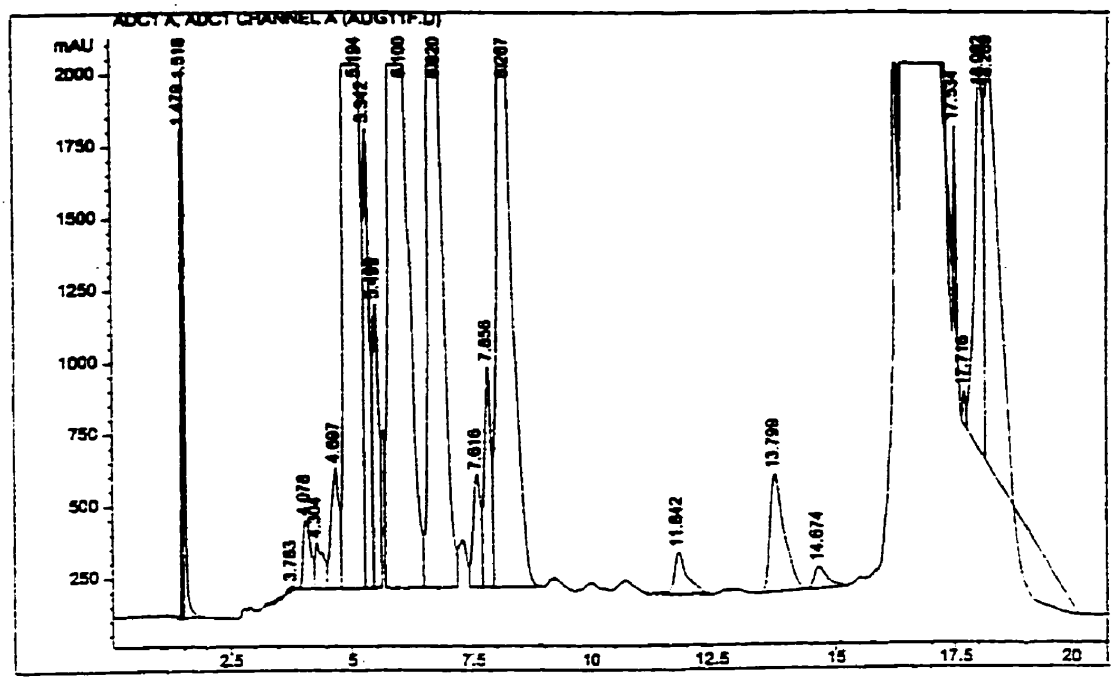
15.917
18.227
19.272
20.169
21.296
22.162
22.627
24.916
25.972
28.370
29.305
29.779
30.698
33.772
33.413
35.250
35.626
5
10
15
20
25
30
35
40

Fig. 9a. Chromatogram of amino acids present in an extract of tissue harvested from canola seeds one hour after incubation at 20°C; prior to incubation, the seeds had been treated with *Enterobacter cloacae* CAL3 for one hour and then rinsed with sterile water. The extract was diluted 1:25 and the AccQ•Tag-labelled amino acids were separated by using a modification of Water's recommended gradient.

Fig. 9b. Chromatogram of amino acids present in the canola seed extract seen in Fig. 9a with the addition of 10 pmoles of ACC. The ACC peak eluted at 7.606 minutes.

a.


ADCT A, ADCT CHANNEL A (AUG11F.D)
mAU
2000
1750
1500
1250
1000
750
500
250
3.783
4.078
4.304
4.697
7.856
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13.799
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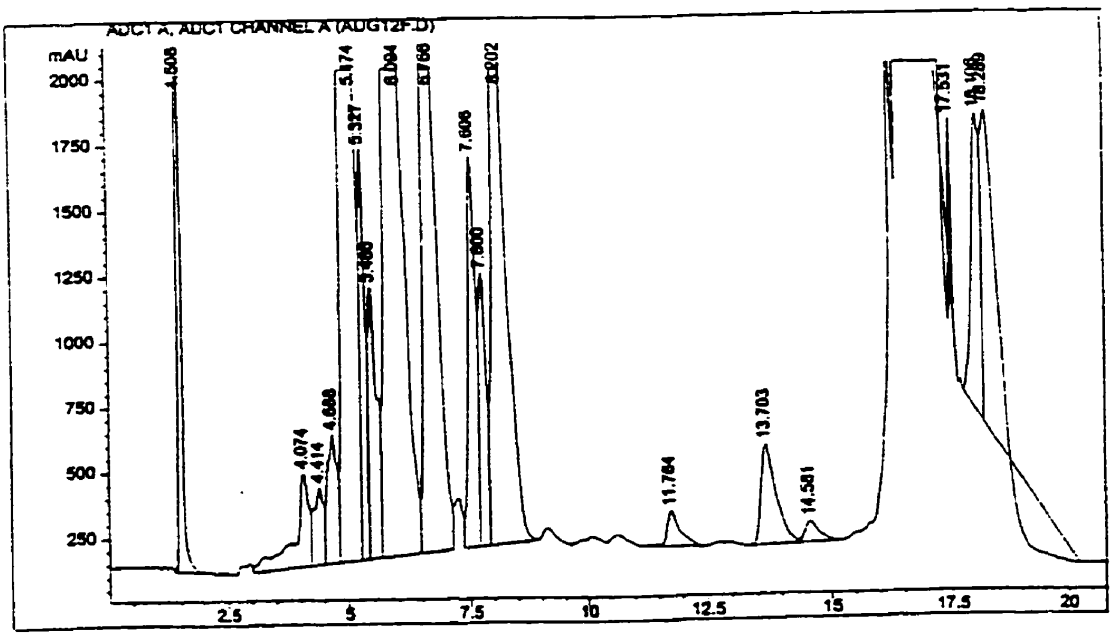
7.5
10
12.5
15
17.5
20

b.


ADCT A, ADCT CHANNEL A (AUG12F.D)
mAU
2000
1750
1500
1250
1000
750
500
250
4.074
4.414
4.688
5.327
5.174
7.606
7.800
11.784
13.703
14.581
17.531
2.5
5
7.5
10
12.5
15
17.5
20

reconditioned for the next sample during the last 12 minutes of the Water's gradient, which had a total run time of 45 minutes. Modification of the Water's gradient resulted in a reduction in the total run time from 45 to 23 minutes and improved separation of the ACC peak from the surrounding peaks.

3.1.3. Standards

Standards were used to account for differences in conditions from run to run, and from day to day. Standard solutions containing either 12.5 pmoles of each of ACC and α-aminobutyric acid or 12.5 pmoles of each of ACC, α-, β-, and γ-aminobutyric acid, which are eluted at approximately 7.6, 8.2, 8.7 and 9.2 minutes respectively, were run daily, in duplicate. In addition to these standards, the by-products of the derivatization process, AMQ and NH_3, eluted at the same time during each run, at approximately 6.0 and 6.7 minutes respectively.

3.1.4. Quantification of ACC

The amount of ACC in samples was quantified by using an ACC standard curve that is linear between 1 and 25 pmoles of ACC per sample. The ACC standard curve was prepared from a fresh stock solution of ACC (0.1 mM) diluted with sterile distilled water to yield between 1 and 25 pmol of ACC per 20-μL injection. The ACC dilutions were derivatized, and following injection, all were eluted at

approximately 7.6 minutes. The peak areas of increasing ACC concentrations were used to generate a standard curve (Fig. 10).

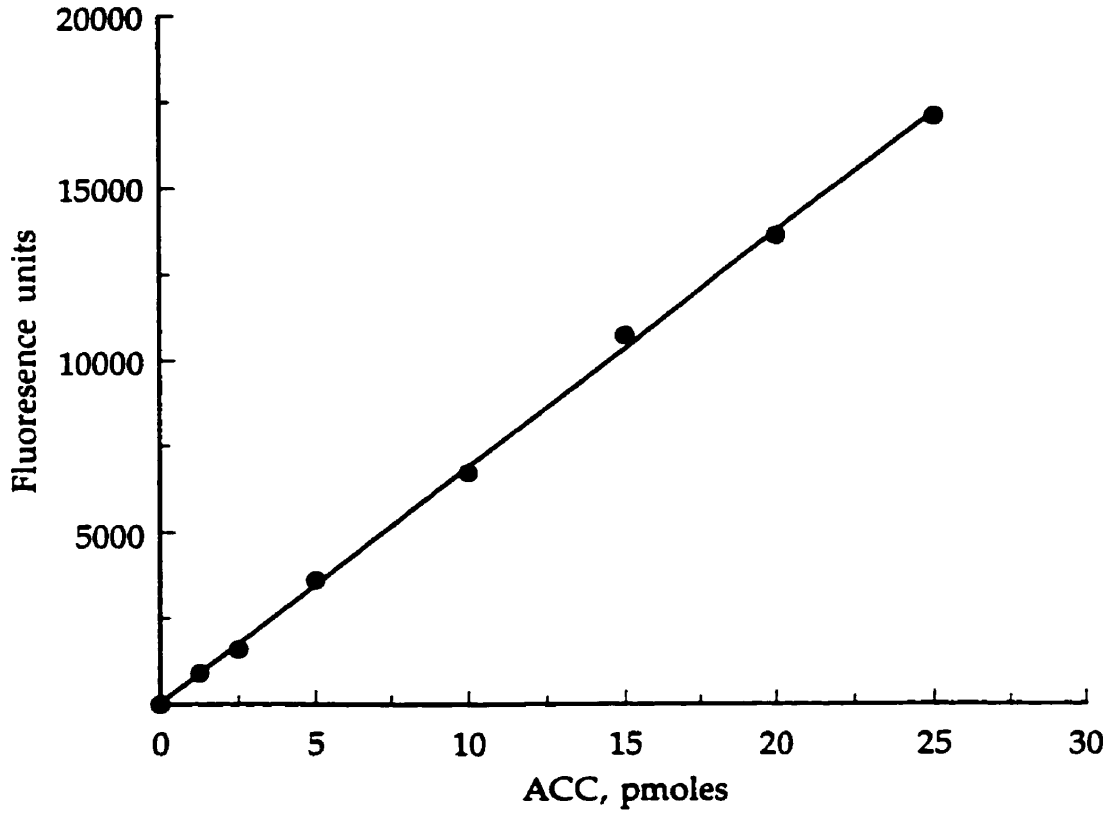
3.2.1. Stability of ACC in solution at 4°C and –20°C

The stability of ACC was assessed at various temperatures including 4°C and –20°C. An ACC stock solution was diluted ten-fold (16 µmoles/L), aliquoted and stored overnight at either 4°C or –20°C. The following morning, one of the aliquots kept at 4°C was further diluted, derivatized and the peak area of the resultant 32-pmole ACC sample was measured; the area of the ACC peak decreased by approximately 5%. After two nights at 4°C a further decrease in peak area was noted and following three nights at this temperature, the chromatogram of the sample showed several new peaks suggesting breakdown of ACC.

Aliquots of the ACC solution (16 µmoles/L) were frozen overnight at –20°C, diluted and the quantity of ACC was measured. The peak area of the 32-pmole ACC sample decreased by approximately 2%, slightly less than the drop seen at 4°C. When aliquots of this ACC stock solution were measured after 1 week, 1 month and 2 months at –20°C, the peak area of the ACC had decreased no more than 10%. However, ACC stored at a concentration lower than 2 pmoles/µL, displayed a drop in peak area of up to 30% when kept at –20°C for a month.

Fig.10. Standard curve of ACC. ACC is quantified in fluorescence units.

Equation: $y = 21.427 + 687.58x$; $R=0.99953$


20000
15000
10000
5000
Fluoresence units
0
5
10
15
20
25
30
ACC, pmoles

3.2.2. Stability of ACC in solution after freezing and thawing

The amount of ACC in a 32-pmole sample was measured following 1, 2, and 3 freeze-thaw cycles at –20°C (Fig. 11). The chromatograms showed a reduction in peak area of approximately 2.25% for each freeze-thaw cycle although the ACC peaks remained well resolved. In the remainder of this study, standard solutions containing ACC were not subjected to more than one freeze thaw cycle. Routinely, solutions of ACC, ACC and α-aminobutyric acid, and ACC, α-, β-, and γ-aminobutyric acid, used as daily standards, were prepared fresh, diluted to produce 12.5 pmoles/20 μL injection and frozen at –20°C; as needed, these samples were thawed, derivatized and injected.

3.2.3. Stability of AccQ•Tag-labelled ACC

The Water's AccQ•Tag Method is dependent on a derivatizing reagent developed specifically for amino acid analysis. It is claimed that standards and samples, once derivatized, are stable for up to 7 days at room temperature. The stability of samples of AccQ•Tag labelled ACC was monitored over a 26-day period (Fig. 12). A number of samples of a standard ACC solution were derivatized with Water's AccQ•Fluor reagent, covered with parafilm and stored at room temperature for up to 26 days. The amount of ACC in the AccQ•Tag-labelled ACC samples was measured after one, four, six and twenty-six days under these conditions. After 4 days, the stability of the derivatized ACC appeared

Fig. 11. Stability of ACC in 32-pmole sample after freezing and thawing. The amount of ACC recovered after one, two and three freeze-thaw cycles at –20°C, expressed as a percentage of the amount of ACC in a fresh solution. The error bars represent standard errors of the means.

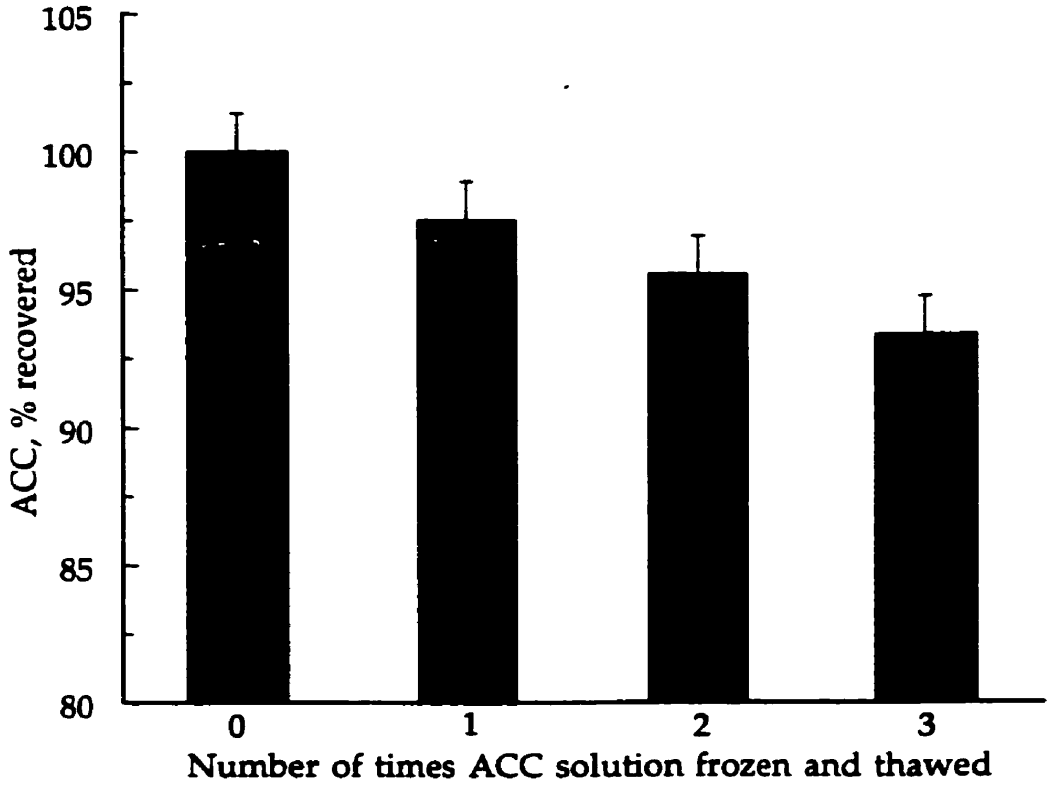
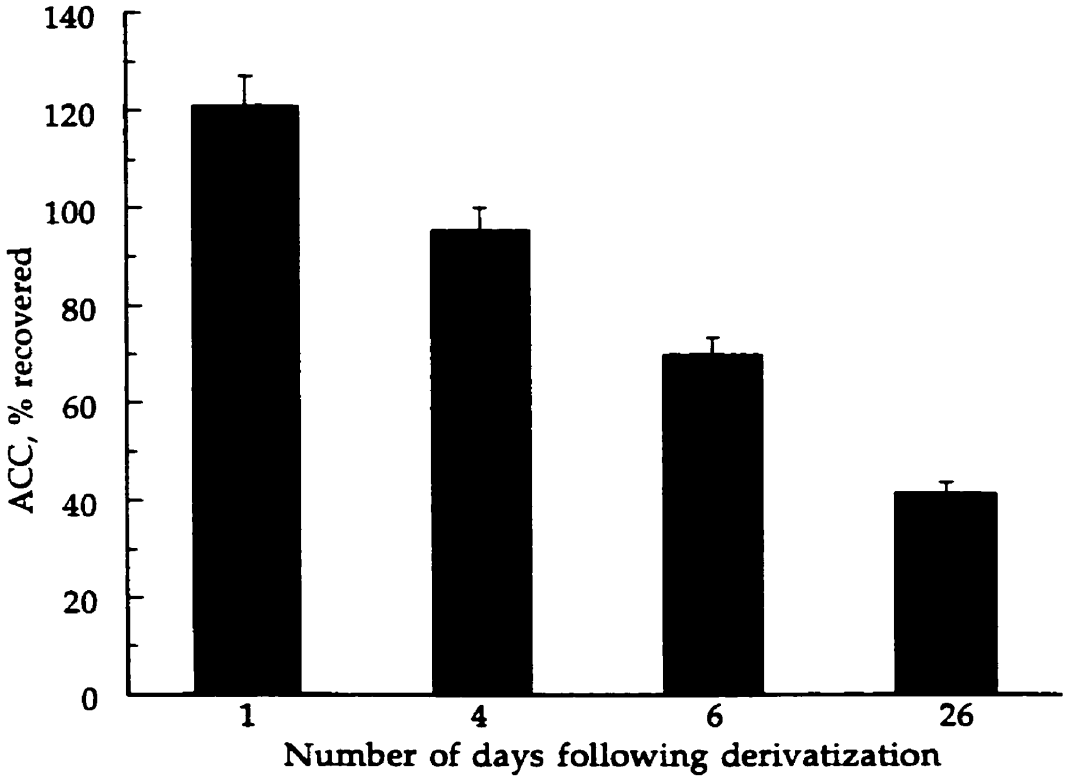

105
100
95
90
85
80
ACC, % recovered
0
1
2
3
Number of times ACC solution frozen and thawed

Fig. 12. Stability of AccQ•Tag-labelled ACC. The amount of ACC recovered from samples of derivatized ACC after one, four, six and twenty-six days at room temperature, expressed as a percentage of the amount of ACC in a fresh solution. The error bars represent standard errors of the means.


140
120
100
80
60
40
20
0
ACC, % recovered
1
4
6
26
Number of days following derivatization

to drop. At 6 days the amount of ACC recovered was 69.9%, athough the high standard error suggests that this number may be low, and by 26 days the ACC content had dropped to 41.6% of the amount measured immediately following derivatization and the chromatogram of the sample also showed several new peaks suggesting breakdown of ACC. In the remainder of this study, all samples were analyzed within one day of being derivatized.

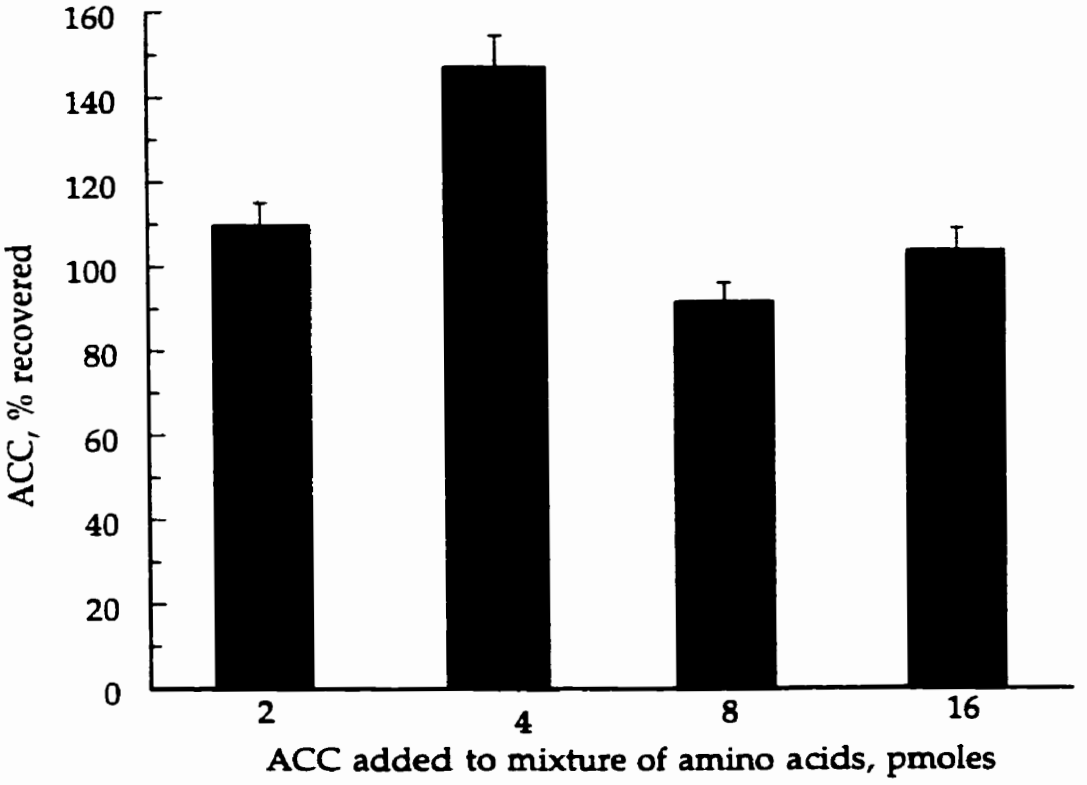
3.3.1. Recovery of ACC added to a standard mixture of amino acids

The recovery of a range of ACC concentrations added to a standard mixture of amino acids was examined. Four concentrations of ACC (2, 4, 8, and 16 pmoles) were added separately to a mixture of amino acid hydrolysates containing 25 pmol of each of 17 hydrolyzed amino acids except cysteine which was 12.5 pmoles. Following derivatization, ACC was eluted along with the amino acids from the AccQ•Tag column at approximately 7.6 minutes. When the ACC peak areas for each of the four concentrations were measured, it was found that between 91.6% and 147.2 % of the ACC was recovered (Fig. 13).

3.3.2. Recovery of ACC added to canola seed exudate

The recovery of ACC added to canola seed exudate was examined. Canola seed exudate was collected from 200 seed-samples. These samples had been treated for one hour with 0.03M $MgSO_4$, rinsed

Fig. 13. Recovery of ACC added to a mixture of amino acid hydrolysates. The amount of ACC recovered from a standard mixture of amino acids containing the addition of 2, 4, 8 or 16 pmoles of ACC, was expressed as a percentage of the amount of ACC added to the mixture. The error bars represent standard errors of the means.


160
140
120
100
80
60
40
20
0
ACC, % recovered
2
4
8
16
ACC added to mixture of amino acids, pmoles

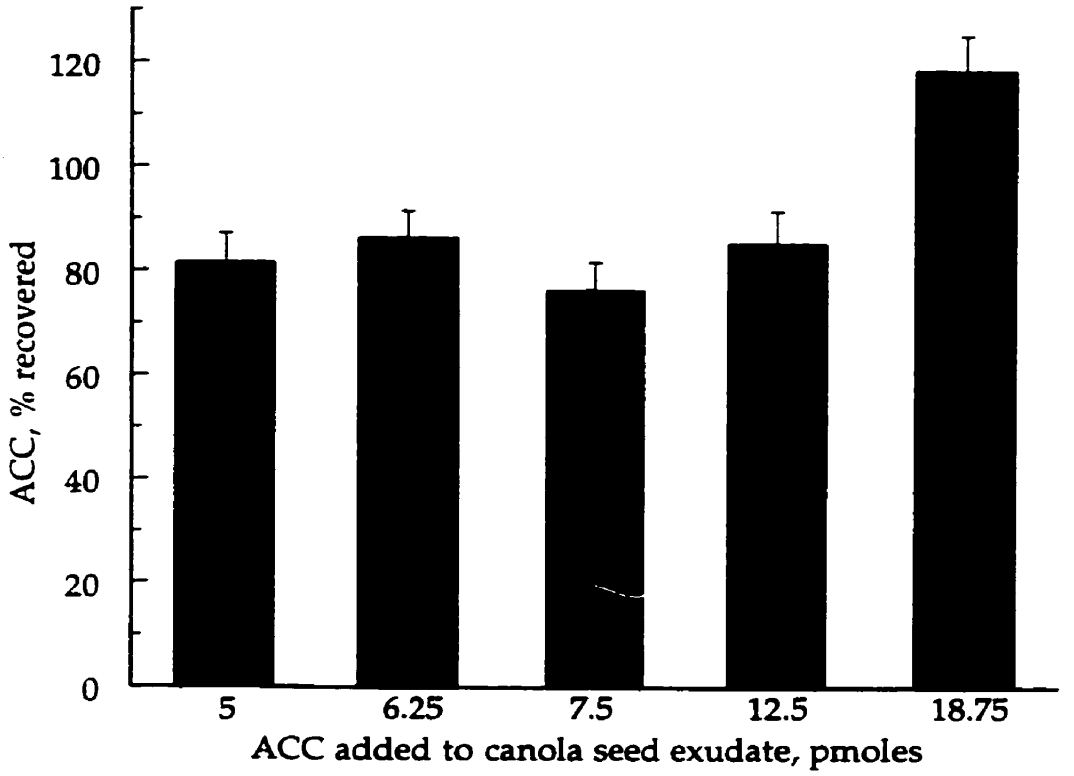
and then transferred to nylon cell strainers set in small petri dishes, to which one mL of sterile distilled water had been added. The samples were incubated in the dark at 20°C for 5 hours, at which time the seed exudate was collected, filtered and frozen.

Five concentrations of ACC (5, 6.25, 7.5, 12.5 and 18.75 pmoles/20-µL injection) were added separately to canola seed exudate. Following derivatization, ACC, along with the other amino acids found in the exudate samples, was eluted from the AccQ•Tag column at approximately 7.6 minutes. When the amount of ACC in the peak areas for each of the five concentrations was measured and compared to the amount added, it was found that between 76.3% and 117.0% of the ACC was recovered (Fig. 14).

3.3.3. Recovery of ACC added to canola seed extract

ACC was added to extracts of canola seed tissue made from the 200-seed samples from which the canola seed exudate had been collected. These samples had been treated for one hour with *Enterobacter cloacae* CAL3 suspended in 0.03M $MgSO_4$ (absorbance of 0.15 at 600 nm). After one hour at 20°C in the dark, the seeds were removed and frozen; the frozen seeds were later pulverized, centrifuged and the resulting supernatants collected and frozen.

Three concentrations of ACC (7.5, 10 and 12.5 pmoles/20-µL injection) were added separately to diluted samples of canola seed


120
100
80
60
40
20
0
ACC, % recovered
5
6.25
7.5
12.5
18.75
ACC added to canola seed exudate, pmoles

extracts and derivatized. The AccQ•Tag-labelled amino acids present in the extracts, including ACC, were eluted from the AccQ•Tag column; ACC was eluted at approximately 7.6 minutes. The amount of ACC in the peak areas for each of the three concentrations was between 96.2% and 110.7% of the amount added to the extract samples (Fig. 15).

3.4.1. Root and shoot lengths of canola seedlings

In earlier work in our lab, a root elongation assay was used to identify and assess the growth-promoting capabilities of bacteria such as *Enterobacter cloacae* CAL3 (Glick et al., 1995). In the present study, the extent of root and shoot elongation, of 4.5-day old plants from seeds inoculated with various treatments, was determined by this assay. The roots and shoots of 4.5-day old canola plants, grown from seeds treated with 0.03M $MgSO_4$, or one of these bacteria: *Enterobacter cloacae* CAL3, *Pseudomonas putida* pRK415, or *Pseudomonas putida* pRKACC, suspended in 0.03M $MgSO_4$ at an absorbance of 0.15 at 600 nm, were measured, excised and frozen at –80°C.

In Fig. 16, it is evident that the shortest roots were seen in the control plants from seeds treated with 0.03M $MgSO_4$ and from seeds inoculated with a *Pseudomonas* strain that does not have an ACC deaminase gene. Roots from seeds treated with *Enterobacter cloacae* CAL3 and a *Pseudomonas* strain transformed with a broad host range plasmid carrying the gene for ACC deaminase, *Pseudomonas putida*

Fig. 15. Recovery of ACC added to canola seed extracts. The amount of ACC recovered from extracts of canola seeds, treated with *Enterobacter cloacae* CAL3 and incubated at 20°C for one hour, that contained the addition of 7.5, 10 or 12.5 pmoles of ACC. The amount of ACC recovered was expressed as a percentage of the amount of ACC added to the mixture. The error bars represent standard errors of the means.

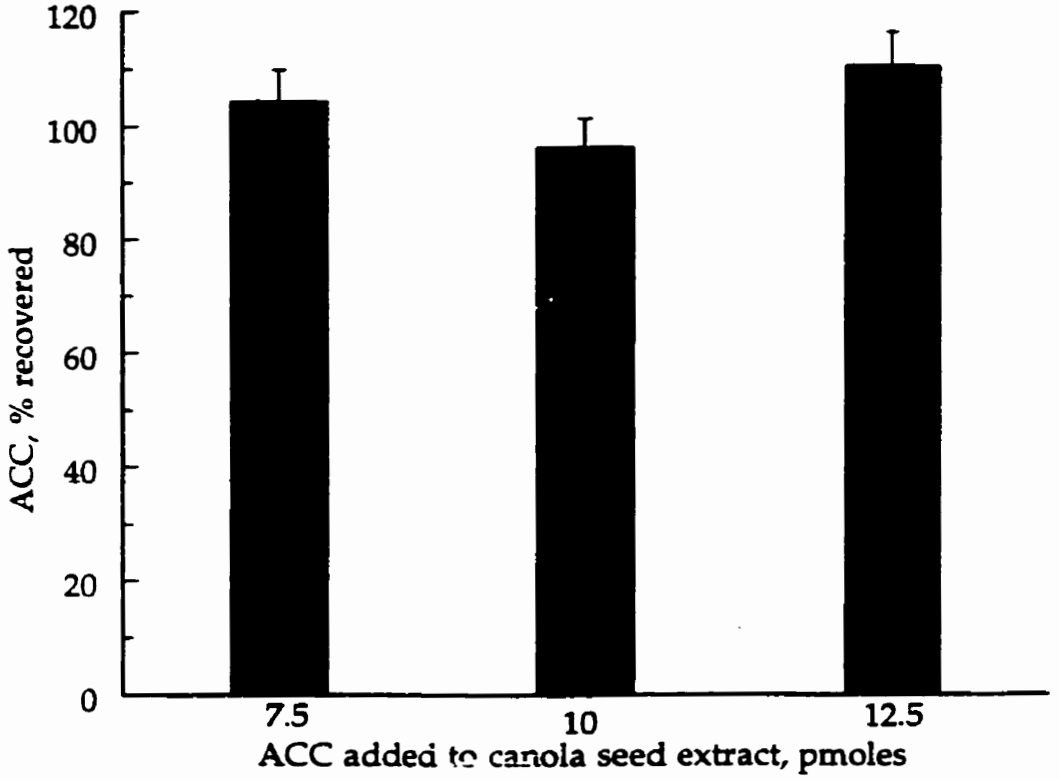
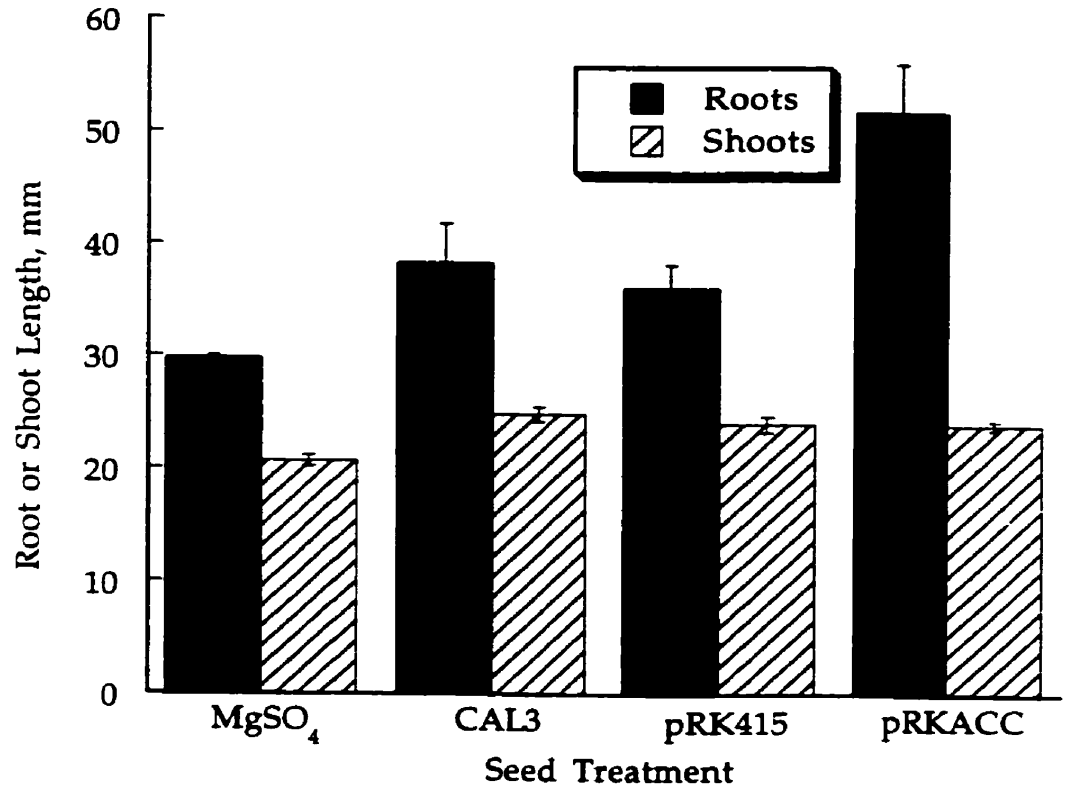

120
100
80
60
40
20
0
ACC, % recovered
7.5
10
12.5
ACC added to canola seed extract, pmoles

Fig. 16. Root and shoot length of canola seedlings. Mean root and shoot lengths of 4.5-day old canola seedlings assessed by means of the root elongation assay from measurements of 60 seedlings (6 seeds/growth pouch; 10 growth pouches/repetition; 2 repetitions). Seed treatment abbreviations: $MgSO_4$, 0.03M $MgSO_4$; CAL3, *Enterobacter cloacae* CAL3, pRK415, *Pseudomonas putida* pRK415; pRKACC, *Pseudomonas putida* pRKACC. The error bars represent standard errors of the means.


60
50
40
30
20
10
0
Root or Shoot Length, mm
Roots
Shoots
MgSO4
CAL3
pRK415
pRKACC
Seed Treatment

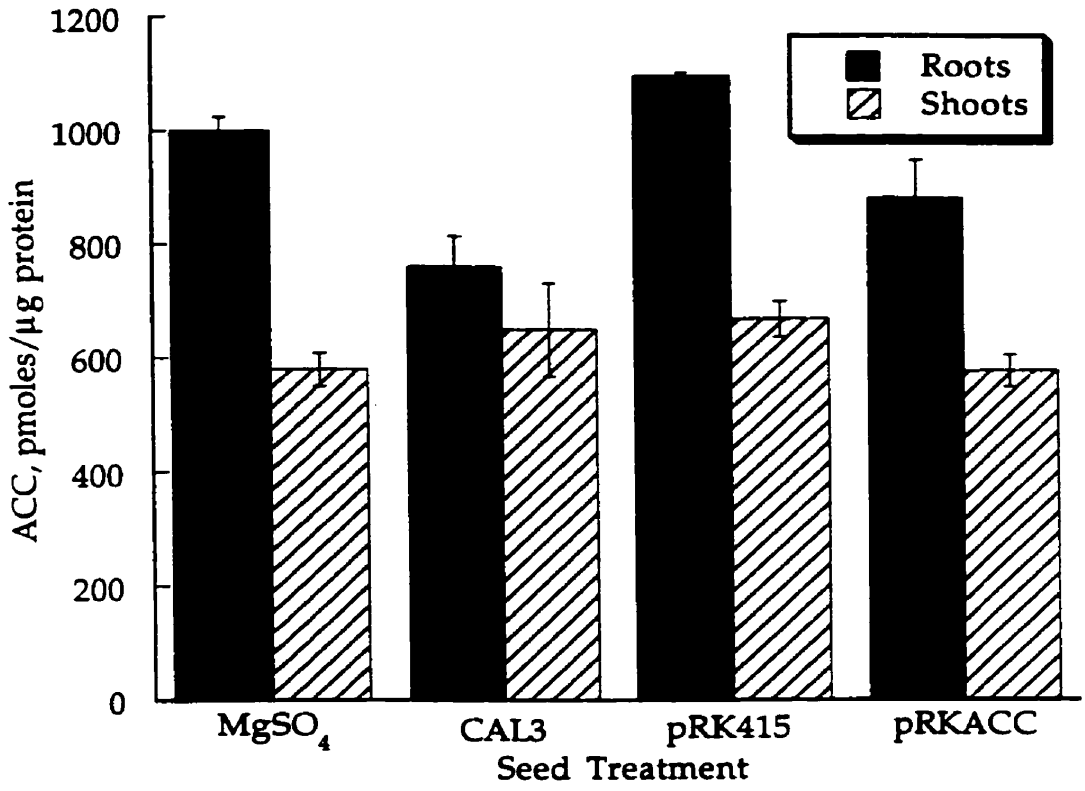
pRKACC, are 28.4% (P value of 0.0001) and 73.8% longer (P value of <0.0001), respectively, than roots from the control plants treated with $MgSO_4$.

Athough the shortest shoots were found in the control seedlings, i.e., from seeds treated with $MgSO_4$, there was very little difference between the shoot lengths of the bacteria-treated plants. The shoots from seeds treated with *Enterobacter cloacae* CAL3, pRK415 and pRKACC were 20.2%, 16.0% and 15.9 % longer respectively than shoots of the control seedlings.

3.4.2. ACC content of canola seedlings

Extracts were made from the roots and shoots of the 4.5-day old canola seedlings grown for the root/shoot elongation assay and the quantity of ACC in these extracts was measured. The roots and shoots, excised and frozen after the elongation assay, were subsequently pulverized, centrifuged and the resulting supernatants collected and frozen. The amount of ACC was measured in these frozen samples after they were thawed once, diluted, and derivatized (Fig. 17). Duplicate measurements were made of extracts containing 60 seedlings/repetition and two repetitions were carried out. The highest level of ACC was seen in the roots of the control seedlings grown from seed treated with *Pseudomonas putida* pRK415. Lower levels of ACC were present in roots

Fig. 17. ACC content of canola seedlings. The amount of ACC measured in the roots and shoots of 4.5-day old canola seedlings grown for the root elongation assay. Seed treatment abbreviations: $MgSO_4$, 0.03M $MgSO_4$; CAL3, *Enterobacter cloacae* CAL3; pRK415, *Pseudomonas putida* pRK415; pRKACC, *Pseudomonas putida* pRKACC. Error bars represent standard errors of the means.


1200
1000
800
600
400
200
0
ACC, pmoles/µg protein
Roots
Shoots
MgSO4
CAL3
pRK415
pRKACC
Seed Treatment

from seeds treated with the plant growth-promoting strain, *Enterobacter cloacae* CAL3, or the *Pseudomonas* strain containing ACC deaminase, on the plasmid, pRKACC. There was a reduction of 31.9% (P value of 0.0008) and 19.2.% (P value of 0.0183) in the level of ACC in roots from seeds treated with *Enterobacter cloacae* CAL3 and *Pseudomonas putida* pRKACC respectively, when compared to the quantity of ACC in the roots of the control seedlings treated with *Pseudomonas putida* pRK415.

The ACC content of the shoots showed a somewhat different pattern than the roots. The lowest levels of ACC were found in the shoots of seedlings treated with *Pseudomonas putida* pRKACC. Shoots from plants treated with 0.03M $MgSO_4$, *Enterobacter cloacae* CAL3 or *Pseudomonas putida* pRK415 were somewhat higher.

3.5. Root elongation and ACC content of canola seedlings under conditions of high and low ACC deaminase activity in bacterial inoculants

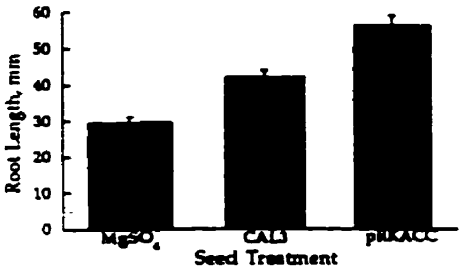
The results of two experiments are shown in Fig. 18. In both experiments 4.5-day old canola plants were grown for a root elongation assay, under the same conditions and from seeds inoculated with the same treatments as those described in section 3.4.1, root and shoot lengths of canola seedlings. The extent of root elongation of the canola seedlings was determined as described in section 3.4.1 and the ACC content was measured in extracts of the excised roots as described in

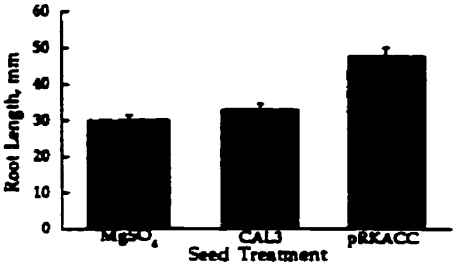
Fig. 18a. Root length of canola seedlings. Mean root length of 4.5-day old canola seedlings was assessed by means of the root elongation assay in Expt 1 (left side) and Expt 2 (right side). Measurements were made of 60 seedlings (6 seedlings/growth pouch; 10 growth pouches). Seed treatment abbreviations: $MgSO_4$, 0.03M $MgSO_4$; CAL3, *Enterobacter cloacae* CAL3; pRKACC, *Pseudomonas putida* pRKACC

Fig. 18b. ACC content of canola seedlings. The amount of ACC measured in the roots 4.5-day old canola seedlings grown for the root elongation assay in Expt 1 (left side) and Expt 2 (right side). Measurements were made in duplicate of extracts containing 60 seedlings.

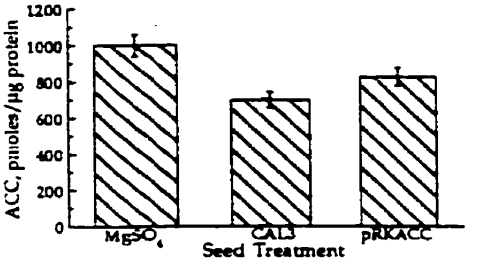
Fig. 18c. ACC deaminase activity of bacterial treatments. ACC deaminase activity of cultures of *Enterobacter cloacae* CAL3 and *Pseudomonas putida* pRKACC used as bacterial seed treatments in Expt 1 (left side) and Expt 2 (right side). Measurements were made in triplicate.

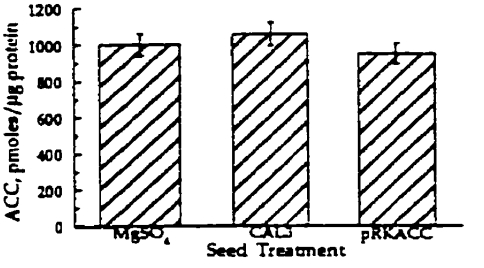
a.


Root Length, mm
0
10
20
30
40
50
60
MgSO4
CAL3
pRKACC
Seed Treatment

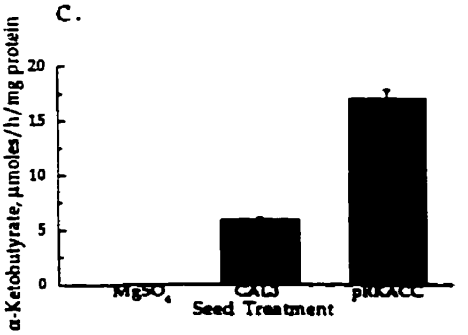

Root Length, mm
0
10
20
30
40
50
60
MgSO4
CAL3
pRKACC
Seed Treatment

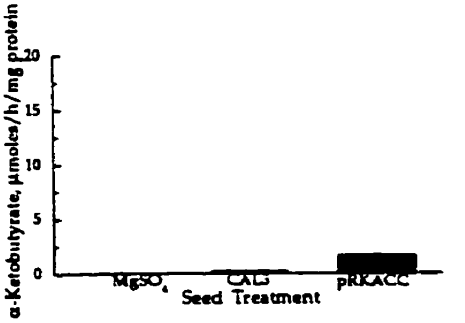
b.


ACC, pmoles/µg protein
0
200
400
600
800
1000
1200
MgSO4
CAL3
pRKACC
Seed Treatment


ACC, pmoles/µg protein
0
200
400
600
800
1000
1200
MgSO4
CAL3
pRKACC
Seed Treatment

c.


α-Ketobutyrate, µmoles/h/mg protein
0
5
10
15
20
MgSO4
CAL3
pRKACC
Seed Treatment

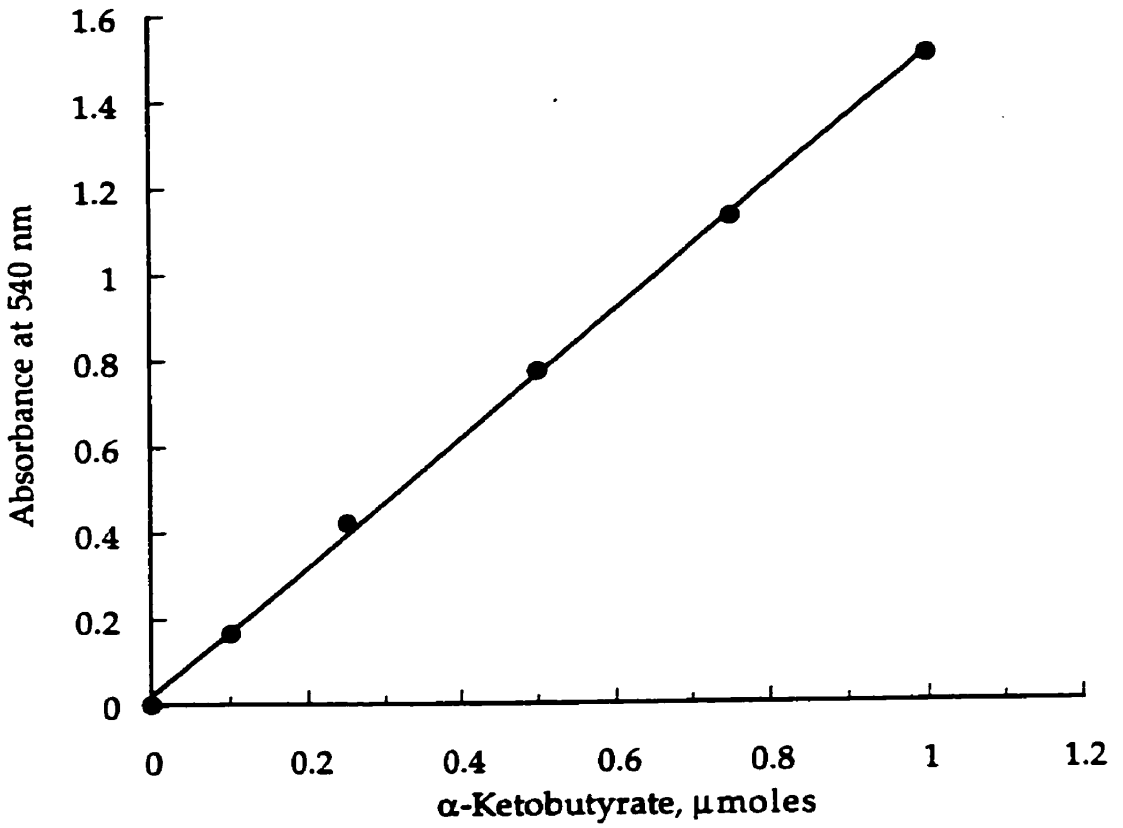

α-Ketobutyrate, µmoles/h/mg protein
0
5
10
15
20
MgSO4
CAL3
pRKACC
Seed Treatment

section 3.4.2, ACC content of canola seedlings. The two experiments were identical with the exception of the ACC deaminase activities of the bacterial seed treatments. ACC deaminase activity was determined by using a modification of the method of Honma and Shimomura (1978) which measures the amount of α-ketobutyrate produced by the enzyme. The number of μmoles of α-ketobutyrate produced by ACC deaminase was determined by comparing the absorbance at 540 nm to a standard curve of α-ketobutyrate (Fig. 19). The enzyme activities of *Enterobacter cloacae* CAL3 and *Pseudomonas putida* pRKACC were greater by 5.5 and 10.4 times, respectively, in Expt 1 than in Expt 2 (Fig. 18c).

The results of the root elongation assay (Fig. 18a) showed that the lengths of the roots of the control plants—those from seeds treated with $MgSO_4$—were the same for both experiments (29.59 and 30.0 mm). The roots from seeds treated with *Enterobacter cloacae* CAL3 or *Pseudomonas putida* pRKACC were 27.5% longer and 18.0% longer, respectively, in Expt 1 than in Expt 2. The level of ACC in the roots from the seeds treated with $MgSO_4$ was the same in both experiments but differed in the roots from seeds exposed to the bacterial treatments. The roots of seeds treated with *Enterobacter cloacae* CAL3 or *Pseudomonas putida* pRKACC were 34.1% and 13.4% lower, respectively, in Expt 1 than in Expt 2 (Fig. 18b). The difference in ACC deaminase activities of the bacterial seed treatments appeared to affect both the extent of root elongation and ACC content of the canola seedlings: a higher level of

Fig. 19. Standard curve of α-ketobutyrate versus absorbance at 540 nm.

Equation: $y = 0.0205 + 1.4927x$; $R = 0.9996$


1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
Absorbance at 540 nm
0
0.2
0.4
0.6
0.8
1
1.2
α-Ketobutyrate, μmoles

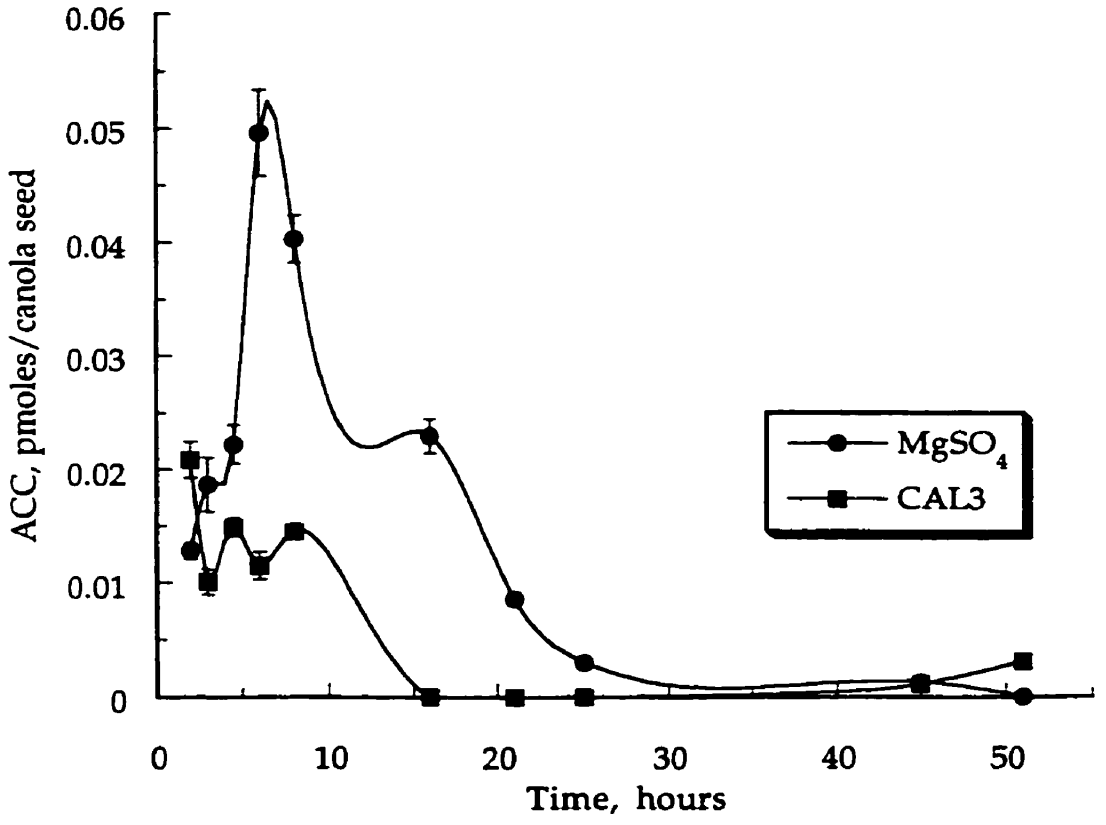
enzyme activity was associated with longer roots and a lower level of ACC.

3.6.1 ACC content in canola seed exudate

Canola seed exudate was collected from 200 seed-samples as described in section 3.3.2. The amount of ACC and other amino acids in these frozen samples was measured after they were thawed once and derivatized (Fig 20). The amount of ACC present in the exudate of canola seeds treated with either *Enterobacter cloacae* CAL3 or $MgSO_4$, was plotted against time intervals that included one hour of seed treatment. A curve through the data points was generated by curve-fitting and may overstate the results. It is possible that a more accurate curve could be generated by joining the data points. Nonetheless, the trend indicated by the fitted curve is consistent with results.

The level of ACC in the exudate collected from the control plants—those grown from seeds treated with 0.03M $MgSO_4$—rose slowly until 3.5 hours following seed treatment, peaked at 5 hours, dropped, peaked again at 15 hours (although a smaller peak than at 5 hours), and slowly decreased to zero at 50 hours. There was more ACC initially—one hour following seed treatment—in the exudate from seeds treated with *Enterobacter cloacae* CAL3 than in the exudate from the control seeds. However, the level of ACC had fallen below that of the control seeds by 2 hours after seed treatment, peaked slightly at 3.5 and

Fig. 20. ACC content of canola seed exudate. The amount of ACC measured in the exudate of canola seeds treated with 0.03M $MgSO_4$ (dark circles) or *Enterobacter cloacae* CAL3 (absorbance of 0.15 at 600 nm) (dark squares) and collected at 1, 2, 3.5, 5, 7, 15, 20, 24, 44 and 50 hours after seed treatment. The points are plotted at time intervals that include one hour of seed treatment. Error bars represent standard errors of the means.


0.06
0.05
0.04
0.03
0.02
0.01
0
ACC, pmoles/canola seed
0
10
20
30
40
50
Time, hours
MgSO4
CAL3

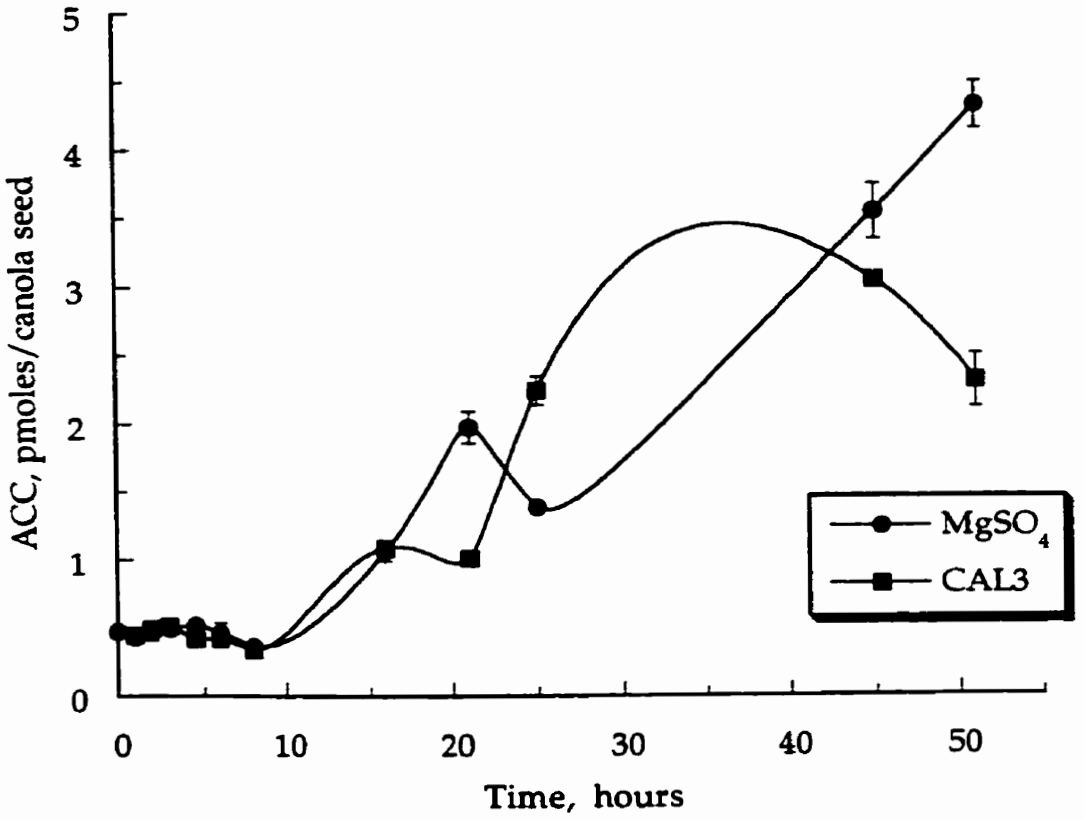
7 hours, and then dropped to zero until 44 hours when it began to increase slightly. Two peaks of ACC were seen in the exudate from both control and bacterium-treated seeds during the first 24 hours following seed treatment and then leveled off. However, the peaks were smaller and fell more rapidly in the exudate from the seed treated with the bacterium, *Enterobacter cloacae* CAL3. It appears that the ACC in the exudate of the bacterium-treated seeds was being hydrolyzed between 2 and 24 hours after seed treatment. There was a slight increase in the ACC level at 44 hours that was maintained up to 50 hours.

3.6.2 ACC content of canola seed extracts

Extracts were made of the canola seed-samples and of 4.5-day old seedlings (described in section 3.6.1). The amount of ACC, measured in these frozen samples after they were thawed once, diluted and derivatized, was plotted against time intervals that included one hour of seed treatment (Fig. 21). Curve-fitting was used to generate curves for the amount of ACC and other amino acids in canola seed extracts. The curve may not be as accurate as possible but a consistent pattern emerged from the curves.

For the first 7 hours following treatment, low levels of ACC were observed in extracts of both $MgSO_4$-treated and *Enterobacter cloacae* CAL3-treated seeds. At this point the amount of ACC began to increase in seeds exposed to both treatments although the ACC peak produced

Fig. 21. ACC content of canola seed extracts. The amount of ACC measured in the extracts of canola seeds treated with 0.03M $MgSO_4$ (dark circles) or *Enterobacter cloacae* CAL3 (absorbance of 0.15 at 600 nm) (dark squares) and collected before treatment and at 0, 1, 2, 3.5, 5, 7, 15, 20, 24, 44 and 50 hours after seed treatment. The points are plotted at time intervals that include one hour of seed treatment. Error bars represent standard errors of the means.


ACC, pmoles/canola seed
5
4
3
2
1
0
0
10
20
30
40
50
Time, hours
MgSO4
CAL3

by the extract from seeds treated with *Enterobacter cloacae* CAL3, occurred earlier and was smaller than the ACC peak in the extract from the control seeds. Another larger peak of ACC was evident in both extracts; however, the peak in the extract of the bacterium–treated seeds, again appeared earlier and contained less ACC than the peak from the $MgSO_4$-treated seeds. Indeed, by 50 hours, the level of ACC in the extract of the control seeds, was increasing just as the amount of the ACC in the extract of the seeds, inoculated with the bacterium, was decreasing.

3.7. Quantification of other amino acids in canola seed exudate and extracts, and in canola seedlings

The levels of some of the amino acids present in the extracts of canola seeds and seedlings and in the exudate of canola seeds were of interest to us. It is possible that amino acids similar in structure to that of ACC, such as α-,β-, and γ-aminobutyric acids, may be derived from ACC. At least one ACC deaminase, isolated by Honma in 1978, is known to be induced by α-aminoisobutyric acid. One of α-,β-, or γ-aminobutyric acids, may induce the enzyme in *Enterobacter cloacae* CAL3, the plant growth-promoting bacterium used in these experiments.

During ethylene biosynthesis SAM is replenished by the methionine cycle (Yang and Hoffman, 1984). Although only a small

amount of SAM, an abundant metabolite in most organisms (Boerjan et al., 1995), is diverted to ethylene synthesis (Yu and Yang, 1979), SAM levels may regulate ethylene production (Fluhr and Mattoo, 1996). The level of methionine during germination may indicate the effect of bacterial ACC deaminase activity on the intermediates of the plant ethylene biosynthetic cycle.

A number of amino acids, including α-, β-and γ-aminobutyric acids, methionine, tyrosine, and valine, were monitored over time, in the exudate and extract of canola seeds, and measured in extracts of roots and shoots of canola seedlings. Canola seed exudate was collected from 200 seed-samples as described in section 3.6.1. The various amino acids were measured in the exudate of canola seeds treated with either *Enterobacter cloacae* CAL3 or $MgSO_4$ after the samples were thawed once and derivatized. The results were plotted against time intervals that included one hour of seed treatment. Extracts were made of the canola seed samples as described in 3.6.2. The levels of the amino acids, present in the extracts of canola seeds treated with either *Enterobacter cloacae* CAL3 or $MgSO_4$, were also plotted against time intervals that included one hour of seed treatment. In some cases, amino acids other than ACC were measured in extracts made from the roots and shoots of 4.5-day old canola seedlings grown for the root elongation assay as described above in 3.4.2. The amino acids in the frozen extracts were

quantified after they were thawed once, diluted, and the amino acids derivatized.

3.7.1. Quantification of α-Aminobutyric acid

The amount of α-aminobutyric acid in samples was quantified by using an α-aminobutyric acid standard curve that is linear between 1 and 25 pmoles of α-aminobutyric acid per sample. The standard curve was prepared from a fresh stock solution of α-aminobutyric acid (0.1 mM) diluted with sterile distilled water to yield between 1 and 25 pmol of α-aminobutyric acid per 20-μL injection. The α-aminobutyric acid dilutions were derivatized, and following injection, all were eluted at approximately 9.4 minutes. The peak areas of increasing α-aminobutyric acid concentrations were used to form a standard curve (Fig. 22).

3.7.2. α-Aminobutyric acid content of canola seed exudate

The level of α-aminobutyric acid in the exudate (Fig. 23) collected from the control seeds—those grown from seeds treated with 0.03M $MgSO_4$ $MgSO_4$—was very low up to 3.5 hours following seed treatment, then rose quickly and peaked at 15 hours. At that point, the amount of α-aminobutyric acid dropped rapidly, peaked at 44 hours although to a lower extent, and continued to fall until 50 hours. The exudate from the seeds treated with *Enterobacter cloacae* CAL3, displayed the same pattern

Fig. 22. Standard curve of α-aminobutyric acid. α-Aminobutyric acid is quantified in fluorescence units. Equation: $y = 3.528 + 895.35x$; $R=0.99674$

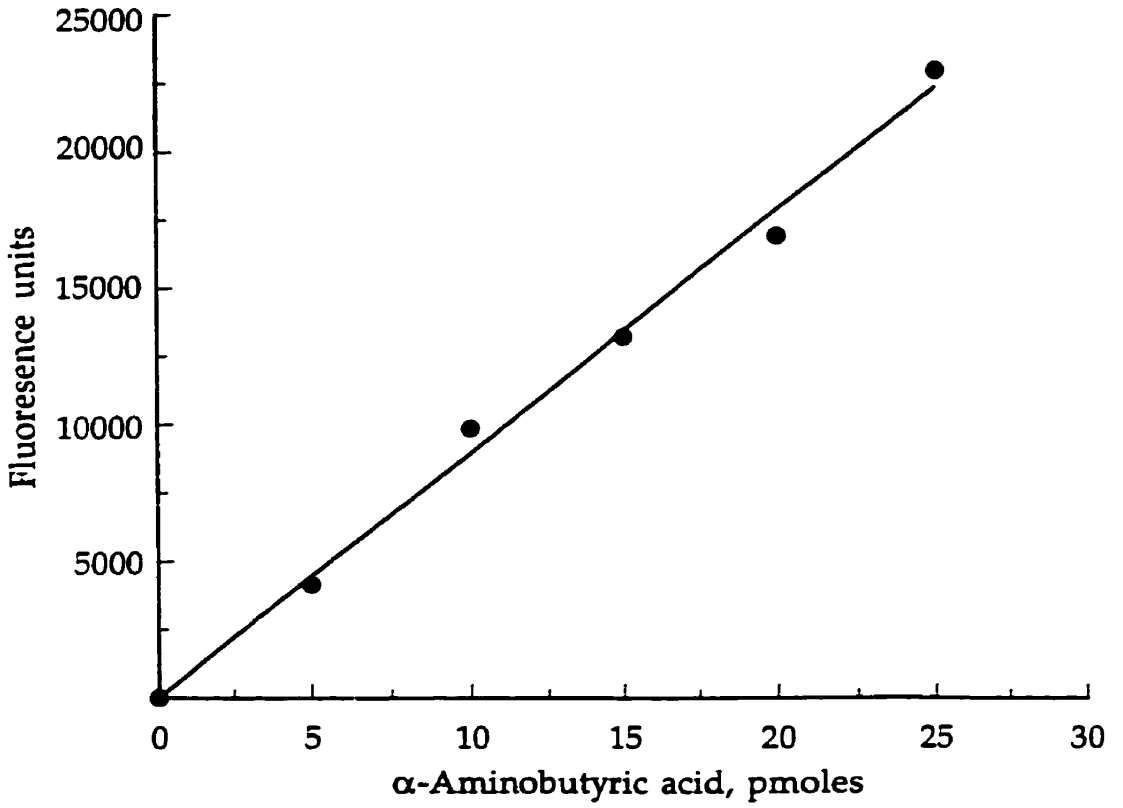
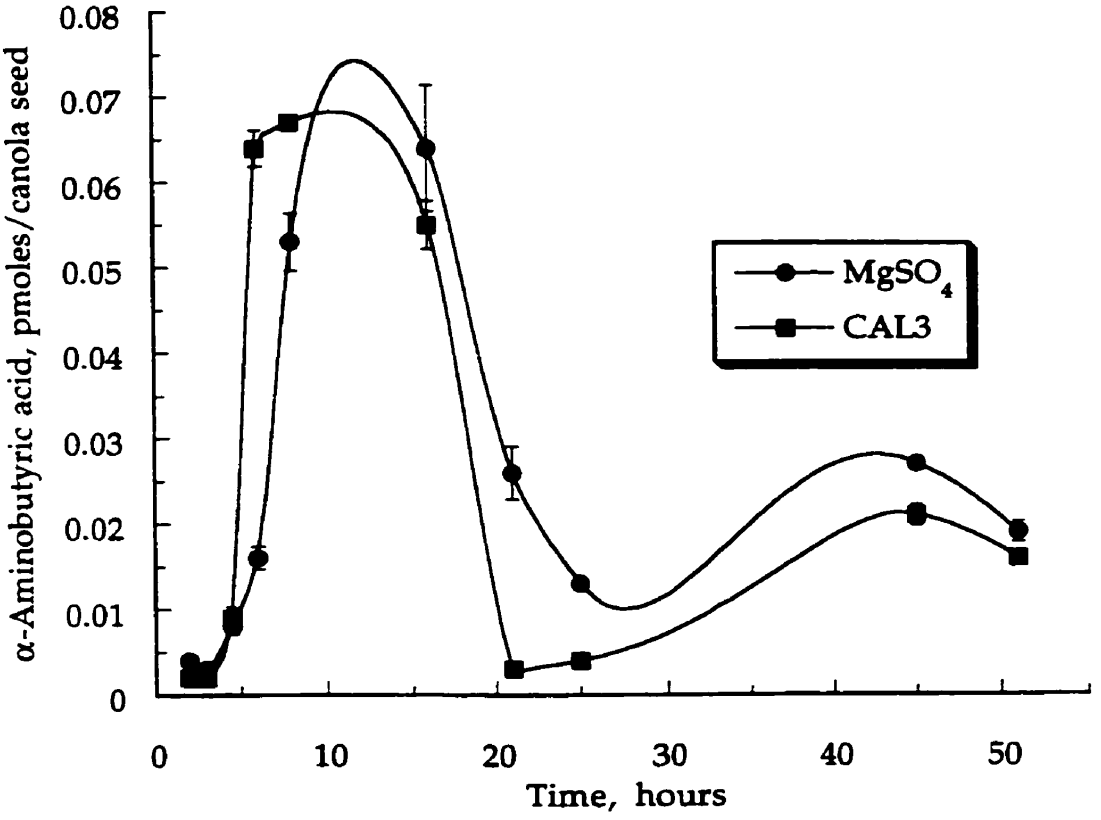

25000
20000
15000
10000
5000
Fluoresence units
0
5
10
15
20
25
30
α-Aminobutyric acid, pmoles

Fig. 23. α-Aminobutyric acid content of canola seed exudate. The amount of α-aminobutyric acid measured in the exudate of canola seeds treated with 0.03M $MgSO_4$ (dark circles) or *Enterobacter cloacae* CAL3 (absorbance of 0.15 at 600 nm) (dark squares) and collected at 1, 2, 3.5, 5, 7, 15, 20, 24, 44 and 50 hours after seed treatment. The points are plotted at time intervals that include one hour of seed treatment. Error bars represent standard errors of the means.


0.08
0.07
0.06
0.05
0.04
0.03
0.02
0.01
0
α-Aminobutyric acid, pmoles/canola seed
MgSO4
CAL3
0
10
20
30
40
50
Time, hours

of α-aminobutyric acid content over time as the exudate from the control seeds except that the peaks from the bacterium-treated seeds were slightly lower and the first peak occurred several hours earlier than the first peak from the $MgSO_4$-treated seed.

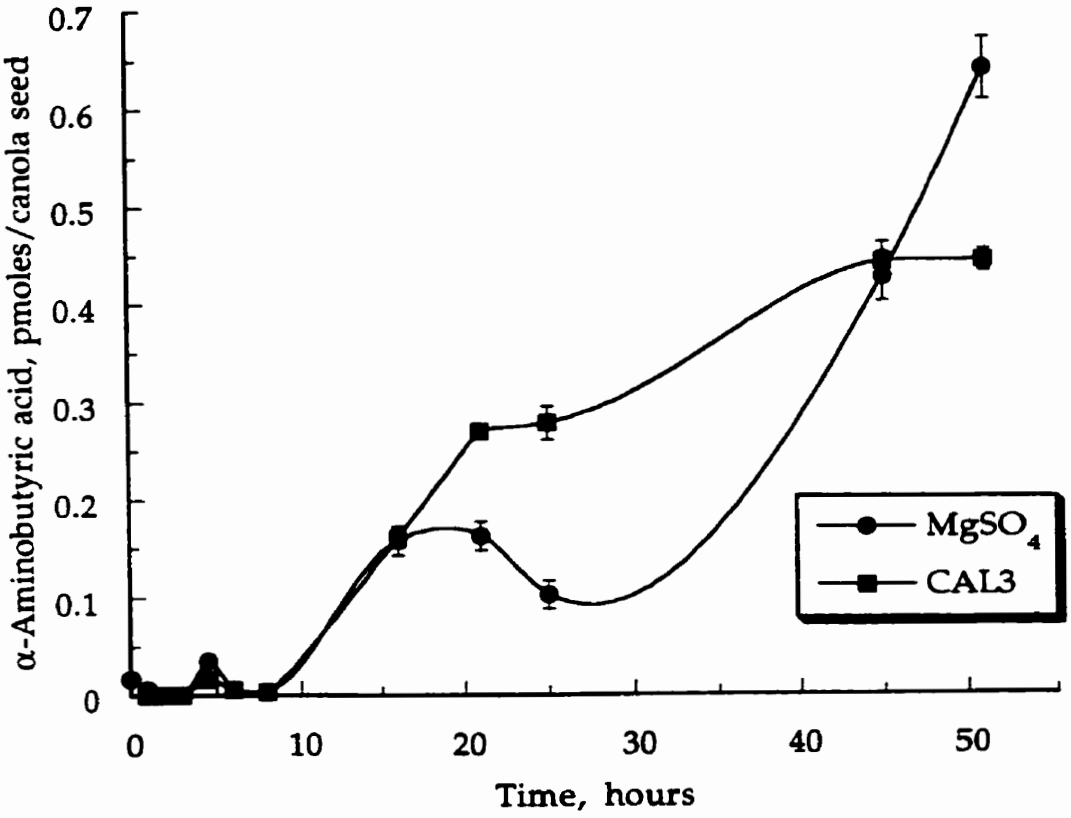
3.7.3. α-Aminobutyric acid content of canola seed extracts

For the first 7 hours following seed treatment, low levels of α-aminobutyric acid were seen in the extracts of both $MgSO_4$-treated and *Enterobacter cloacae* CAL3-treated seeds (Fig. 24) with a slight increase in both extracts at 3.5 hours. At this point, the amount of α-aminobutyric acid began to increase at approximately the same rate in seeds exposed to both treatments. The level of α-aminobutyric acid dropped for several hours, in the extract from control seeds, peaked at approximately 20 hours, and then began to increase. The amount of α-aminobutyric acid, contained in the extract of seeds treated with *Enterobacter cloacae* CAL3 rose quickly until 20 hours, slowed down and reached a plateau after 44 hours, at a level 30% lower than the level reached in the extract from $MgSO_4$-treated seeds.

3.8.1. Quantification of γ-aminobutyric acid

The amount of γ-aminobutyric acid in samples was quantified by using a standard curve that is linear between 1 and 25 pmoles of γ-aminobutyric acid per sample. The γ-aminobutyric acid standard

Fig. 24. α-Aminobutyric acid content of canola seed extracts. The amount of α-aminobutyric acid measured in the extracts of canola seeds treated with 0.03M $MgSO_4$ (dark circles) or *Enterobacter cloacae* CAL3 (absorbance of 0.15 at 600 nm) (dark squares) and collected before treatment and at 0, 1, 2, 3.5, 5, 7, 15, 20, 24, 44 and 50 hours after seed treatment. The points are plotted at time intervals that include one hour of seed treatment. Error bars represent standard errors of the means.


α-Aminobutyric acid, pmoles/canola seed
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
10
20
30
40
50
Time, hours
MgSO4
CAL3

curve was prepared from a fresh stock solution of γ-aminobutyric acid (0.1 mM) diluted with sterile distilled water to yield between 1 and 25 pmol of γ-aminobutyric acid per 20-μL injection. The γ-aminobutyric acid dilutions were derivatized, and following injection, all were eluted at approximately 8.3 minutes. The peak areas of increasing γ-aminobutyric acid concentrations were used to form a standard curve (Fig. 25).

3.8.2. γ-Aminobutyric acid content of canola seed exudate

γ-Aminobutyric acid in the exudate (Fig. 26) collected from the control seeds was present at a low level; it appeared to peak twice in the first 5 hours—a peak at 2 hours and a larger peak at 7 hours—and then slowly dropped to a very low level by 50 hours. Although the initial level was higher, γ-aminobutyric acid in the exudate from seeds treated with *Enterobacter cloacae* CAL3 showed some similarity to the pattern of γ-aminobutyric acid in the exudate of the $MgSO_4$-treated seed; γ-aminobutyric acid peaked twice within the first 12 hours—at 3.5 hours and a larger peak at approximately 11 hours, and then fell to a low level by 24 hours. Unlike the control seeds, γ-aminobutyric acid in the exudate of the bacterium-treated seeds began to increase after 24 hours and continued to do so until 50 hours.

Fig. 25. Standard curve of γ-aminobutyric acid. γ-Aminobutyric acid is quantified in fluorescence units. Equation: y = -253.88 + 1273.5x; R=0.99548

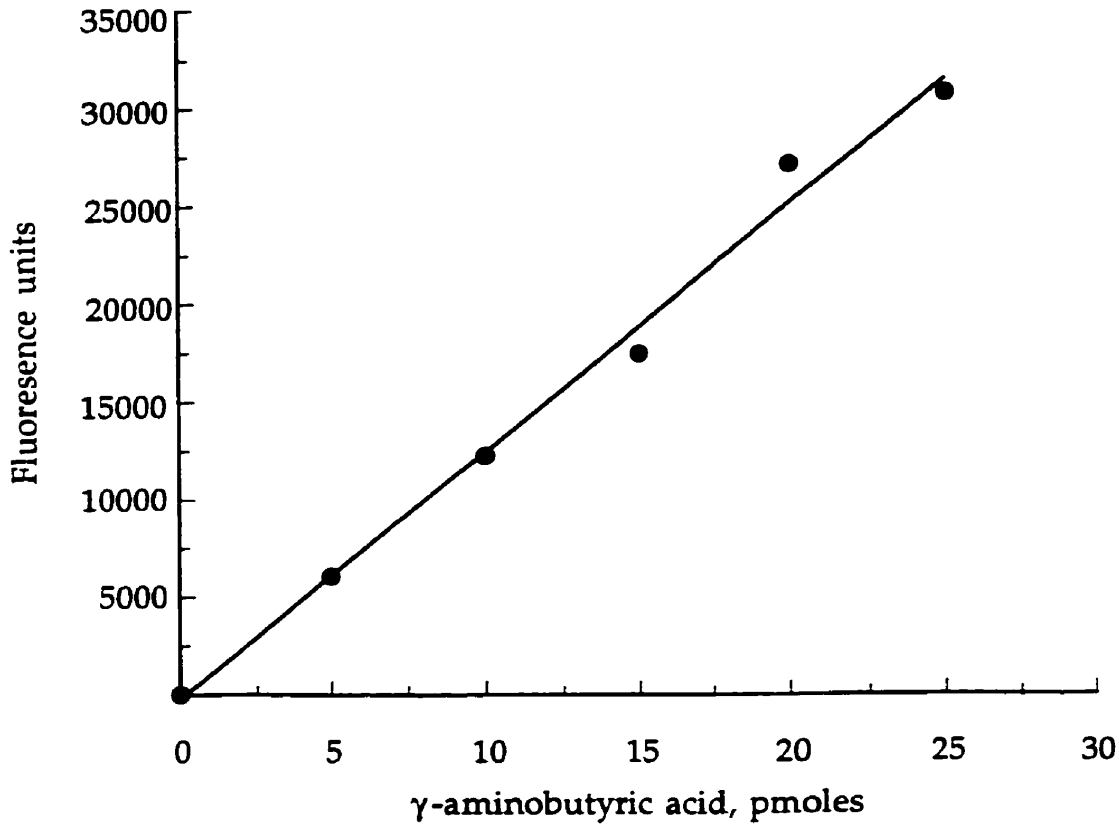
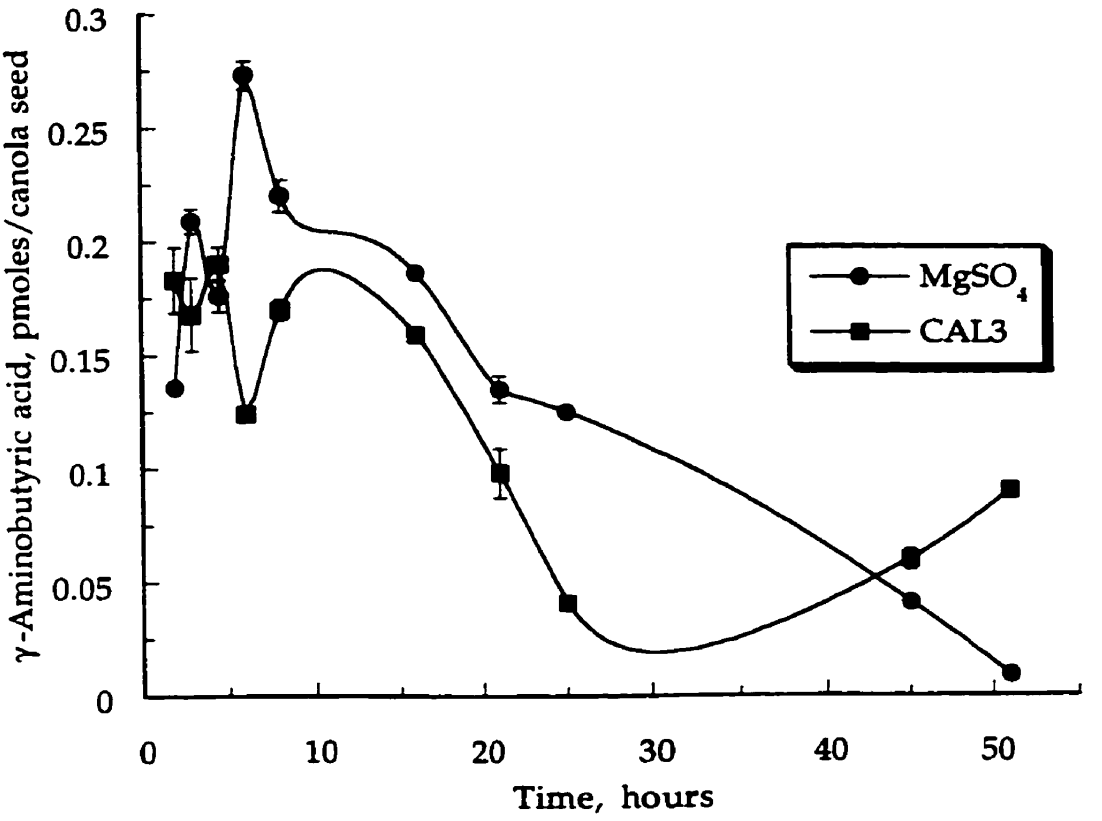

35000
30000
25000
20000
15000
10000
5000
Fluoresence units
0
5
10
15
20
25
30
γ-aminobutyric acid, pmoles

Fig. 26. γ-Aminobutyric acid content of canola seed exudate. The amount of γ-aminobutyric acid measured in the exudate of canola seeds treated with 0.03M $MgSO_4$ (dark circles) or *Enterobacter cloacae* CAL3 (absorbance of 0.15 at 600 nm) (dark squares) and collected at 1, 2, 3.5, 5, 7, 15, 20, 24, 44 and 50 hours after seed treatment. The points are plotted at time intervals that include one hour of seed treatment. Error bars represent standard errors of the means.


0.3
0.25
0.2
0.15
0.1
0.05
0
γ-Aminobutyric acid, pmoles/canola seed
0
10
20
30
40
50
Time, hours
MgSO4
CAL3

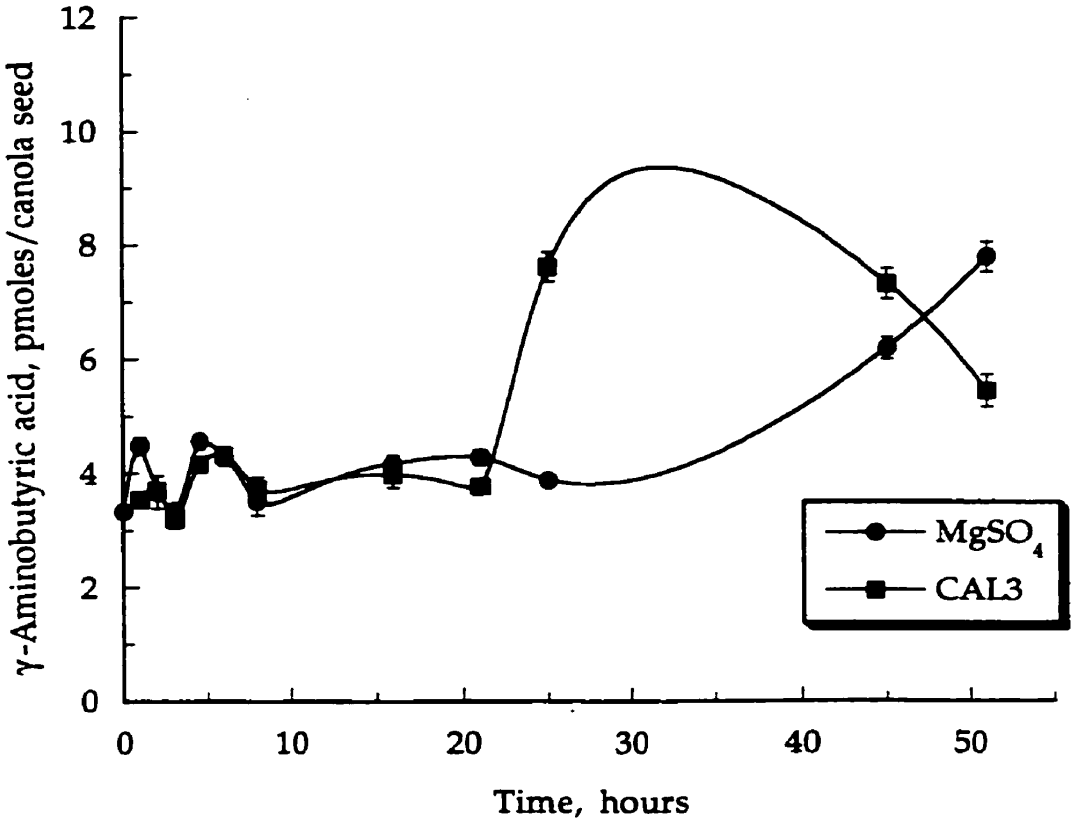
3.8.3 γ-Aminobutyric acid content of canola seed extract

Up until 20 hours after seed treatment, the appearance of γ-aminobutyric acid in the extracts of control and bacterium-treated seeds (Fig. 27) was almost identical: two peaks were seen within the first 5 hours (the peaks from the bacterium-treated seeds were slightly smaller) and then leveled off at approximately 3.1 pmoles/seed. γ-Aminobutyric acid continued at that level for another four hours in the exudate of the $MgSO_4$-treated seed and then increased to almost 7.8 pmoles/seed at 50 hours. When compared with ACC, the amount of γ-aminobutyric acid measured at 50 hours in the extracts of both the control and bacterium-treated seeds, was almost twice as much as the amount of ACC in the same extracts.

3.8.4. β-Aminobutyric acid content of canola seedlings

β-aminobutyric acid was present only in the shoot extracts of 4.5-day old canola seedlings and was eluted at approximately 8.76 minutes. The low levels of β-aminobutyric acid seen in the shoot extracts of the seeds treated with $MgSO_4$, *Pseudomonas putida* pRK415, *Pseudomonas putida* pRKACC or *Enterobacter cloacae* CAL3 were very similar. There appeared to be no appreciable difference in the level of this compound as a result of seed treatment.

Fig. 27. γ-Aminobutyric acid content of canola seed extracts. The amount of γ-aminobutyric acid measured in the extracts of canola seeds treated with 0.03M $MgSO_4$ (dark circles) or *Enterobacter cloacae* CAL3 (absorbance of 0.15 at 600 nm) (dark squares) and collected before treatment and at 0, 1, 2, 3.5, 5, 7, 15, 20, 24, 44 and 50 hours after seed treatment. The points are plotted at time intervals that include one hour of seed treatment. Error bars represent standard errors of the means.


γ-Aminobutyric acid, pmoles/canola seed
12
10
8
6
4
2
0
0
10
20
30
40
50
Time, hours
MgSO4
CAL3

3.9.1. Quantification of tyrosine

The amount of tyrosine in samples was quantified by using a tyrosine standard curve that is linear between 1 and 25 pmoles of tyrosine per sample. The standard curve was prepared from a fresh stock solution of tyrosine (0.1 mM) diluted with sterile distilled water to yield between 1 and 25 pmoles of tyrosine per 20 µL injection. The tyrosine dilutions were derivatized, and following injection, all were eluted at approximately 7.6 minutes. The peak areas of increasing tyrosine concentrations were used to form a standard curve (Fig. 28).

3.9.2. Tyrosine content of canola seed exudate

The pattern of tyrosine content in the exudate of canola seeds (Fig. 29) resembled that of ACC. During the first 7 hours after seed treatment tyrosine appeared as two peaks and then dropped in the exudate of both the $MgSO_4$-treated and *Enterobacter cloacae* CAL3-treated seeds. However, the initial amount of tyrosine in the exudate of the bacterium-treated seeds was higher, the peaks smaller, and it dropped to zero four hour earlier than the control seeds.

3.9.3. Tyrosine content of canola seed extracts

Although the absolute amounts of tyrosine in canola seed extracts differed with those of ACC during germination—ACC reached higher levels than tyrosine in the extracts of both the $MgSO_4$-treated and

Fig. 28. Standard curve of tyrosine. Tyrosine is quantified in fluorescence units. Equation: $y = 106.6 + 627.3x$; $R=0.99978$

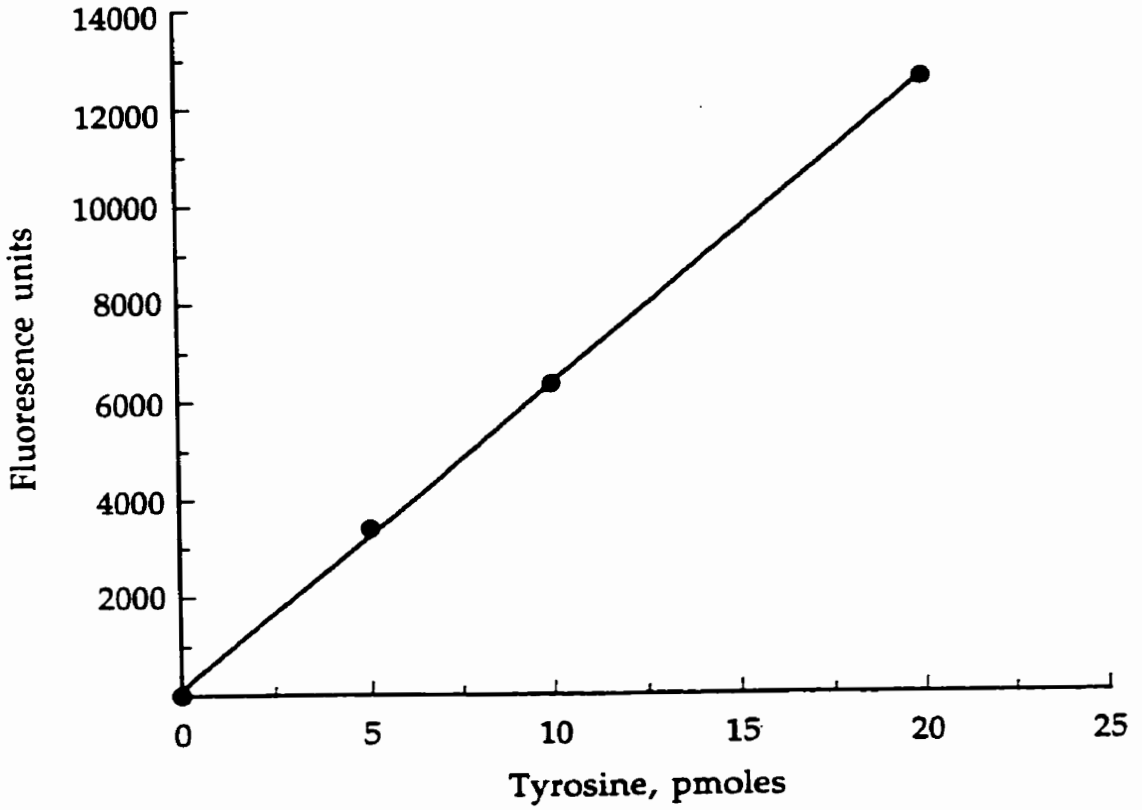
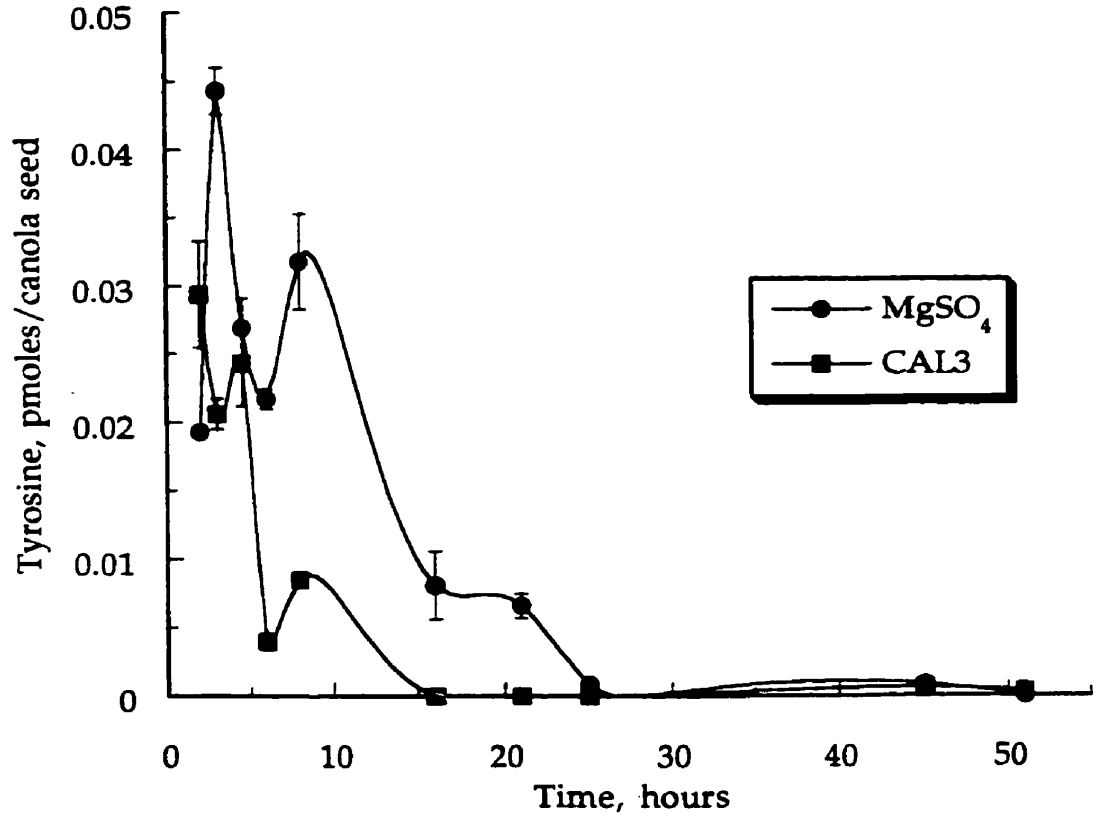

14000
12000
10000
8000
6000
4000
2000
Fluoresence units
0
5
10
15
20
25
Tyrosine, pmoles

Fig. 29. Tyrosine content of canola seed exudate. The amount of tyrosine measured in the exudate of canola seeds treated with 0.03M $MgSO_4$ (dark circles) or *Enterobacter cloacae* CAL3 (absorbance of 0.15 at 600 nm) (dark squares) and collected at 1, 2, 3.5, 5, 7, 15, 20, 24, 44 and 50 hours after seed treatment. The points are plotted at time intervals that include one hour of seed treatment. Error bars represent standard errors of the means.


0.05
0.04
0.03
0.02
0.01
0
Tyrosine, pmoles/canola seed
0
10
20
30
40
50
Time, hours
MgSO4
CAL3

Enterobacter cloacae CAL3-treated seeds—the pattern of tyrosine content in both the control and bacterium-treated seeds appeared to be very similar to that of ACC (Fig. 30).

During the first 7 hours following seed treatment, low levels of tyrosine including a small peak at 3.5 hours, were seen in extracts of both $MgSO_4$-treated and *Enterobacter cloacae* CAL3-treated seeds. A small peak was visible at 3.5 hours, which was slightly larger in the extract of the control seeds. The amount of tyrosine began to increase in seeds exposed to either treatments and continued to increase in the control seeds, after a small dip at 24 hours, up to 50 hours. However, there was a rapid increase in the level of tyrosine in the extract of the bacterium-treated seeds after 20 hours, which peaked at approximately 30 hours and then dropped by 50 hours.

3.10.1. Quantification of valine

The amount of valine in samples was quantified by using a valine standard curve that is linear between 1 and 25 pmoles of valine per sample. The standard curve was prepared from a fresh stock solution of valine (0.1 mM) diluted with sterile distilled water to yield between 1 and 25 pmoles of valine per 20-µL injection. The valine dilutions were derivatized, and following injection, all were eluted at approximately 14.1 minutes. The peak areas of increasing valine concentrations were used to form a standard curve (Fig. 31).

Fig. 30. Tyrosine content of canola seed extracts. The amount of tyrosine measured in the extracts of canola seeds treated with 0.03M $MgSO_4$ (dark circles) or *Enterobacter cloacae* CAL3 (absorbance of 0.15 at 600 nm) (dark squares) and collected before treatment and at 0, 1, 2, 3.5, 5, 7, 15, 20, 24, 44 and 50 hours after seed treatment. The points are plotted at time intervals that include one hour of seed treatment. Error bars represent standard errors of the means.

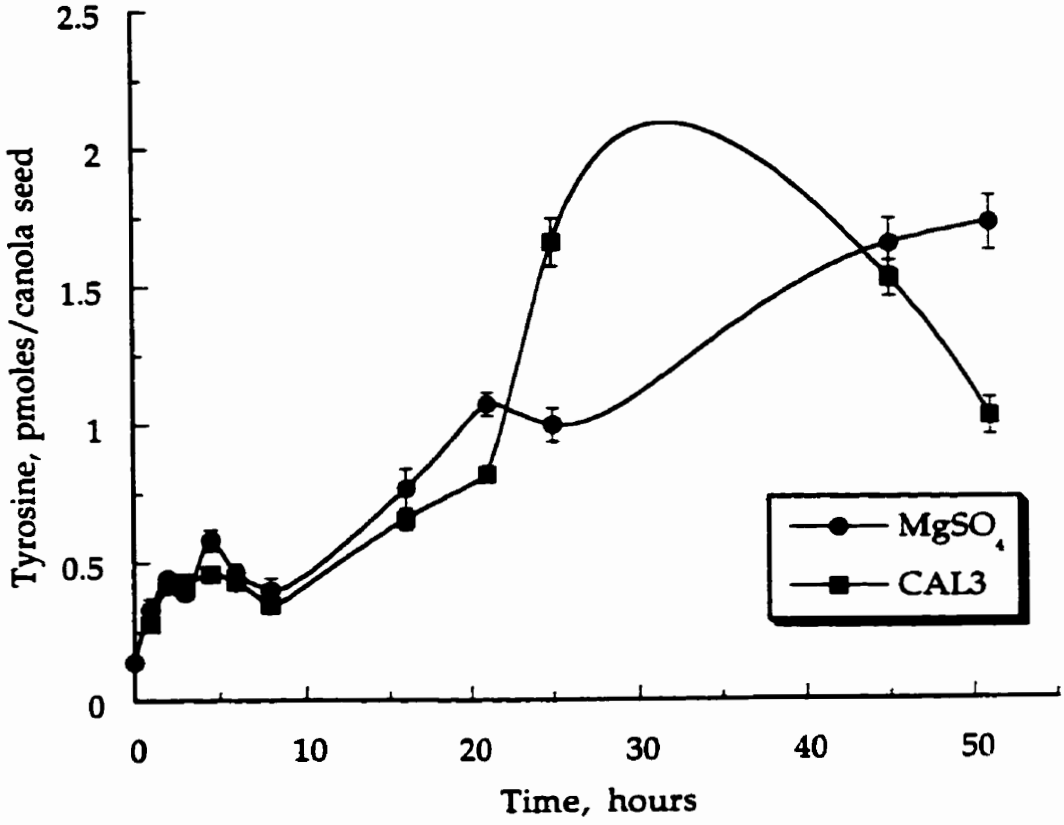
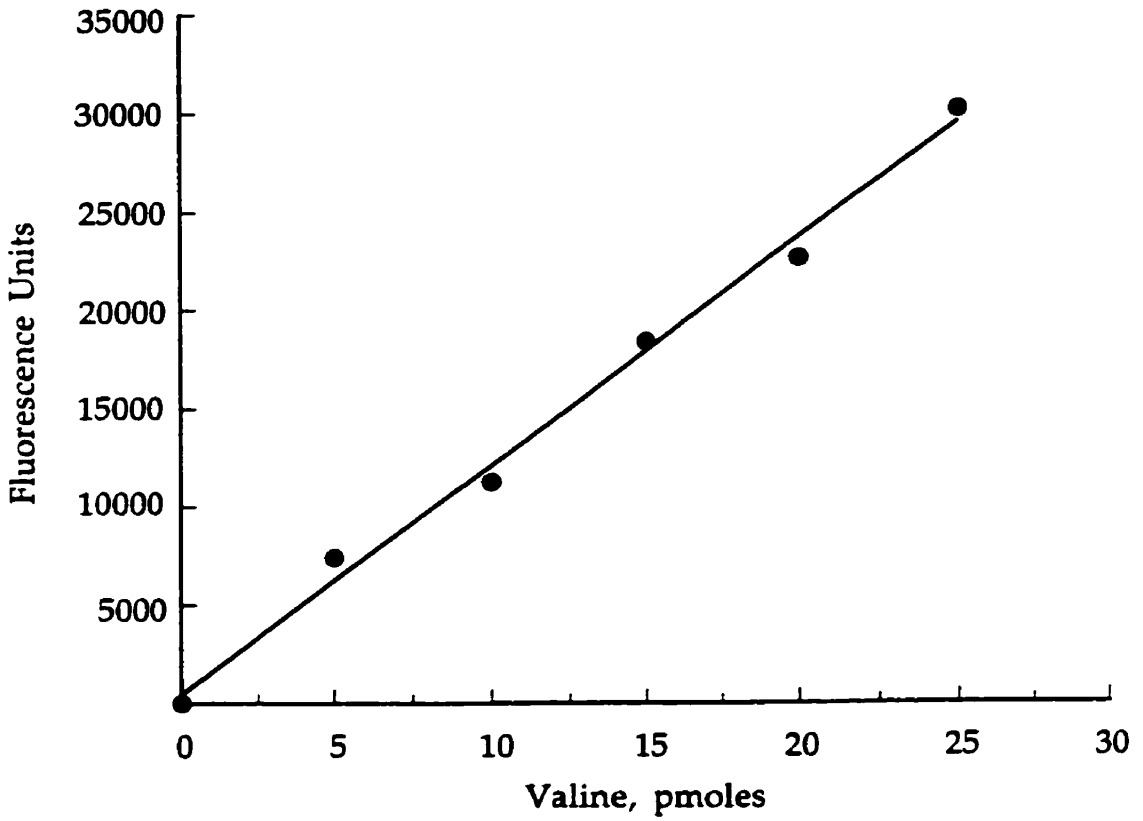

2.5
2
1.5
1
0.5
0
Tyrosine, pmoles/canola seed
0
10
20
30
40
50
Time, hours
MgSO4
CAL3

Fig. 31. Standard curve of valine. Valine is quantified in fluorescence units. Equation: $y = 423.3 + 1166.2x$; $R=0.99662$


35000
30000
25000
20000
15000
10000
5000
Fluorescence Units
0
5
10
15
20
25
30
Valine, pmoles

3.10.2. Valine content of canola seed exudate

Valine appeared in the exudate of both the $MgSO_4$-treated and *Enterobacter cloacae* CAL3-treated seeds, at high levels, in the first 24 hours following seed treatment and then almost disappeared (Fig. 32). The pattern of valine content was similar in the exudate of both the control seeds and bacterium-treated seeds—two early peaks followed by a rapid drop in the level of valine. However, the quantity of valine in the peaks from the control seed exudate was much higher than the amount of valine in the equivalent peaks from the bacterium-treated seeds. As well, the initial level of valine was approximately 30% higher in the exudate of the seeds inoculated with the bacterium, *Enterobacter cloacae* CAL3, than in the exudate from the control seeds.

3.10.3. Valine content of canola seed extract

The quantity of valine increased over the 50 hours following seed treatment in extracts from seeds treated with either $MgSO_4$ or *Enterobacter cloacae* CAL3 (Fig. 33). Two peaks occurred in both extracts within the first 24 hours: a small peak at 3.5 hours, and a larger peak, at 15 hours in the bacterium-treated seeds and at 20 hours in the control seeds. The amount of valine was lower in both peaks of the seeds inoculated with the bacterium than in the equivalent peaks from the seeds treated with $MgSO_4$. As well, the extract of the bacterium-treated

Fig. 32. Valine content of canola seed exudate. The amount of valine measured in the exudate of canola seeds treated with 0.03M $MgSO_4$ (dark circles) or *Enterobacter cloacae* CAL3 (absorbance of 0.15 at 600 nm) (dark squares) and collected at 1, 2, 3.5, 5, 7, 15, 20, 24, 44 and 50 hours after seed treatment. The points are plotted at time intervals that include one hour of seed treatment. Error bars represent standard errors of the means.

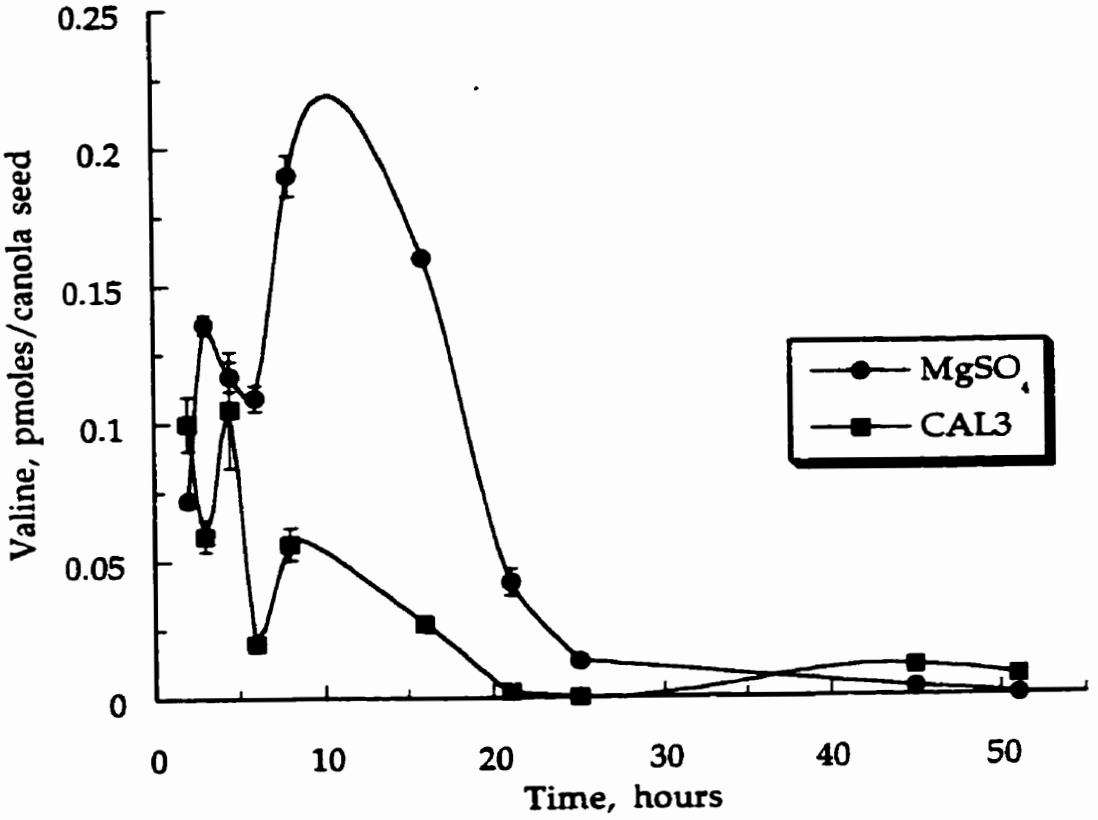
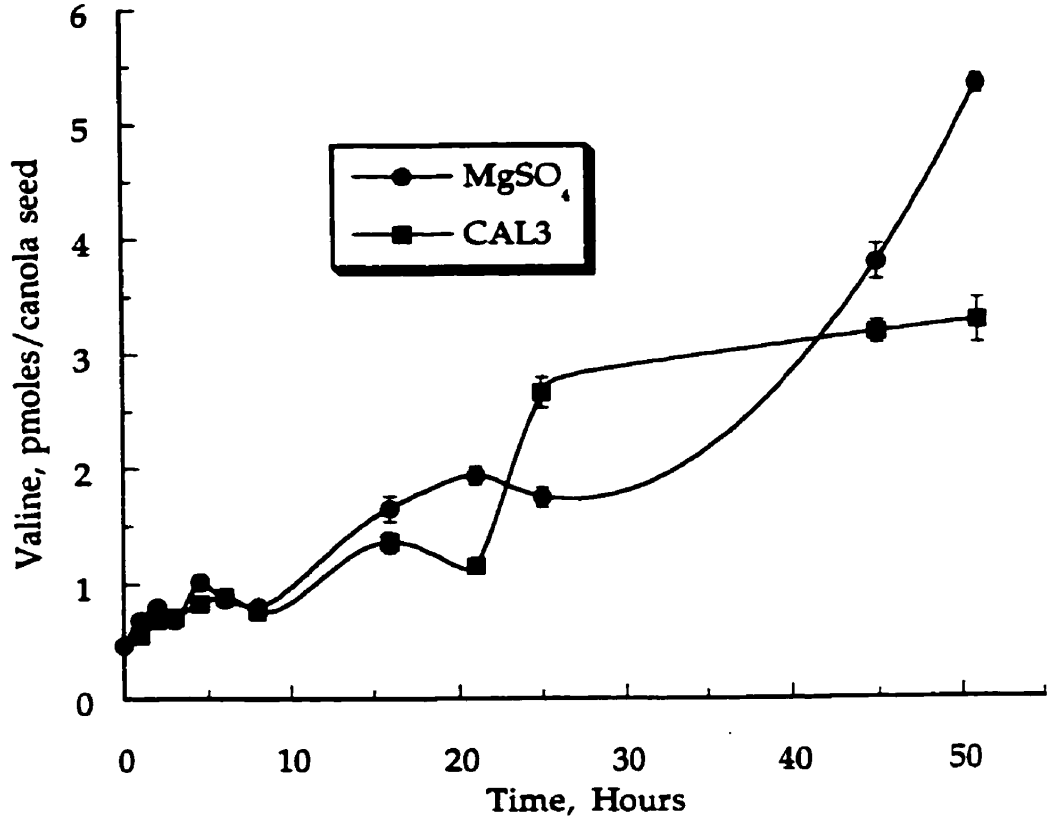

0.25
0.2
0.15
0.1
0.05
0
Valine, pmoles/canola seed
0
10
20
30
40
50
Time, hours
MgSO4
CAL3

Fig. 33. Valine content of canola seed extracts. The amount of valine measured in the extracts of canola seeds treated with 0.03M $MgSO_4$ (dark circles) or *Enterobacter cloacae* CAL3 (absorbance of 0.15 at 600 nm) (dark squares) and collected before treatment and at 0, 1, 2, 3.5, 5, 7, 15, 20, 24, 44 and 50 hours after seed treatment. The points are plotted at time intervals that include one hour of seed treatment. Error bars represent standard errors of the means.


6
5
4
3
2
1
0
Valine, pmoles/canola seed
MgSO4
CAL3
0
10
20
30
40
50
Time, Hours

seeds contained a lower level of valine at 50 hours following seed treatment.

3.10.4. Valine content of canola seedlings

At 4.5 days after seed treatment, valine was present in roots and shoots of canola seedlings (Fig. 34) at levels about 50% lower than those of ACC. The valine content was considerably higher in the extract of roots from $MgSO_4$-treated seeds than in the extracts from roots of seeds inoculated with *Enterobacter cloacae* CAL3, *Pseudomonas putida* pRK415 or *Pseudomonas putida* pRKACC—approximately 100% higher than seeds treated with *Enterobacter cloacae* CAL3 or *Pseudomonas putida* pRKACC, bacteria containing ACC deaminase. However, the levels of valine were very similar in the shoots of canola seeds despite their treatment.

3.11.1. Quantification of methionine

The amount of methionine in samples was quantified by using a standard curve that is linear between 1 and 25 pmoles of methionine per sample. The standard curve was prepared from a fresh stock solution of methionine (0.1 mM) diluted with sterile distilled water to yield between 1 and 25 pmol of methionine per 20 µL injection. The dilutions were derivatized, and following injection, all were eluted at approximately 15.0 minutes. The peak areas of increasing methionine concentrations were used to form a standard curve (Fig. 35).

Fig. 34. Valine content of canola seedlings. The amount of valine measured in the roots and shoots of 4.5-day old canola seedlings grown for the root elongation assay. Measurements were made on 60 seedlings (6 seeds/growth pouch; 10 growth pouches. Seed treatment abbreviations: $MgSO_4$, 0.03M $MgSO_4$; CAL3, *Enterobacter cloacae* CAL3; pRK415, *Pseudomonas putida* pRK415; pRKACC, *Pseudomonas putida* pRKACC. Error bars represent standard errors of the means.

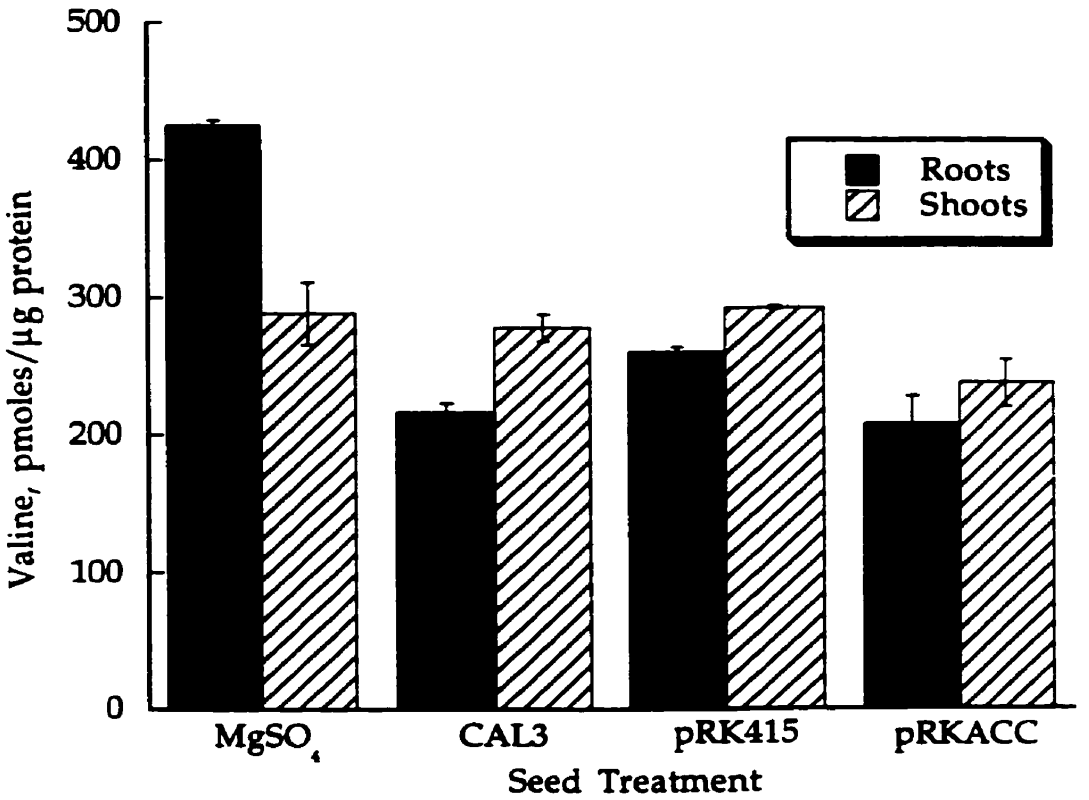
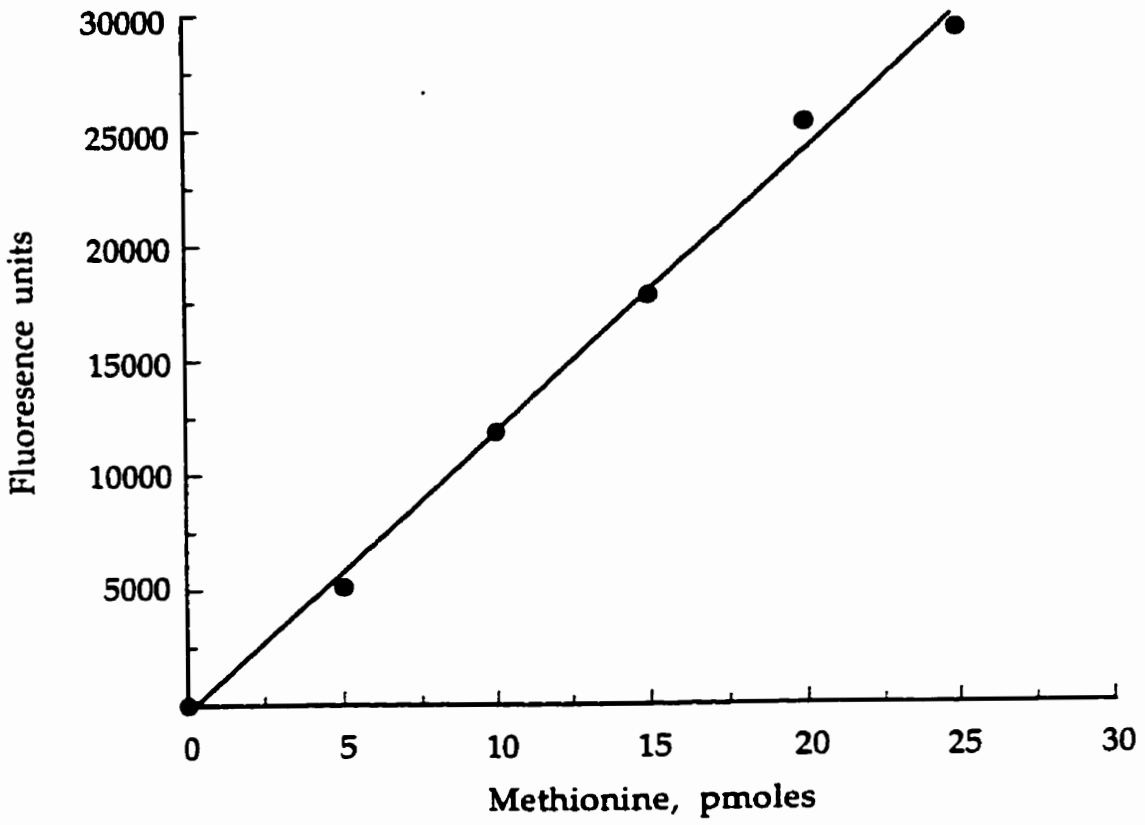

500
400
300
200
100
0
Valine, pmoles/µg protein
Roots
Shoots
MgSO4
CAL3
pRK415
pRKACC
Seed Treatment

Fig. 35. Standard curve of methionine. Methionine is quantified in fluorescence units. Equation: $y = -327.38 + 1221.3x$; $R = 0.99799$


30000
25000
20000
15000
10000
5000
Fluoresence units
0
5
10
15
20
25
30
Methionine, pmoles

3.11.2. Methionine content of canola seed exudate

The levels of methionine seen in canola seed exudate were quite low in comparison with levels of ACC (Fig. 36). Interestingly, the amount of methionine at one hour after treatment was higher in the $MgSO_4$-treated seeds than in the bacterium-treated seeds. In the exudate from both seed treatments the quantity of methionine peaked twice in the first 24 hours after seed treatment, dropped to zero, and then began to increase. However, when compared with the exudate from the control seeds, the methionine peaks in the exudate from seed treated with *Enterobacter cloacae* CAL3 were much smaller, and the other events, which included the occurrence of the second peak, the drop to zero level and the onset of the increase, happened much earlier. By 50 hours, the amount of methionine had begun to drop in the exudate from the bacterium-treated seeds but was still rising in the exudate of the control seeds.

3.11.3. Methionine content of canola seed extracts

Methionine was present in the canola seed extracts at a very low level (Fig. 37). There appeared to be three methionine peaks in the control seed extracts—the first two peaks were within 3.5 hours of seed treatment, and the third was 20 hours following seed treatment—after which the level of methionine increased up to 50 hours. The first two of the four methionine peaks seen in the extracts of the *Enterobacter cloacae*

CAL3-treated seeds were very similar to those from the $MgSO_4$-treated seeds. The third peak occurred about 5 hours before the third peak in the control seed extract, and a large fourth peak appeared at approximately 30 hours following seed treatment; by 50 hours, the level of methionine had started to fall.

Fig. 36. Methionine content of canola seed exudate. The amount of methionine measured in the exudate of canola seeds treated with 0.03M $MgSO_4$ (dark circles) or *Enterobacter cloacae* CAL3 (absorbance of 0.15 at 600 nm) (dark squares) and collected at 1, 2, 3.5, 5, 7, 15, 20, 24, 44 and 50 hours after seed treatment. The points are plotted at time intervals that include one hour of seed treatment. Error bars represent standard errors of the means.

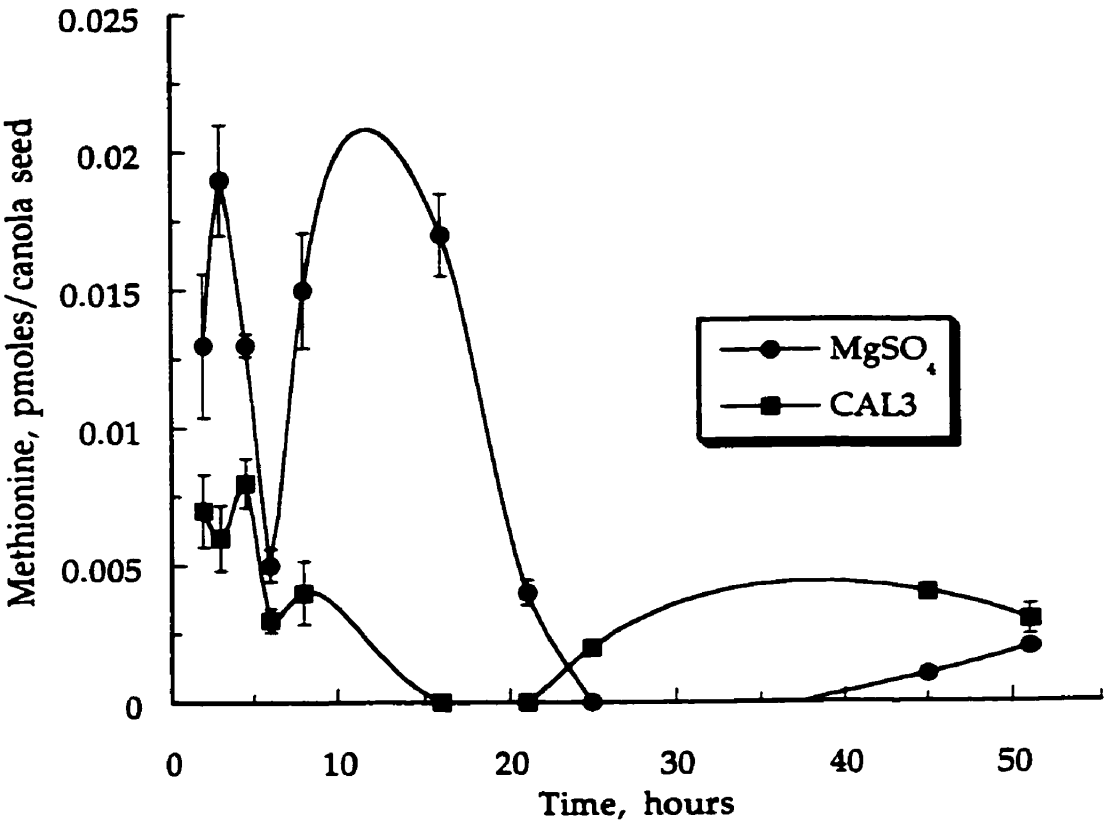
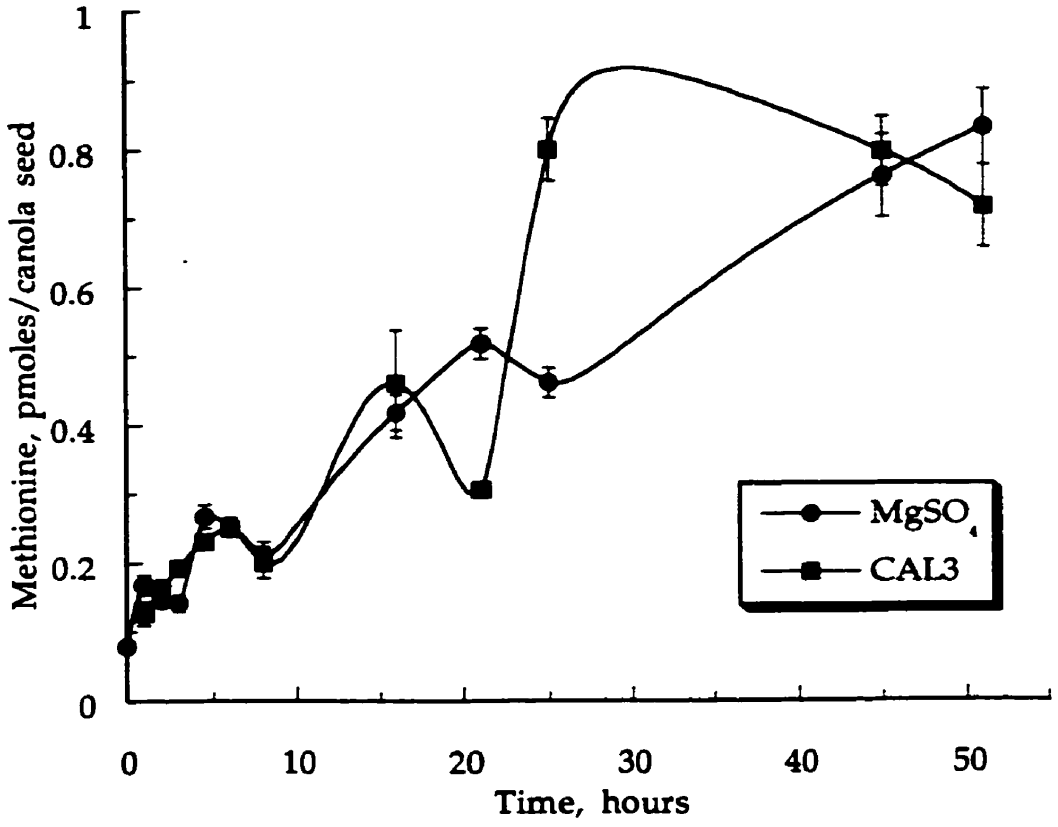

0.025
0.02
0.015
0.01
0.005
0
Methionine, pmoles/canola seed
0
10
20
30
40
50
Time, hours
MgSO4
CAL3

Fig. 37. Methionine content of canola seed extracts. The amount of methionine measured in the extracts of canola seeds treated with 0.03M $MgSO_4$ (dark circles) or *Enterobacter cloacae* CAL3 (absorbance of 0.15 at 600 nm) (dark squares) collected before treatment and at 0, 1, 2, 3.5, 5, 7, 15, 20, 24, 44 and 50 hours after seed treatment. The points are plotted at time intervals that include one hour of seed treatment. Error bars represent standard errors of the means.


Methionine, pmoles/canola seed
1
0.8
0.6
0.4
0.2
0
0
10
20
30
40
50
Time, hours
MgSO4
CAL3

3.12. Isolation and characterization of genes cloned from *Enterobacter cloacae* CAL3

3.12.1. Characterization of *Enterobacter cloacae* CAL3

In 1978, Honma discovered that several microorganisms could utilize ACC as a sole source of nitrogen and that these microorganisms contained the enzyme, ACC deaminase. Since then, this enzyme has been found in many organisms (Penrose and Glick, 1997). A number of rhizosphere soil samples were collected from several locations in Waterloo, Ontario, Canada and in California, U.S.A. and screened in our laboratory for the presence of ACC deaminase. From these samples, seven strains were isolated on the basis of their growth on a minimal medium containing ACC as a sole source of nitrogen. After a more complete characterization, it was found that all seven strains contained ACC deaminase activity, although with varying levels of specific activity. The bacterium used in this study, *Enterobacter cloacae* CAL3, showed a slow rate of growth on DF minimal medium containing ACC (Table 12) and a low level of ACC deaminase activity, when compared with the other six strains (Table 13) (Shah et al., 1997).

Although *Enterobacter cloacae* CAL3 shared some similarities with the other strains–they were all Gram-negative bacteria with inducible ACC deaminase activity whose growth was inhibitied by low levels of kanamycin and tetracycline–it appeared to be fundamentally different than the others. *Enterbacter cloacae* CAL3 secreted a relatively high level

Table 12. Characterization of seven strains for the ability to utilize ACC as a sole source of nitrogen. Growth was monitored on plates containing DF minimal medium plus 3 mM ACC.

Strain	Growth on DF plates plus ACC
Pseudomonas putida UW1	++
Enterobacter cloacae UW2	++
Pseudomonas putida UW3	+++
Enterobacter cloacae UW4	++++
Pseudomonas fluorescens CAL1	±
Enterobacter cloacae CAL2	++++
Enterobacter cloacae CAL3	++

Symbols used: -, no growth; ±, very low level of growth; +, ++, +++, ++++, increasing–from low to high–levels of growth.

Table 13. Physiological characteristics of seven plant growth promoting strains. The measurements of each trait for *Enterobacter cloacae* CAL3 are in bold face type. Adapted from Shah et al., 1997.

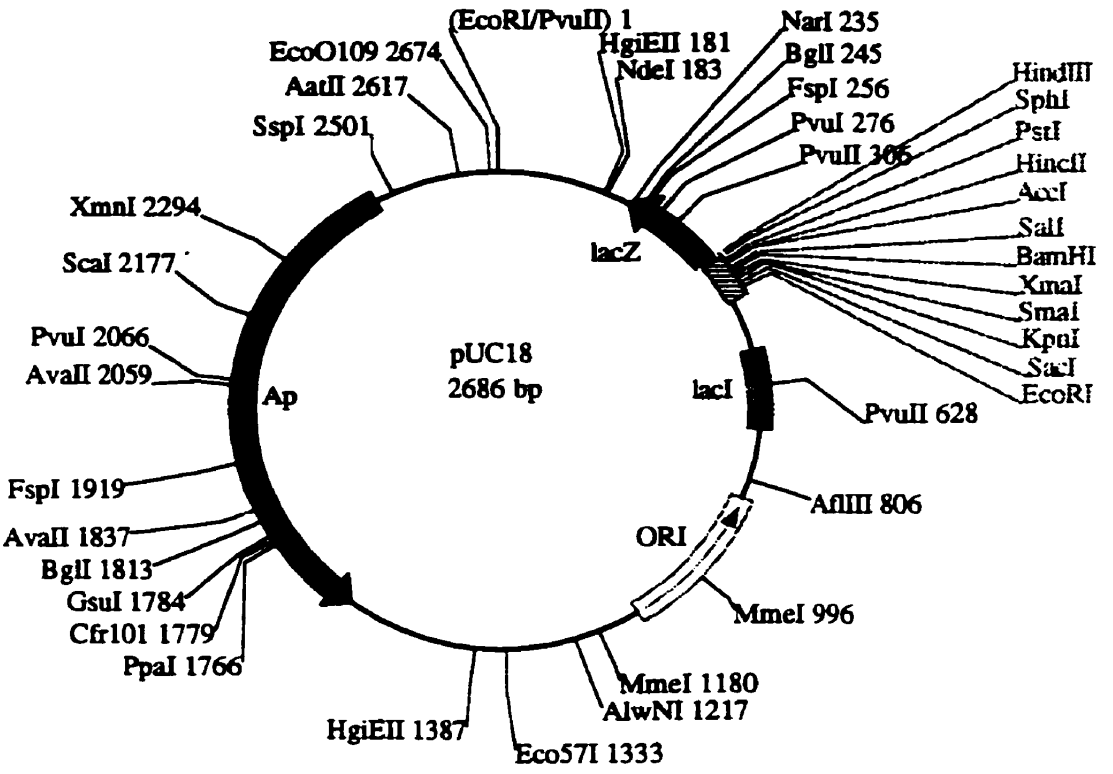
	Strain #						
Trait	UW1	UW2	UW3	UW4	CAL1	CAL2	**CAL3**
Associated plant	beans	clover	corn	reeds	oats	tomato	**cotton**
Genus and species	*P. putida*	*E. cloacae*	*P. putida*	*E. cloacae*	*P. fluorescens*	*E. cloacae*	***E. cloacae***
Siderophore activity, μmoles/10^9 cells	16.7	15.2	5.7	6.9	13.8	11.2	**13.0**
IAA produced, μg/mL	1.1	1.5	1.7	0.9	0.5	0.3	**16.3**
ACC deaminase activity, μmoles/mg protein/h	2.56	2.56	–	12.29	11.50	25.03	**5.84**

of siderophores and could produce a high level of IAA (indole acetic acid) when grown in the presence of a high level tryptophan. Despite its potential to synthesize a large amount of IAA, in the absence of tryptophan, this strain produced very little of it.

3.12.2. Isolation of putative ACC deaminase genes from *Enterobacter cloacae* CAL3

Total genomic DNA was isolated from *Enterobacter cloacae* CAL3 and partially digested with *Sau*3A. Nearly all of the partially digested fragments were between 1 kb and 10 kb in size. After ligation of the fragments into plasmid pUC18 predigested with *Bam*HI and bacterial alkaline phosphatase (Fig. 38), the recombinant ligation products were used to transform competent *Escherichia coli* DH5α cells. Transformed cells that grew on selective agar (LB medium containing 100 μg ampicillin/mL) were further screened by growth on M9 minimal medium containing 3 mM ACC as a sole source of nitrogen. After the third screening, 12 positive clones were isolated from the approximately 7000 clones of the *Enterobacter cloacae* CAL3 library. The plasmid from each of the 12 clones was isolated, and designated as pUC318, pUC319, pUC320, pUC321, pUC322, pUC323, pUC324, pUC325, pUC326, pUC327, pUC328 or pUC329 and digested with *Eco*RI and *Hind*III. The DNA inserts, released following digestion, were separated by agarose gel electrophoresis. The results of the agarose gel electrophoresis (data

Fig. 38. Restriction map of the *Escherichia coli* cloning vector, pUC18; restriction sites are as labelled. Chromosomal *Enterobacter cloacae* CAL3 DNA, partially digested with *Sau*3A, was inserted into *Bam*HI of the multiple cloning site of pUC18.


(EcoRI/PvuII) 1
HgiEII 181
NdeI 183
NarI 235
BglI 245
FspI 256
PvuI 276
PvuII 306
HindIII
SphI
PstI
HincII
AccI
SalI
BamHI
XmaI
SmaI
KpnI
SacI
EcoRI
EcoO109 2674
AatII 2617
SspI 2501
XmnI 2294
ScaI 2177
PvuI 2066
AvaII 2059
FspI 1919
AvaII 1837
BglI 1813
GsuI 1784
Cfr101 1779
PpaI 1766
HgiEII 1387
Eco57I 1333
MmeI 1180
AlwNI 1217
MmeI 996
ORI
AflIII 806
PvuII 628
lacI
lacZ
Ap
pUC18
2686 bp

not shown) confirmed the presence of an insert in each plasmid: the size of the inserts ranged from less than 1000 bp to approximately 6500 bp.

3.12.3. Characterization of the *Enterobacter cloacae* CAL3 clones

Four of the twelve clones: pUC319, pUC320, pUC324 andpUC327, were selected for further analyses based on their growth in M9 minimal medium with ACC as the sole source of nitrogen and on their level of ACC deaminase activity. The growth of the twelve clones in M9 minimal medium containing 3 mM ACC, at either 30°C or 37°C, was monitored over 5 days and compared with growth of the control strains under the same conditions (Tables 14 and 15). The positive control, *Escherichia coli* with the ACC deaminase gene into the broadhost range plasmid, pRK415, grew very quickly at both temperaures in both types of media. As expected, the negative control, *Escherichia coli* containing the plasmid pRK415, grew very slowly in liquid cultures at both temperatures. However, this control initially grew more quickly than expected on plates–perhaps due to the presence of a low level of nitrogen in the agar. The twelve *Enterobacter cloacae* CAL3 clones varied in the level of growth and the temperature at which they grew best. Clone pUC324 grew very rapidly in both liquid cultures and on plates, at both temperatures.

Table 14. Characterization of the growth of *Escherichia coli* clones in liquid cultures of M9 minimal medium plus 3 mM ACC at 30°C or 37°C; pRK415 serves as a negative control (i.e., no ACC deaminase gene), pRKACC serves as a positive control (i.e., contains a cloned ACC deaminase gene).

Plasmid Name	Liquid 30°C 1 day	Liquid 30°C 2 days	Liquid 30°C 5 days	Liquid 37°C 1 day	Liquid 37°C 2 days	Liquid 37°C 5 days
pRK415	±	+	+	+	+	+
pRKACC	++	+++	++++	+++	+++	++++
pUC319	+	++	++	+	+	++
pUC320	+	++	++	+	++	++++
pUC324	+	+++	+++	+	+	+++
pUC327	+	++	+++	+	+	+++

Symbols used: ±, very low level of growth; +, ++, +++, ++++, increasing–from low to high–levels of growth.

Table 15. Characterization of the growth of *Escherichia coli* clones on plates of M9 minimal medium plus 3 mM ACC at 30°C or 37°C; pRK415 serves as a negative control (i.e., no ACC deaminase gene), pRKACC serves as a positive control (i.e., contains a cloned ACC deaminase gene).

Plasmid Name	Agar 30°C 1 day	Agar 30°C 2 days	Agar 30°C 5 days	Agar 37°C 1 day	Agar 37°C 2 days	Agar 37°C 5 days
pRK415	++	++	++	++	++	++
pRKACC	++	+++	++++	+++	++++	++++
pUC319	+	++	++	+	++	++
pUC320	+	++	+++	+	++	+++
pUC324	+	++	+++	++	+++	+++
pUC327	+	+	+++	++	++	+++

Symbols used: +, ++, +++, ++++, increasing–from low to high–levels of growth.

Another clone, pUC327, also grew well at both temperatures, but not as quickly as pUC324. Two other clones, pUC319 and pUC320, grew very well on plates at 30°C but showed slower growth in liquid cultures at this temperature. At 37°C, pUC319 grew slowly in liquid cultures and on plates, whereas pUC320 grew very quickly in both liquid and solid media.

The ACC deaminase activity of the clones was measured and compared with that of the control strains (Table 16). The enzyme activity of both the positive control, *Escherichia coli* with pRKACC, and the negative control, *Escherichia coli* with pRK415, were consistently high and low, respectively. However, ACC deaminase activity of the clones and *Enterobacter cloacae* CAL3 fluctuated slightly. The ACC deaminase activity of *Enterobacter cloacae* CAL3 is lower than in some of the other plant growth-promoting strains isolated in our lab, i.e., about 50% lower than the activity in *Enterobacter cloacae* UW4 or *Pseudomonas fluorescens* CAL1 and approximately 80% lower than the activity in *Enterobacter cloacae* CAL2. Nonetheless, the ACC deaminase activity found in *Enterobacter cloacae* CAL3 was still higher than the activity in any of the clones from the genomic library of *Enterobacter cloacae* CAL3. The highest level of ACC deaminase activity was seen in pUC324. Several clones, such as pUC319 and pUC320 showed about 50% of the activity seen in *Enterobacter cloacae* CAL3, and the rest of the clones, including pUC327, had lower levels of enzyme activity.

Table 16: Characterization of the *Escherichia control* strains, *Enterobacter cloacae* CAL3 and four *Enterobacter cloacae* CAL3 clones for their level of ACC deaminase activity.

Sample name	ACC deaminase activity μmoles α-ketobutyrate/h/mg protein
E. coli + pRK415	–0.26 ± 0.03
E. coli + pRKACC	18.67 ±0.29
Enterobacter cloacae CAL3	5.86 ± .10
E. coli + pUC 319	2.65 ± 0.12
E. coli + pUC 320	2.49 ± 0.04
E. coli + pUC 324	3.67 ± 0.07
E. coli + pUC 327	1.54 ± 0.15

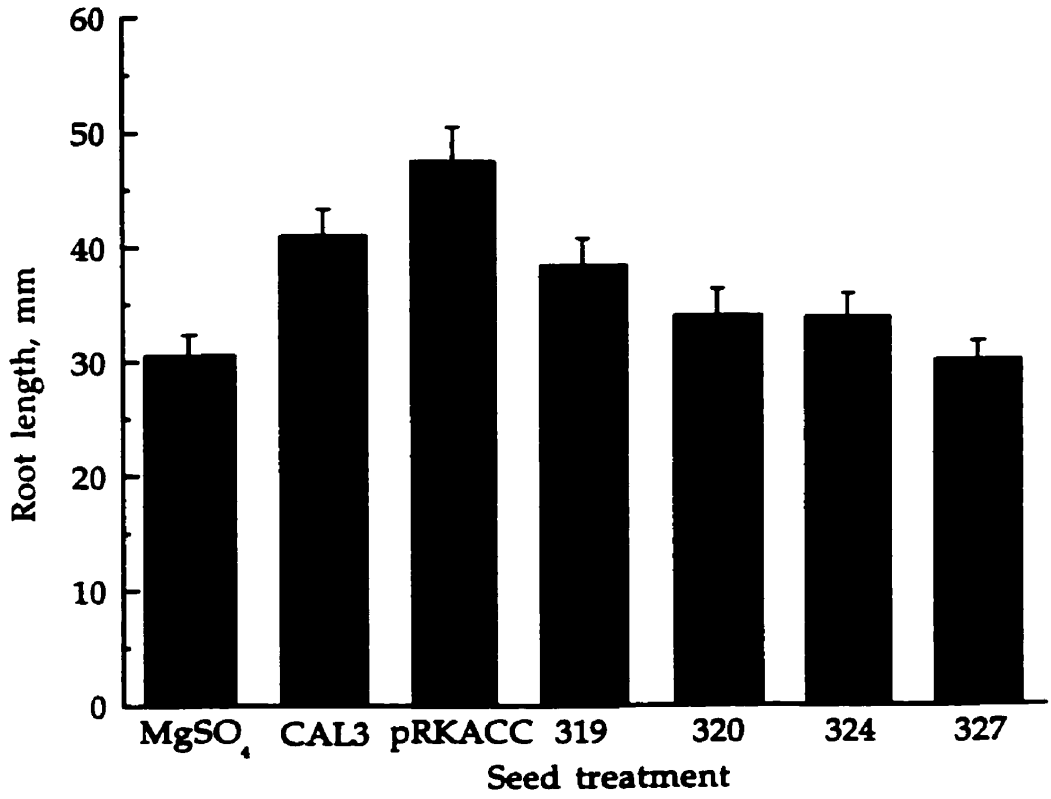
The measurements include standard errors of the means.

Each of the four selected clones was assayed for plant growth-promotion. A root elongation assay was used to assess the growth-promotion cabilities of these clones. The extent of root elongation, of 4.5-day old plants from seeds inoculated with various treatments, were determined by this assay. The roots of canola seedlings, grown from seeds treated with 0.03M $MgSO_4$, or one of these bacteria: *Enterobacter cloacae* CAL3, *Escherichia coli* pRKACC, pUC319, pUC320, pUC324 or pUC327 suspended in 0.03M $MgSO_4$ at an absorbance of 0.15 at 600 nm, were measured. In Fig. 39, it is evident that the shortest roots were seen in the control plants from seeds treated with 0.03M $MgSO_4$. Roots from seeds treated with *Enterobacter cloacae* CAL3 and an *Escherichia coli* strain transformed with a broad host range plasmid carrying the gene for ACC deaminase, *Escherichia coli* pRKACC, were 28.5% (P value of 0.0001), and 37.4% (P value of <0.0001) longer, respectively than roots from the control plants. The roots from seeds treated with the clone pUC319 were significantly longer (22.6%; P value of 0.0117) than those from the control seeds, but the roots of the seeds treated with pUC320, pUC324 or pUC327, were not appreciably longer (12.6%, 12.2%, and 1.3% respectively with P values of 0.2570, 0.6762 and 0.8750 respectively).

3.12.4. Analysis of the DNA sequences

The DNA inserts of the three clones: pUC319, pUC320 and pUC324 were sequenced in both directions. Both ends of the insert from

Fig. 39. Root and shoot length of canola seedlings. Mean root and shoot lengths of 4.5-day old canola seedlings assessed by means of the root elongation assay. Measurements were made from 60 seedlings (6 seeds/growth pouch, 10 growth pouches). Seed treatment abbreviations: $MgSO_4$, 0.03M $MgSO_4$; CAL3, *Enterobacter cloacae* CAL3; pRKACC, *Pseudomonas putida* pRKACC; pUC319, pUC320, pUC324 and pUC327, clones of *Enterobacter cloacae* CAL3 DNA cloned into pUC18. The error bars represent standard errors of the means.


60
50
40
30
20
10
0
Root length, mm
MgSO4
CAL3
pRKACC
319
320
324
327
Seed treatment

pUC327, which was much larger than the others–approximately 6.5 kb in size–have been sequenced. The computer program, DNA Strider 1.2, (Christian Marck, Service de Biochimie et de Génétique Moléculaire, France) was used to derive amino acid sequences from the DNA sequences and the deduced amino acid sequences were aligned with similar sequences using a BLAST search (http://www.ncbi.nim.nih.gov/blast/blast.cgi?)

3.12.4.1. Analysis of the DNA sequence from clone pUC319

Clone pUC319 contained 2256 bp of DNA (Fig. 40) which was inserted into B*am*HI of the multiple cloning site of pUC18, in the direction, 5'to 3', from *Eco*RI to *Hind* III. This clone appeared to have two open reading frames beginning at 286 bp and 1383 bp (Fig. 41). There were 1094 bp in the first open reading frame which suggested a protein of 365 amino acids. Results from a BLAST search indicated a similarity (identities = 300/365, 82%; positives = 313/365, 85%) with the flagellar basal body P-ring protein precursor from *Salmonella typhimurium* (Fig. 42). The second open reading frame contained 854 bp which appeared to include the first 90% of a gene that encoded the flagellar protein flgJ (316 amino acids in length) similar to that of *Salmonella typhimurium* (identities = 217/285, 76%; positives = 238/285, 85%) (Fig. 43). It is quite likely that the gene for this protein and the

Fig. 40. Nucleotide sequence of pUC319, a positive clone screened from the *Enterobacter cloacae* CAL3 genomic library.

GTTCCGCTCCTGCCTGCAGGTCGACTCTAGAGGATCCTGGGCGCGGTGTT 50
TGGCCATACCGTGCTGAATCTGATGAAGATCGCCATTAACCAGGGCACTG 100
AGTTCATTCGCTTCTCCGGTGtGGTTAACCCTCGCACCATCAGCGGCACC 150
AACACCGTACCGTCCACCCAGGTGGCGGATGCGCGCATTGAATACGTCGG 200
TAACGGCTATATCAATGAAGCGCAAAATATGGGCTGGCTGCAGCGTTTCT 250
TCCTTAACTTATCGCCGATGTAAGCGAGGTGACCCATGTTTAAAACGATC 300
TTCGCCGTGGCGCTGGCGCTGGTGGCAACCTTCGCTCAGGCTGACCGTAT 350
CCGCGACCTGACCAGCGTTCAGGGCGTGCGCGAAAACTCGCTGATTGGCT 400
ACGGCCTGGTGGTGGGGCTGGATGGTACGGGCGACCAGACCACCCAGACG 450
CCGTTCACCACCCAAAGCCTGAACAACATGCTTTCTCAGCTCGGTATTAC 500
CGTCCCTGCGGGAACCAACATGCAGCTGAAAAACGTGGCTGCGGTGATGG 550
TCACCGCGTCCTATCCGGCGTTCGCGCGTCAGGGGCAGACCATCGACGTG 600
GTGGTCTCTTCCATGGGTAACGCCAAAAGCCTGCGCGGCGGCACGCTGTT 650
GATGACGCCGCTTAAGGGCGTCGACAGCCAGGTCTATGCCCTGGCGCAGG 700
GTAACATTCTGGTTGGCGGTGCGGGTGCCTCTGCGGGCGGGAGCAGCGTG 750
CAgGTTAACCAGCTGAACGGCGGGCGCATCACCAACGGGGCCATCATTGA 800
GCGCGAGCTGCCGACCCAGTTTGGCTCAGGAAACACCATCAACCTGCAGC 850
TCAACAATGAAGACTTCACGATGGCGCAGCAAATTGCCGATACCATCAAC 900
CGCAGCCGcGGCTACGGCAGCGCCACGGCGCTGGACGCGCGTACCGTGCA 950
GATCCGGACCTCTTCGGGTAGCAGTAACCAGGTGCGCATGCTGGCAGATA 1000
TCCAGAATATGGAAGTGAACGTGCCTGTTCAGGATGCCAAAGTCATCATC 1050
AACTCACGCACCGGGTCGGTGGTGATGAACCGCGAGGTGTCGCTGGACAG 1100
CTGTGCCGTGGCGCAGGGTAACCTCTCCGTGACGGTGAACCGCTCTGCCA 1150
ACGTCAGCCAGCCGGACACGCCGTTTGGTGGCGGTCAGACGGTGGTGACA 1200
CCGCAAACGCAGATTGATTTGCGCCAGAGCGGCGGCTCGCTGCAAAGCGT 1250
GCGTTCCAGCGCGAACCTGAACAGCGTGGTGCGCGCCCTGAATGCGCTGG 1300
GCGCGACGCCGATGGATCTGATGTCGATCCTGCAATCAATGCAAAGTGCA 1350
GGCTGCCTGCGCGCGAAGCTGGAAATCATCTAATGCTGACCGATAGCAAA 1400
CTGCTGACCGGTGCCGCCTGGGATGCCCAGTCGCTTAACGAACTGAAAAC 1450
CAAAGCAGGTAAAGACCCGGCGGCGAATATCCGCCCGGTCGCCCGCCAGG 1500
TGGAGGGGATGTTCGTGCAGATGATGCTGAAAAGCATGCGTGAAACCCTG 1550
CCGAAAGACGGGATGTTCAGCAGCGATTCCACGCGGCTTTACACCAGCAT 1600
GTATGACCAGCAGATTGCGCAGCAGATGACCGCCGGTAAAGGACTCGGTC 1650
TGGCTGACATGATTGTCAAACAGACCGCTGCCGCACAGGGTATTCAGCCT 1700
GAGGATCAGCCGCAGCAGGTGCCAATGAAGTTCGACATCGAAACGGTGAC 1750
CAGTTATCAGAACCAGGCGCTGACGCAGATGGTGCGCAAGGCGATGCCGA 1800
AGCCCGCAGAGACGCGCGACGAGCCGCTCTCCGGCGACAGTAAGGACTTC 1850
CTTGCGCAGCTTTCTCTGCCTGCGCGGCTGGCCAGCGAGCAGAGCGGGGT 1900
ACCGCATCACCTGATCCTGGCCCAGGCGGCGCTGGAGTCGGGCTGGGGCC 1950
AGCGTCAGATCCGTAAGGAAAACGGCGAGCCGAGCTTCAACATCTTTGGC 2000
GTGAAAGCGACCTCCAGCTGGAAGGGGCCGACAACCGAGATCACCACCAC 2050
CGAATACGAAAACGGCGCGGCAGTGAAGGTTAAAGCCAAATTCCGCGTTT 2100
ACAGCTCCTACCTTGAAGCATTGTCAGATTACGTCGGCCTGTTAAGCCGT 2150
AACCCGCGCTATACCGCCGTGACGCAGGCATCGACGCCGGAGCAGGGCGC 2200
GCAGGCATTGCAGAATGCGGGATACGCTACCGATCCCCGGGTACCGAGTG 2250
GATTTG 2256

Fig. 41. The open reading frames of pUC319 that correspond to *Salmonella typhimuium* flagellar basal body P-ring protein precursor (1094 bp) and *Salmonella typhimuium* flagellar basal body protein flgJ (854 bp) are shown in bold face type and underlined bold face type, respectively.

```
1/1                                                 31/11
GTT CCG CTC CTG CCT GCA GGT CGA CTC TAG AGG ATC CTG GGC GCG GTG TTT GGC CAT ACC
V   P   L   L   P   A   G   R   L   *   R   I   L   G   A   V   F   G   H   T
 F   R   S   C   L   Q   V   D   S   R   G   S   W   A   R   C   L   A   I   P
  S   A   P   A   C   R   S   T   L   E   D   P   G   R   G   V   W   P   Y   R
61/21                                               91/31
GTG CTG AAT CTG ATG AAG ATC GCC ATT AAC CAG GGC ACT GAG TTC ATT CGC TTC TCC GGT
V   L   N   L   M   K   I   A   I   N   Q   G   T   E   F   I   R   F   S   G
 C   *   I   *   *   R   S   P   L   T   R   A   L   S   S   F   A   S   P   V
  A   E   S   D   E   D   R   H   *   P   G   H   *   V   H   S   L   L   R   C
121/41                                              151/51
GtG GTT AAC CCT CGC ACC ATC AGC GGC ACC AAC ACC GTA CCG TCC ACC CAG GTG GCG GAT
V   V   N   P   R   T   I   S   G   T   N   T   V   P   S   T   Q   V   A   D
 W   L   T   L   A   P   S   A   A   P   T   P   Y   R   P   P   R   W   R   M
  G   *   P   S   H   H   Q   R   H   Q   H   R   T   V   H   P   G   G   G   C
181/61                                              211/71
GCG CGC ATT GAA TAC GTC GGT AAC GGC TAT ATC AAT GAA GCG CAA AAT ATG GGC TGG CTG
A   R   I   E   Y   V   G   N   G   Y   I   N   E   A   Q   N   M   G   W   L
 R   A   L   N   T   S   V   T   A   I   S   M   K   R   K   I   W   A   G   C
  A   H   *   I   R   R   *   R   L   Y   Q   *   S   A   K   Y   G   L   A   A
241/81                                              271/91
CAG CGT TTC TTC CTT AAC TTA TCG CCG ATG TAA GCG AGG TGA CCC ATG TTT AAA ACG ATC
Q   R   F   F   L   N   L   S   P   M   *   A   R   *   P   M   F   K   T   I
 S   V   S   S   L   T   Y   R   R   C   K   R   G   D   P   C   L   K   R   S
  A   F   L   P   *   L   I   A   D   V   S   E   V   T   H   V   *   N   D   L
301/101                                             331/111
TTC GCC GTG GCG CTG GCG CTG GTG GCA ACC TTC GCT CAG GCT GAC CGT ATC CGC GAC CTG
F   A   V   A   L   A   L   V   A   T   F   A   Q   A   D   R   I   R   D   L
 S   P   W   R   W   R   W   W   Q   P   S   L   R   L   T   V   S   A   T   *
  R   R   G   A   G   A   G   G   N   L   R   S   G   *   P   Y   P   R   P   D
361/121                                             391/131
ACC AGC GTT CAG GGC GTG CGC GAA AAC TCG CTG ATT GGC TAC GGC CTG GTG GTG GGG CTG
T   S   V   Q   G   V   R   E   N   S   L   I   G   Y   G   L   V   V   G   L
 P   A   F   R   A   C   A   K   T   R   *   L   A   T   A   W   W   W   G   W
  Q   R   S   G   R   A   R   K   L   A   D   W   L   R   P   G   G   G   A   G
421/141                                             451/151
GAT GGT ACG GGC GAC CAG ACC ACC CAG ACG CCG TTC ACC ACC CAA AGC CTG AAC AAC ATG
D   G   T   G   D   Q   T   T   Q   T   P   F   T   T   Q   S   L   N   N   M
 M   V   R   A   T   R   P   P   R   R   R   S   P   P   K   A   *   T   T   C
  W   Y   G   R   P   D   H   P   D   A   V   H   H   P   K   P   E   Q   H   A
481/161                                             511/171
CTT TCT CAG CTC GGT ATT ACC GTC CCT GCG GGA ACC AAC ATG CAG CTG AAA AAC GTG GCT
L   S   Q   L   G   I   T   V   P   A   G   T   N   M   Q   L   K   N   V   A
 F   L   S   S   V   L   P   S   L   R   E   P   T   C   S   *   K   T   W   L
  F   S   A   R   Y   Y   R   P   C   G   N   Q   H   A   A   E   K   R   G   C
541/181                                             571/191
GCG GTG ATG GTC ACC GCG TCC TAT CCG GCG TTC GCG CGT CAG GGG CAG ACC ATC GAC GTG
A   V   M   V   T   A   S   Y   P   A   F   A   R   Q   G   Q   T   I   D   V
 R   *   W   S   P   R   P   I   R   R   S   R   V   R   G   R   P   S   T   W
  G   D   G   H   R   V   L   S   G   V   R   A   S   G   A   D   H   R   R   G
601/201                                             631/211
GTG GTC TCT TCC ATG GGT AAC GCC AAA AGC CTG CGC GGC GGC ACG CTG TTG ATG ACG CCG
V   V   S   S   M   G   N   A   K   S   L   R   G   G   T   L   L   M   T   P
 W   S   L   P   W   V   T   P   K   A   C   A   A   A   R   C   *   *   R   R
  G   L   F   H   G   *   R   Q   K   P   A   R   R   H   A   V   D   D   A   A
661/221                                             691/231
CTT AAG GGC GTC GAC AGC CAG GTC TAT GCC CTG GCG CAG GGT AAC ATT CTG GTT GGC GGT
L   K   G   V   D   S   Q   V   Y   A   L   A   Q   G   N   I   L   V   G   G
 L   R   A   S   T   A   R   S   M   P   W   R   R   V   T   F   W   L   A   V
  *   G   R   R   Q   P   G   L   C   P   G   A   G   *   H   S   G   W   R   C
```

```
721/241                                                 751/251
GCG GGT GCC TCT GCG GGC GGG AGC AGC GTG CAg GTT AAC CAG CTG AAC GGC GGG CGC ATC
A  G  A  S  A  G  G  S  S  V  Q  V  N  Q  L  N  G  G  R  I
 R  V  P  L  R  A  G  A  A  C  R  L  T  S  *  T  A  G  A  S
  G  C  L  C  G  R  E  Q  R  A  G  *  P  A  E  R  R  A  H  H
781/261                                                 811/271
ACC AAC GGG GCC ATC ATT GAG CGC GAG CTG CCG ACC CAG TTT GGC TCA GGA AAC ACC ATC
T  N  G  A  I  I  E  R  E  L  P  T  Q  F  G  S  G  N  T  I
 P  T  G  P  S  L  S  A  S  C  R  P  S  L  A  Q  E  T  P  S
  Q  R  G  H  H  *  A  R  A  A  D  P  V  W  L  R  K  H  H  Q
841/281                                                 871/291
AAC CTG CAG CTC AAC AAT GAA GAC TTC ACG ATG GCG CAG CAA ATT GCC GAT ACC ATC AAC
N  L  Q  L  N  N  E  D  F  T  M  A  Q  Q  I  A  D  T  I  N
 T  C  S  S  T  M  K  T  S  R  W  R  S  K  L  P  I  P  S  T
  P  A  A  Q  Q  *  R  L  H  D  G  A  A  N  C  R  Y  H  Q  P
901/301                                                 931/311
CGC AGC CGc GGC TAC GGC AGC GCC ACG GCG CTG GAC GCG CGT ACC GTG CAG ATC CGG ACC
R  S  R  G  Y  G  S  A  T  A  L  D  A  R  T  V  Q  I  R  T
 A  A  A  A  T  A  A  P  R  R  W  T  R  V  P  C  R  S  G  P
  Q  P  R  L  R  Q  R  H  G  A  G  R  A  Y  R  A  D  P  D  L
961/321                                                 991/331
TCT TCG GGT AGC AGT AAC CAG GTG CGC ATG CTG GCA GAT ATC CAG AAT ATG GAA GTG AAC
S  S  G  S  S  N  Q  V  R  M  L  A  D  I  Q  N  M  E  V  N
 L  R  V  A  V  T  R  C  A  C  W  Q  I  S  R  I  W  K  *  T
  F  G  *  Q  *  P  G  A  H  A  G  R  Y  P  E  Y  G  S  E  R
1021/341                                                1051/351
GTG CCT GTT CAG GAT GCC AAA GTC ATC ATC AAC TCA CGC ACC GGG TCG GTG GTG ATG AAC
V  P  V  Q  D  A  K  V  I  I  N  S  R  T  G  S  V  V  M  N
 C  L  F  R  M  P  K  S  S  S  T  H  A  P  G  R  W  *  *  T
  A  C  S  G  C  Q  S  H  H  Q  L  T  H  R  V  G  G  D  E  P
1081/361                                                1111/371
CGC GAG GTG TCG CTG GAC AGC TGT GCC GTG GCG CAG GGT AAC CTC TCC GTG ACG GTG AAC
R  E  V  S  L  D  S  C  A  V  A  Q  G  N  L  S  V  T  V  N
 A  R  C  R  W  T  A  V  P  W  R  R  V  T  S  P  *  R  *  T
  R  G  V  A  G  Q  L  C  R  G  A  G  *  P  L  R  D  G  E  P
1141/381                                                1171/391
CGC TCT GCC AAC GTC AGC CAG CCG GAC ACG CCG TTT GGT GGC GGT CAG ACG GTG GTG ACA
R  S  A  N  V  S  Q  P  D  T  P  F  G  G  G  Q  T  V  V  T
 A  L  P  T  S  A  S  R  T  R  R  L  V  A  V  R  R  W  *  H
  L  C  Q  R  Q  P  A  G  H  A  V  W  W  R  S  D  G  G  D  T
1201/401                                                1231/411
CCG CAA ACG CAG ATT GAT TTG CGC CAG AGC GGC GGC TCG CTG CAA AGC GTG CGT TCC AGC
P  Q  T  Q  I  D  L  R  Q  S  G  G  S  L  Q  S  V  R  S  S
 R  K  R  R  L  I  C  A  R  A  A  A  R  C  K  A  C  V  P  A
  A  N  A  D  *  F  A  P  E  R  R  L  A  A  K  R  A  F  Q  R
1261/421                                                1291/431
GCG AAC CTG AAC AGC GTG GTG CGC GCC CTG AAT GCG CTG GGC GCG ACG CCG ATG GAT CTG
A  N  L  N  S  V  V  R  A  L  N  A  L  G  A  T  P  M  D  L
 R  T  *  T  A  W  C  A  P  *  M  R  W  A  R  R  R  W  I  *
  E  P  E  Q  R  G  A  R  P  E  C  A  G  R  D  A  D  G  S  D
1321/441                                                1351/451
ATG TCG ATC CTG CAA TCA ATG CAA AGT GCA GGC TGC CTG CGC GCG AAG CTG GAA ATC ATC
M  S  I  L  Q  S  M  Q  S  A  G  C  L  R  A  K  L  E  I  I
 C  R  S  C  N  Q  C  K  V  Q  A  A  C  A  R  S  W  K  S  S
  V  D  P  A  I  N  A  K  C  R  L  P  A  R  E  A  G  N  H  L
1381/461                                                1411/471
TAA TGC TGA CCG ATA GCA AAC TGC TGA CCG GTG CCG CCT GGG ATG CCC AGT CGC TTA ACG
*  C  *  P  I  A  N  C  *  P  V  P  P  G  M  P  S  R  L  T
 N  A  D  R  *  Q  T  A  D  R  C  R  L  G  C  P  V  A  *  R
 M  L  T  D  S  K  L  L  T  G  A  A  W  D  A  Q  S  L  M  E
```

1441/481 1471/491

AAC TGA AAA CCA AAG CAG GTA AAG ACC CGG CGG CGA ATA TCC GCC CGG TCG CCC GCC AGG

N * K P K Q V K T R R R I S A R S P A R

T E N Q S R * R P G G E Y P P G R P P G

L K T K A G K D P A A N I R P V A R Q V

1501/501 1531/511

TGG AGG GGA TGT TCG TGC AGA TGA TGC TGA AAA GCA TGC GTG AAA CCC TGC CGA AAG ACG

W R G C S C R * C * K A C V K P C R K T

G G D V R A D D A E K H A * N P A E R R

E G M F V Q M M L K S M R E T L P K D G

1561/521 1591/531

GGA TGT TCA GCA GCG ATT CCA CGC GGC TTT ACA CCA GCA TGT ATG ACC AGC AGA TTG CGC

G C S A A I P R G F T P A C M T S R L R

D V Q Q R F H A A L H Q H V * P A D C A

M F S S D S T R L Y T S M Y D Q Q I A Q

1621/541 1651/551

AGC AGA TGA CCG CCG GTA AAG GAC TCG GTC TGG CTG ACA TGA TTG TCA AAC AGA CCG CTG

S R * P P V K D S V W L T * L S N R P L

A D D R R * R T R S G * H D C Q T D R C

Q M T A G K G L G L A D M I V K Q T A A

1681/561 1711/571

CCG CAC AGG GTA TTC AGC CTG AGG ATC AGC CGC AGC AGG TGC CAA TGA AGT TCG ACA TCG

P H R V F S L R I S R S R C Q * S S T S

R T G Y S A * G S A A A G A N E V R H R

A Q G I Q P E D Q P Q Q V P M K F D I E

1741/581 1771/591

AAA CGG TGA CCA GTT ATC AGA ACC AGG CGC TGA CGC AGA TGG TGC GCA AGG CGA TGC CGA

K R * P V I R T R R * R R W C A R R C R

N G D Q L S E P G A D A D G A Q G D A E

T V T S Y Q M Q A L T Q M V R K A M P K

1801/601 1831/611

AGC CCG CAG AGA CGC GCG ACG AGC CGC TCT CCG GCG ACA GTA AGG ACT TCC TTG CGC AGC

S P Q R R A T S R S P A T V R T S L R S

A R R D A R R A A L R R Q * G L P C A A

P A E T R D E F L S G D S K D F L A Q L

1861/621 1891/631

TTT CTC TGC CTG CGC GGC TGG CCA GCG AGC AGA GCG GGG TAC CGC ATC ACC TGA TCC TGG

F L C L R G W P A S R A G Y R I T * S W

F S A C A A G Q R A E R G T A S P D P G

S L P A R L A S E Q S G V P H H L I L A

1921/641 1951/651

CCC AGG CGG CGC TGG AGT CGG GCT GGG GCC AGC GTC AGA TCC GTA AGG AAA ACG GCG AGC

P R R R W S R A G A S V R S V R K T A S

P G G A G V G L G P A S D P * G K R R A

Q A A L E S G W G Q R Q I R K E M G E F

1981/661 2011/671

CGA GCT TCA ACA TCT TTG GCG TGA AAG CGA CCT CCA GCT GGA AGG GGC CGA CAA CCG AGA

R A S T S L A * K R P P A G R G R Q P R

E L Q H L W R E S D L Q L E G A D N R D

S F N I F G V K A T S S W K G P T T E I

2041/681 2071/691

TCA CCA CCA CCG AAT ACG AAA ACG GCG CGG CAG TGA AGG TTA AAG CCA AAT TCC GCG TTT

S P P P N T K T A R Q * R L K P N S A F

H H H R I R K R R G S E G * S Q I P R L

T T T E Y E M G A A V K V K A K F R V Y

2101/701 2131/711

ACA GCT CCT ACC TTG AAG CAT TGT CAG ATT ACG TCG GCC TGT TAA GCC GTA ACC CGC GCT

T A P T L K H C Q I T S A C * A V T R A

Q L L P * S I V R L R R P V K P * P A L

S S Y L E A L S D Y V G L L S R M P R Y

```
2161/721                                                2191/731
ATA CCG CCG TGA CGC AGG CAT CGA CGC CGG AGC AGG GCG CGC AGG CAT TGC AGA ATG CGG
I   P   P   *   R   R   H   R   R   R   S   R   A   R   R   H   C   R   M   R
 Y   R   R   D   A   G   I   D   A   G   A   G   R   A   G   I   A   E   C   G
  T   A   V   T   Q   A   S   T   P   E   Q   G   A   Q   A   L   Q   M   A   G
2221/741                                                2251/751
GAT ACG CTA CCG ATC CCC GGG TAC CGA GTG GAT TTG
D   T   L   P   I   P   G   Y   R   V   D   L
 I   R   Y   R   S   P   G   T   E   W   I
  Y   A   T   D   P   R   V   P   S   G   F
```

Fig. 42. Alignment of the deduced amino acid sequence from pUC319 with the sequence of *Salmonella typhimurium* flagellar basal body P-ring protein precursor obtained from GenBank. The X's represent stretches of derived amino acids where the BLAST program finds no consensus with the amino acid sequences of the subject; the + represents a deduced amino acid that is similar but not identical to the amino acid in the subject sequence and that is not essential for the proper strucure of the protein. The numbers beside the sequences indicate the nucleotide numbers in each sequence.

Query: pUC319 from *Enterobacter cloacae* CAL3

Sbjct: Flagellar basal body P-ring protein precursor from *Salmonella typhimurium*, P19530 (Jones et al., 1989)

```
Query: 286  MFKTIXXXXXXXXXXXXXXXXDRIRDLTSVQGVRENSLIGYGLVVGLXXXXXXXXXXXXXXXXX 465
             M K +               +RIRDLTSVQGVR+NSLIGYGLVVGL
Sbjct: 1    MIKFLSALILLLVTTAAQAERIRDLTSVQGVRQNSLIGYGLVVGLDGTGDQTTQTPFTTQ 60

Query: 466  SLNNMLSQLGITVPAGTNMQLKNVAAVMVTASYPAFARQGQTIDVVVSSMGNAKSLRGGT 645
             +LNNMLSQLGITVP GTNMQLKNVAAVMVTAS P F RQGQTIDVVVSSMGNAKSLRGGT
Sbjct: 61   TLNNMLSQLGITVPTGTNMQLKNVAAVMVTASLPPFGRQGQTIDVVVSSMGNAKSLRGGT 120

Query: 646  LLMTPLKGVDSQVYALAQGNILVXXXXXXXXXXXXVQVNQLNGGRITNGAIIERELPTQFG 825
             LLMTPLKGVDSQVYALAQGNILV            VQVNQLNGGRITNGA+IERELP+QFG
Sbjct: 121  LLMTPLKGVDSQVYALAQGNILVGGAGASAGGSSVQVNQLNGGRITNGAVIERELPSQFG 180

Query: 826  SGNTINLQLNNEDFTMAQQIADTINRSRGYGSATALDARTVQIRTSSGSSNQVRMLADIQ 1005
              GNT+NLQLN+EDF+MAQQIADTINR RGYGSATALDART+Q+R   SG+S+QVR LADIQ
Sbjct: 181  VGNTLNLQLNDEDFSMAQQIADTINRVRGYGSATALDARTIQVRVPSGNSSQVRFLADIQ 240

Query: 1006 NMEVNVPVQDAKVIINSRTGSVVMNREVSLDSCAVAQGNLSVTVNRSANVSQPDTPFGGG 1185
             NM+VNV  QDAKV+INSRTGSVVMNREV+LDSCAVAQGNLSVTVNR ANVSQPDTPFGGG
Sbjct: 241  NMQVNVTPQDAKVVINSRTGSVVMNREVTLDSCAVAQGNLSVTVNRQANVSQPDTPFGGG 300

Query: 1186 QTVVTPQTQIDLRQSGGSLQSVRSSANLNSVVRALNALGATPMDLMSILQSMQSAGCLRA 1365
             QTVVTPQTQIDLRQSGGSLQSVRSSA+LN+VVRALNALGATPMDLMSILQSMQSAGCLRA
Sbjct: 301  QTVVTPQTQIDLRQSGGSLQSVRSSASLNNVVRALNALGATPMDLMSILQSMQSAGCLRA 360

Query: 1366 KLEII 1380
             KLEII
Sbjct: 361  KLEII 365
```

Fig. 43. Alignment of the deduced amino acid sequence from pUC319 with the sequence of *Salmonella typhimurium* flagellar basal body flgJ obtained from GenBank. The X's represent stretches of derived amino acids where the BLAST program finds no consensus with the amino acid sequences of the subject; the + represents a deduced amino acid that is similar but not identical to the amino acid in the subject sequence and that is not essential for the proper structure of the protein. The numbers beside the sequences indicate the nucleotide numbers in each sequence.

Query: pUC319 from *Enterobacter cloacae* CAL3

Sbjct: Flagellar basal body protein from *Salmonella typhimurium*, P19531. (Jones et al., 1989)

```
Query: 1383 MLTDSKLLTGAAWDAQSLNELKTQSXXXXXXXXXXXXXXXXXXMFVQMMLKSMRETLPKDG 1562
            M++DSKLL  AAWDAQSLNELK ++                   MFVQMMLKSMR+ LPKDG
Sbjct: 1    MISDSKLLASAAWDAQSLNELKAKAGEDPAANIRPVARQVEGMFVQMMLKSMRDALPKDG 60

Query: 1563 MFSSDSTRLYTSMYDQQIAQQMTAGKGLGLADMIVKQTAAAQGIQPEDQPQQVPMKFDIE 1742
            +FSS+ TRLYTSMYDQQIAQQMTAGKGLGLA+M+VKQ    Q + PE+     PMKF +E
Sbjct: 61   LFSSEHTRLYTSMYDQQIAQQMTAGKGLGLAEMMVKQMTPEQPL-PEESTPAAPMKFPLE 119

Query: 1743 TVTSYQNQALTQMVRKAMPKPAETRDEPLSGDSKDFLAQLSLPARLASEQSGVPHHLILA 1922
            TV  YQNQAL+Q+V+KA+P+     D+ L GDSK FLAQLSLPA+LAS+QSGVPHHLILA
Sbjct: 120  TVVRYQNQALSQLVQKAVPR---NYDDSLPGDSKAFLAQLSLPAQLASQQSGVPHHLILA 176

Query: 1923 QAALESGWGQRQIRKENGEPSFNIFGVKATSSWKGPTTEITTTEYENGAAVKVKAKFRVY 2102
            QAALESGWGQRQIR+ENGEPS+N+FGVKA+ +WKGP TEITTTEYENG A KVKAKFRVY
Sbjct: 177  QAALESGWGQRQIRRENGEPSYNLFGVKASGNWKGPVTEITTTEYENGEAKKVKAKFRVY 236

Query: 2103 SSYLEALSDYVGLLSRNPRYTAVTQASTPEQGAQALQNAGYATDP 2237
            SSYLEALSDYVGLL+RNPRY AVT A++ EQGAQALQ+AGYATDP
Sbjct: 237  SSYLEALSDYVGLLTRNPRYAAVTTAASAEQGAQALQDAGYATDP 281
```

gene for the flagellar basal body P-ring protein precursor are part of the same operon.

3.12.4.2. Analysis of the DNA sequence from clone pUC320

There was an insert 1978 bp long inserted in the *Bam*HI site of pUC18 in the 5′ to 3′ direction of *Eco*RI to *Hind*III (Fig. 44). Two open reading frames, from 2 to 770 bp and from 787 to 1978 bp, that suggested proteins of 256 and 324 amino acids in length, respectively, were indicated by the DNA Strider 1.2 program (Fig. 45), but there was no match with known DNA sequences when GenBank was searched.

3.12.4.3. Analysis of the DNA sequence from clone pUC324

Following agarose gel electrophoresis, the DNA insert appeared to be appproximately 2.6 kb in length, very close to the size of pUC18. After sequencing, it was evident that the inserted DNA was only 318 bp in length (Fig. 46). The DNA, which was inserted 5′ end to 3′ end, in the multiple cloning site, in the *Hind*III to *Eco*RI direction, appeared to be one open reading frame (Fig. 47). The deduced amino acid sequence was a good match for a portion of a gene that encoded uracil phosphoribosyltransferase from *Escherichia coli* (identities = 94/99, 94%; positives = 99/99, 99%) (Fig. 48). However, of the 217 amino acids encoded by uracil phosphoribosyltransferase, the nucleotides which

Fig. 44. Nucleotide sequence of pUC320, a positive clone screened from the *Enterobacter cloacae* CAL3 genomic library.

CTTGCTTGCCTGCAGGTCGACTCTAGAGGATCCTAAGCTATTAGACCCTA 50
TACTTGAACAGCTTTGCACCCTCATTGAAAGCGATAATGAAAAACAAAGT 100
GCTGTAGCTACTTATTGCCTTGCTGAAATATTTCGACATTCAGCAATGAC 150
TATTCATAAAAAAGATACCTATGCATTCCCTGCGCAAGCCAATGTGGATG 200
GGTACTTTGAAAAAATACAACATTGTGCCGCGACATTCATTAATAAGCGC 250
AGCGCCTCTGACAACGAAACTTGGAATCTGTTAATTAATCAGGCTAGTTT 300
TCTGTTGCTTGTGCGTTTAGATAATACATTAGAAAAAAATGGCACTGATG 350
CCAGGCATGATCTTATCTTAAAACTGGCATCAGGCTTTAGAACAATTACA 400
CTTCCCACTAAAATGGATAGCAAGACTATAGCCTCATGTATTTTGCTGGC 450
TAGTCAATTAGTTAAAGATAACAAACCATTTATTCGCTCATGTGCTTCTT 500
TGTGCGAAGGAATTTATGACAAAGAACACGTCATAAAATTGAAGAAAATA 550
GTTAGCATAATATCACATCAAAACTTATCATTGTTTAAATCCTTAGTTTA 600
TCATTCACGACCTTTACAACAGAAGTGGCTAAACTCAGACTCCGTGAAAA 650
CAATAATTAATGAATGCCATATAGATATACAACCTTTGGCGACTTCTTTA 700
GGCATGATAAAAAGTAGTCACTCATTACTTAGAATCTATCAAGATCTGAT 750
AACCCATTTTCCAATGAGATAATGGCATTAAAACTGATGCAAGCCCTTTT 800
ATTGGACAGGATTGTTTGCCTGGATAATAAAAAAGATTATCAAATAAGTG 850
TAGCAAACACTAAAGTGACGTTTCATAACTACTCCAACCCTCCAACATCG 900
AATGTCTTCGATGCAGGAATGGATGTGGATGCAAAATTATTCAAATCATC 950
GGGATGGGTCGATTCTATTTTCACGGATGATGCAGACACTCAAATATTGT 1000
ATAGAGTTGCCATGTGCATCCGATCAGTACTACTCGGCAAACAAGACTGG 1050
ACAGATTTTGGTCAAGCAATTTCCCCCAAACAGGGTTATCGGGGTATTAA 1100
AACTAGTAGAGACAAACGTCAATTGGGGATGATGACAACACCTGAGTCCA 1150
TTGCCGGTGAGAACTCTCAGGTTTCTGGTTGGCTTACCACACTCTTATCC 1200
AAGTTGCTTGCCTGGCCGGGAATTTCAGTGGGTGATAATGGATATCAATG 1250
GCCAGCAATTTTTACAGTAGATGCTGTCAGAAAACTAGTTGATGCTCGGC 1300
TGAGTAAGCTTAAGCAGGATTACTGCAAACTATCAGGAACTCCGGGACTT 1350
ACAGAAAAAATACAGTTCGACTGGTCTGACTCGAAAAAAGCCCTAACAGT 1400
TGCTATGGTCCAGTCAAAACTGCCTGCAACGAAAGATTTTGCCAGCCATG 1450
GACTTCTTTTAAACTCCGCAAAATACAGAGTGATTCATCGCAGACATGTT 1500
GCTGAAGTGGCTGATTTAGTTGTAAAACACACGCTTGCACAAAAAACAAC 1550
TCAACGAACTCATGGTGAAAAAATAGAGAACATTGATTTAATAGTATGGC 1600
CTGAGCTCGCTGTACATAGTGACGATTTGGATGTACTCATCGCCTTATCT 1650
AGAAAAACCAATGCAATCATATACTCGGGCCTGACATTTATTGAGCAACC 1700
TGGAATCAAAGGACCAAATAATTGTGCCGTTTGGATTGTCCCACCTAAAA 1750
GCAATAGCAGCCAGAAAGAAATGATAAGACTTCAAGGCAAGCATAATATG 1800
ATGGAAGATGAGAAAGGCCGGGTTGAACCCTGGAGACCATACCAATTGAT 1850
GCTTGAaCTTGTTCACCCACAATTCCCTGATAAAAAAGGATTTATTCTCA 1900
CAGGCTCCATTTGTTATGACGCAACCGACATCGCACTAAGTGCAGATCCC 1950
CGGGTACCGAGCCGAATTCGTATCCGAA 1978

Fig. 45. The two open reading frames of pUC320 are shown in bold face type.

```
1/1                                                 31/11
CCT GCT TGC CTG CAG GTC GAC TCT AGA GGA TCC TAA GCT ATT AGA CCC TAT ACT TGA ACA
L   A   C   L   Q   V   D   S   R   G   S   *   A   I   R   P   Y   T   *   T
 L   L   A   C   R   S   T   L   E   D   P   K   L   L   D   P   I   L   E   Q
  C   L   P   A   G   R   L   *   R   I   L   S   Y   *   T   L   Y   L   N   S
61/21                                               91/31
GCT TTG CAC CCT CAT TGA AAG CGA TAA TGA AAA ACA AAG TGC TGT AGC TAC TTA TTG CCT
A   L   H   P   H   *   K   R   *   *   K   T   K   C   C   S   Y   L   L   P
 L   C   T   L   I   E   S   D   M   E   K   Q   S   A   V   A   T   Y   C   L
  F   A   P   S   L   K   A   I   M   K   N   K   V   L   *   L   L   I   A   L
121/41                                              151/51
TGC TGA AAT ATT TCG ACA TTC AGC AAT GAC TAT TCA TAA AAA AGA TAC CTA TGC ATT CCC
C   *   N   I   S   T   F   S   N   D   Y   S   *   K   R   Y   L   C   I   P
 A   E   I   F   R   H   S   A   M   T   I   H   K   K   D   T   Y   A   F   P
  L   K   Y   F   D   I   Q   Q   *   L   F   I   K   K   I   P   M   H   S   L
181/61                                              211/71
TGC GCA AGC CAA TGT GGA TGG GTA CTT TGA AAA AAT ACA ACA TTG TGC CGC GAC ATT CAT
C   A   S   Q   C   G   W   V   L   *   K   N   T   T   L   C   R   D   I   H
 A   Q   A   M   V   D   G   Y   F   E   K   I   Q   H   C   A   A   T   F   I
  R   K   P   M   W   M   G   T   L   K   K   Y   N   I   V   P   R   H   S   L
241/81                                              271/91
TAA TAA GCG CAG CGC CTC TGA CAA CGA AAC TTG GAA TCT GTT AAT TAA TCA GGC TAG TTT
*   *   A   Q   R   L   *   Q   R   N   L   E   S   V   N   *   S   G   *   F
 M   K   R   S   A   S   D   M   E   T   W   M   L   L   I   M   Q   A   S   F
  I   S   A   A   P   L   T   T   K   L   G   I   C   *   L   I   R   L   V   F
301/101                                             331/111
TCT GTT GCT TGT GCG TTT AGA TAA TAC ATT AGA AAA AAA TGG CAC TGA TGC CAG GCA TGA
S   V   A   C   A   F   R   *   Y   I   R   K   K   W   H   *   C   Q   A   *
 L   L   L   V   R   L   D   M   T   L   E   K   M   G   T   D   A   R   H   D
  C   C   L   C   V   *   I   I   H   *   K   K   M   A   L   M   P   G   M   I
361/121                                             391/131
TCT TAT CTT AAA ACT GGC ATC AGG CTT TAG AAC AAT TAC ACT TCC CAC TAA AAT GGA TAG
S   Y   L   K   T   G   I   R   L   *   N   N   Y   T   S   H   *   N   G   *
 L   I   L   K   L   A   S   G   F   R   T   I   T   L   P   T   K   M   D   S
  L   S   *   N   W   H   Q   A   L   E   Q   L   H   F   P   L   K   W   I   A
421/141                                             451/151
CAA GAC TAT AGC CTC ATG TAT TTT GCT GGC TAG TCA ATT AGT TAA AGA TAA CAA ACC ATT
Q   D   Y   S   L   M   Y   F   A   G   *   S   I   S   *   R   *   Q   T   I
 K   T   I   A   S   C   I   L   L   A   S   Q   L   V   K   D   M   K   P   F
  R   L   *   P   H   V   F   C   W   L   V   N   *   L   K   I   T   N   H   L
481/161                                             511/171
TAT TCG CTC ATG TGC TTC TTT GTG CGA AGG AAT TTA TGA CAA AGA ACA CGT CAT AAA ATT
Y   S   L   M   C   F   F   V   R   R   N   L   *   Q   R   T   R   H   K   I
 I   R   S   C   A   S   L   C   E   G   I   Y   D   K   E   H   V   I   K   L
  F   A   H   V   L   L   C   A   K   E   F   M   T   K   N   T   S   *   N   *
541/181                                             571/191
GAA GAA AAT AGT TAG CAT AAT ATC ACA TCA AAA CTT ATC ATT GTT TAA ATC CTT AGT TTA
E   E   N   S   *   H   N   I   T   S   K   L   I   I   V   *   I   L   S   L
 K   K   I   V   S   I   I   S   H   Q   M   L   S   L   F   K   S   L   V   Y
  R   K   *   L   A   *   Y   H   I   K   T   Y   H   C   L   N   P   *   F   I
601/201                                             631/211
TCA TTC ACG ACC TTT ACA ACA GAA GTG GCT AAA CTC AGA CTC CGT GAA AAC AAT AAT TAA
S   F   T   T   F   T   T   E   V   A   K   L   R   L   R   E   N   N   N   *
 H   S   R   P   L   Q   Q   K   W   L   M   S   D   S   V   K   T   I   I   M
  I   H   D   L   Y   N   R   S   G   *   T   Q   T   P   *   K   Q   *   L   M
661/221                                             691/231
TGA ATG CCA TAT AGA TAT ACA ACC TTT GGC GAC TTC TTT AGG CAT GAT AAA AAG TAG TCA
*   M   P   Y   R   Y   T   T   F   G   D   F   F   R   H   D   K   K   *   S
 E   C   H   I   D   I   Q   P   L   A   T   S   L   G   M   I   K   S   S   H
  N   A   I   *   I   Y   N   L   W   R   L   L   *   A   *   *   K   V   V   T
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721/241                                                 751/251
CTC ATT ACT TAG AAT CTA TCA AGA TCT GAT AAC CCA TTT TCC AAT GAG ATA ATG GCA TTA
L   I   T   *   N   L   S   R   S   D   N   P   F   S   N   E   I   M   A   L
 S   L   L   R   I   Y   Q   D   L   I   T   H   F   F   M   R   *   W   H   *
  H   Y   L   E   S   I   K   I   *   *   P   I   F   Q   *   D   N   G   I   K
781/261                                                 811/271
AAA CTG ATG CAA GCC CTT TTA TTG GAC AGG ATT GTT TGC CTG GAT AAT AAA AAA GAT TAT
K   L   M   Q   A   L   L   L   D   R   I   V   C   L   D   N   K   K   D   Y
 N   *   C   K   P   F   Y   W   T   G   L   F   A   W   I   I   K   K   I   I
  T   D   A   S   P   F   I   G   Q   D   C   L   P   G   *   *   K   R   L   S
841/281                                                 871/291
CAA ATA AGT GTA GCA AAC ACT AAA GTG ACG TTT CAT AAC TAC TCC AAC CCT CCA ACA TCG
Q   I   S   V   A   N   T   K   V   T   F   H   N   Y   S   N   P   P   T   S
 K   *   V   *   Q   T   L   K   *   R   F   I   T   T   P   T   L   Q   H   R
  N   K   C   S   K   H   *   S   D   V   S   *   L   L   Q   P   S   N   I   E
901/301                                                 931/311
AAT GTC TTC GAT GCA GGA ATG GAT GTG GAT GCA AAA TTA TTC AAA TCA TCG GGA TGG GTC
N   V   F   D   A   G   M   D   V   D   A   K   L   F   K   S   S   G   W   V
 M   S   S   M   Q   E   W   M   W   M   Q   N   Y   S   N   H   R   D   G   S
  C   L   R   C   R   N   G   C   G   C   K   I   I   Q   I   I   G   M   G   R
961/321                                                 991/331
GAT TCT ATT TTC ACG GAT GAT GCA GAC ACT CAA ATA TTG TAT AGA GTT GCC ATG TGC ATC
D   S   I   F   T   D   D   A   D   T   Q   I   L   Y   R   V   A   M   C   I
 I   L   F   S   R   M   M   Q   T   L   K   Y   C   I   E   L   P   C   A   S
  F   Y   F   H   G   *   C   R   H   S   N   I   V   *   S   C   H   V   H   P
1021/341                                                1051/351
CGA TCA GTA CTA CTC GGC AAA CAA GAC TGG ACA GAT TTT GGT CAA GCA ATT TCC CCC AAA
R   S   V   L   L   G   K   Q   D   W   T   D   F   G   Q   A   I   S   P   K
 D   Q   Y   Y   S   A   N   K   T   G   Q   I   L   V   K   Q   F   P   P   N
  I   S   T   T   R   Q   T   R   L   D   R   F   W   S   S   N   F   P   Q   T
1081/361                                                1111/371
CAG GGT TAT CGG GGT ATT AAA ACT AGT AGA GAC AAA CGT CAA TTG GGG ATG ATG ACA ACA
Q   G   Y   R   G   I   K   T   S   R   D   K   R   Q   L   G   M   M   T   T
 R   V   I   G   V   L   K   L   V   E   T   N   V   N   W   G   *   *   Q   H
  G   L   S   G   Y   *   N   *   *   R   Q   T   S   I   G   D   D   D   N   T
1141/381                                                1171/391
CCT GAG TCC ATT GCC GGT GAG AAC TCT CAG GTT TCT GGT TGG CTT ACC ACA CTC TTA TCC
P   E   S   I   A   G   E   N   S   Q   V   S   G   W   L   T   T   L   L   S
 L   S   P   L   P   V   R   T   L   R   F   L   V   G   L   P   H   S   Y   P
  *   V   H   C   R   *   E   L   S   G   F   W   L   A   Y   H   T   L   I   Q
1201/401                                                1231/411
AAG TTG CTT GCC TGG CCG GGA ATT TCA GTG GGT GAT AAT GGA TAT CAA TGG CCA GCA ATT
K   L   L   A   W   P   G   I   S   V   G   D   N   G   Y   Q   W   P   A   I
 S   C   L   P   G   R   E   F   Q   W   V   I   M   D   I   N   G   Q   Q   F
  V   A   C   L   A   G   N   F   S   G   *   *   W   I   S   M   A   S   N   F
1261/421                                                1291/431
TTT ACA GTA GAT GCT GTC AGA AAA CTA GTT GAT GCT CGG CTG AGT AAG CTT AAG CAG GAT
F   T   V   D   A   V   R   K   L   V   D   A   R   L   S   K   L   K   Q   D
 L   Q   *   M   L   S   E   N   *   L   M   L   G   *   V   S   L   S   R   I
  Y   S   R   C   C   Q   K   T   S   *   C   S   A   E   *   A   *   A   G   L
1321/441                                                1351/451
TAC TGC AAA CTA TCA GGA ACT CCG GGA CTT ACA GAA AAA ATA CAG TTC GAC TGG TCT GAC
Y   C   K   L   S   G   T   P   G   L   T   E   K   I   Q   F   D   W   S   D
 T   A   N   Y   Q   E   L   R   D   L   Q   K   K   Y   S   S   T   G   L   T
  L   Q   T   I   R   N   S   G   T   Y   R   K   N   T   V   R   L   V   *   L
1381/461                                                1411/471
TCG AAA AAA GCC CTA ACA GTT GCT ATG GTC CAG TCA AAA CTG CCT GCA ACG AAA GAT TTT
S   K   K   A   L   T   V   A   M   V   Q   S   K   L   P   A   T   K   D   F
 R   K   K   P   *   Q   L   L   W   S   S   Q   N   C   L   Q   R   K   I   L
  E   K   S   P   N   S   C   Y   G   P   V   K   T   A   C   N   E   R   F   C
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1441/481                                                1471/491
GCC AGC CAT GGA CTT cTT TTA AAC TCC GCA AAA TAC AGA GTG ATT CAT CGC AGA CAT GTT
A   S   H   G   L   L   L   N   S   A   K   Y   R   V   I   H   R   R   H   V
 P   A   M   D   F   F   *   T   P   Q   N   T   E   *   F   I   A   D   M   L
  Q   P   W   T   S   F   K   L   R   K   I   Q   S   D   S   S   Q   T   C   C
1501/501                                                1531/511
GCT GAA GTG GCT GAT TTA GTT GTA AAA CAC ACG CTT GCA CAA AAA ACA ACT CAA CGA ACT
A   E   V   A   D   L   V   V   K   H   T   L   A   Q   K   T   T   Q   R   T
 L   K   W   L   I   *   L   *   N   T   R   L   H   K   K   Q   L   N   E   L
  *   S   G   *   F   S   C   K   T   H   A   C   T   K   N   N   S   T   N   S
1561/521                                                1591/531
CAT GGT GAA AAA ATA GAG AAC ATT GAT TTA ATA GTA TGG CCT GAG CTC GCT GTA CAT AGT
H   G   E   K   I   E   N   I   D   L   I   V   W   P   E   L   A   V   H   S
 M   V   K   K   *   R   T   L   I   *   *   Y   G   L   S   S   L   Y   I   V
  W   *   K   N   R   E   H   *   F   N   S   M   A   *   A   R   C   T   *   *
1621/541                                                1651/551
GAC GAT TTG GAT GTA CTC ATC GCC TTA TCT AGA AAA ACC AAT GCA ATC ATA TAC TCG GGC
D   D   L   D   V   L   I   A   L   S   R   K   T   N   A   I   I   Y   S   G
 T   I   W   M   Y   S   S   P   Y   L   E   K   P   M   Q   S   Y   T   R   A
  R   F   G   C   T   H   R   L   I   *   K   N   Q   C   N   H   I   L   G   P
1681/561                                                1711/571
CTG ACA TTT ATT GAG CAA CCT GGA ATC AAA GGA CCA AAT AAT TGT GCC GTT TGG ATT GTC
L   T   F   I   E   Q   P   G   I   K   G   P   N   N   C   A   V   W   I   V
 *   H   L   L   S   N   L   E   S   K   D   Q   I   I   V   P   F   G   L   S
  D   I   Y   *   A   T   W   N   Q   R   T   K   *   L   C   R   L   D   C   P
1741/581                                                1771/591
CCA CCT AAA AGC AAT AGC AGC CAG AAA GAA ATG ATA AGA CTT CAA GGC AAG CAT AAT ATG
P   P   K   S   N   S   S   Q   K   E   M   I   R   L   Q   G   K   H   N   M
 H   L   K   A   I   A   A   R   K   K   *   *   D   F   K   A   S   I   I   *
  T   *   K   Q   *   Q   P   E   R   N   D   K   T   S   R   Q   A   *   Y   D
1801/601                                                1831/611
ATG GAA GAT GAG AAA GGC CGG GTT GAA CCC TGG AGA CCA TAC CAA TTG ATG CTT GAa CTT
M   E   D   E   K   G   R   V   E   P   W   R   P   Y   Q   L   M   L   E   L
 W   K   M   R   K   A   G   L   N   P   G   D   H   T   N   *   C   L   N   L
  G   R   *   E   R   P   G   *   T   L   E   T   I   P   I   D   A   *   T   C
1861/621                                                1891/631
GTT CAC CCA CAA TTC CCT GAT AAA AAA GGA TTT ATT CTC ACA GGC TCC ATT TGT TAT GAC
V   H   P   Q   F   P   D   K   K   G   F   I   L   T   G   S   I   C   Y   D
 F   T   H   N   S   L   I   K   K   D   L   F   S   Q   A   P   F   V   M   T
  S   P   T   I   P   *   *   K   R   I   Y   S   H   R   L   H   L   L   *   R
1921/641                                                1951/651
GCA ACC GAC ATC GCA CTA AGT GCA GAT CCC CGG GTA CCG AGC CGA ATT CGt ATC CGA A
A   T   D   I   A   L   S   A   D   P   R   V   P   S   R   I   R   I   R
 Q   P   T   S   H   *   V   Q   I   P   G   Y   R   A   E   F   V   S   E
  N   R   H   R   T   K   C   R   S   P   G   T   E   P   N   S   Y   P
```

Fig. 46. Nucleotide sequence of pUC324, a positive clone screened from the *Enterobacter cloacae* CAL3 genomic library.

```
GCTGCCCTCGGTACCCGGGGATCAAAGGTAAGAAAATTACCGTTGTGCCA      50
ATCCTGCGTGCCGGTCTCGGCATGATGGAAGGCGTGCTGGAGCACGTCCC     100
AAGCGCACGTATCAGCGTGGTGGGGATCTACCGTAACGAAGAAACCCTTG     150
AGCCGGTTCCTTACTTCCAGAAGCTGGTGTCCAACATCGACGAGCGTATG     200
GCGCTGGTGGTTGACCCAATGCTGGCAACCGGCGGATCTATGATCGCCAC     250
CATCGACCTGCTGAAAAAAGCGGGCTGCAGCAGCATCAAAGTGCTGGTGC     300
TGGTAGCGGCACCGGAAG                                     318
```

Fig. 47. The open reading frame of pUC324 (302 bp) that corresponds to *Escherichia coli* uracil phosphoribosyltransferase is shown in bold face type.

```
1/1                                                 31/11
GCT GCC CTC GGT ACC cGG GGA TCA AAG GTA AGA AAA TTA CCG TTG TGC CAA TCC TGC GTG
A   A   L   G   T   R   G   S   K   V   R   K   L   P   L   C   Q   S   C   V
 L   P   S   V   P   G   D   Q   R   *   E   N   Y   R   C   A   N   P   A   C
  C   P   R   Y   P   G   I   K   G   K   K   I   T   V   V   P   I   L   R   A
61/21                                               91/31
CCG GTC TCG GCA TGA TGG AAG GCG TGC TGG AGC ACG TCC CAA GCG CAC GTa TcA GCG TGG
P   V   S   A   *   W   K   A   C   W   S   T   S   Q   A   H   V   S   A   W
 R   S   R   H   D   G   R   R   A   G   A   R   P   K   R   T   Y   Q   R   G
  G   L   G   M   M   E   G   V   L   E   H   V   P   S   A   R   I   S   V   V
121/41                                              151/51
TGG GGA TCT ACC GTA ACG AAG AAA CCC TTG AGC CGG TTC CTT ACT TCC AGA AGC TGG TGT
W   G   S   T   V   T   K   K   P   L   S   R   F   L   T   S   R   S   W   C
 G   D   L   P   *   R   R   N   P   *   A   G   S   L   L   P   E   A   G   V
  G   I   Y   R   M   E   E   T   L   E   P   V   P   Y   F   Q   K   L   V   S
181/61                                              211/71
CCA ACA TCG ACG AGC GTA TGG CGC TGG TGG TTG ACC CAA TGC TGG CAA CCG GCG GAT CTA
P   T   S   T   S   V   W   R   W   W   L   T   Q   C   W   Q   P   A   D   L
 Q   H   R   R   A   Y   G   A   G   G   *   P   N   A   G   N   R   R   I   Y
  N   I   D   E   R   M   A   L   V   V   D   P   M   L   A   T   G   G   S   M
241/81                                              271/91
TGA TCG CCA CCA TCG ACC TGC TGA AAA AAG CGG GCT GCA GCA GCA TCA AAG TGC TGG TGC
*   S   P   P   S   T   C   *   K   K   R   A   A   A   A   S   K   C   W   C
 D   R   H   H   R   P   A   E   K   S   G   L   Q   Q   H   Q   S   A   G   A
  I   A   T   I   D   L   L   K   K   A   G   C   S   S   I   K   V   L   V   L
301/101
TGG TAG CGG CAC CGG AAG
W   *   R   H   R   K
 G   S   G   T   G
  V   A   A   P   E
```

Fig. 48. Alignment of the deduced amino acid sequence from pUC324 with the sequence of *Escherichia coli* uracil phosphoribosyltransferase obtained from GenBank. The + represents a derived amino that is similar but not identical to the amino acid in the subject amino acid sequence and that is not essential for the proper protein structure. The numbers beside the sequences indicate the nucleotide numbers in each sequence.

Query: pUC324 from *Enterobacter cloacae* CAL3

Sbjct: Uracil phosphoribosyltransferase from *Escherichia coli*, A65026. (Andersen et al., 1992)

```
Query: 21  IKGKKITVVPILRAGLGMMEGVLEHVPSARISVVGIYRNEETLEPVPYFQKLVSNIDERM 200
           IKGKKITVVPILRAGLGMM+GVLE+VPSARISVVG+YRNEETLEPVPYFQKLVSNIDERM
Sbjct: 75  IKGKKITVVPILRAGLGMMDGVLENVPSARISVVGMYRNEETLEPVPYFQKLVSNIDERM 134

Query: 201 ALVVDPMLATGGSMIATIDLLKKAGCSSIKVLVLVAAPE 317
           AL+VDPMLATGGS+IATIDLLKKAGCSSIKVLVLVAAPE
Sbjct: 135 ALIVDPMLATGGSVIATIDLLKKAGCSSIKVLVLVAAPE 173
```

encoded the first 75 amino acids and the last 44 amino acids were missing from the DNA insert in pUC324.

3.12.4.4. Analysis of the DNA sequence from clone pUC327

The rather large DNA insert in pUC327 contained 5000 bp (Fig. 49) and was sequenced from both ends. The *Enterobacter cloacae* CAL3 DNA was inserted into the cloning vector, 5′ to 3′, in the *Eco*RI to *Hind*III direction. There were two main reading frames present in pUC327, from 306 to 1790 bp (Fig. 50) and from 3104 to 4318 bp (Fig. 51). The first open reading frame contained a sequence that was a close match with a protein 495 amino acids in length, altronate hydrolase from *Escherichia coli* (identities = 435/495, 87%; positives = 464/495, 92%), and the entire gene appeared to be present in the insert (Fig. 52).

The other reading frame, from 3104 to 4318 bp Fig. 53) was very similar to a 43.5 kD hypothetical protein in the ebgC-exuT intergenic region of *Escherichia coli* strain K-12 (identities = 289/405, 71%; positives = 309/405, 75%; gaps = 5/405, 1%) (Fig. 55). The insert contained almost the entire sequence that encoded this protein, 414 amino acids in length.

Fig. 49. Nucleotide sequence of pUC327, a positive clone screened from the *Enterobacter cloacae* CAL3 genomic library.

CTCGCTGCCTGCAGGTCGACTCTAGAGGATCAGAAAGACGGCATGGAGCG 50
TCAAATGACGCAGCTGGCGCAGCTTGGCCTGCTGAGTCGCTTTGTCGGTA 100
TGCTGACCGACAGCCGCAGCTTCCTGTCCTATACCCGCCATGAATACTTC 150
CGCCGCATTCTGTGCCAGATGATTGGCCGCTGGGTGCACGCGGGCGAAGC 200
GCCAGCGGATATCCAGCTGCTGGGCGAAATGGTGAAAAACATCTGCTTTA 250
ATAATGCGCGTGACTACTTCGCCATTGAACTGAACTAAGGCCGTCTGAGG 300
TTGATATGCAATACATCAAAATCCATTCGCTGGATAACGTGGCCGTCGCG 350
CTGGCTGATTTAACCGAAGGAACGGACGTCACGTTTGACGGTCAGTCGGT 400
CACGTTGCGCCAGGCCATTGGGCGTGGGCACAAGTTTGCCCTGCTCCCCA 450
TTGCGAAAGGGGAGAACGTGGTGAAGTACGGTTTGCCCATCGGTCACGCG 500
CTGGCGGATATTGCGCCGGGTGAATATATTCACTCCCACAATACCCGCAC 550
CAATCTGAGCGATCTGGACGAGTACAGCTATCAACCTGATTTCCAGGCGG 600
AGGCCGGACAGGCAGCGGATCGTGAAGTGCAGATCTACCGTCGCGCCAGC 650
GGCGAGGTGGGGATCCGCAACGAACTGTGGATCCTCCCGACCGTCGGCTG 700
CGTGAATGGGATTGCGCGTCAGATCCAGACGCGTTTCCTGGAAaGAGACC 750
CGCGACGCCGAACGCACTGACGGCGTGCATCTGTTCAGCCATACCTATGG 800
CTGCTCGCAGCTCGGCGACGACCACATCAATACCCGCACCATGCTGCAAA 850
ACATGGTGCGCCACCCGAACGCGGGCGCGGTGCTGGTGATTGGCCTCGGC 900
TGCGAGAACAATCAGGTTGACGCTTTCCGCGACACGCTGGGCGAGTTCGA 950
TCCTGAGCGCGTGCATTTTATGGTGTGTCAGCATCAGGATGACGAAGTGG 1000
AAGCGGGCGTTGAGCAGCTTCATCAGTTGTATGACGTCATGCGCCATGAC 1050
AAGCGCGAGCCGGGCAAGCTGAGCGAGCTGAAGTTTGGTCTCGAGTGTGG 1100
CGGATCTGATGGCCTTTCCGGCATTACCGCTAACCCGATGCTCGGCCGTT 1150
TCTCCGACTACGTTATCGCTAACGGCGGCACCACGGTGCTGACCGAAGTG 1200
CCGGAAATGTTCGGCGCGGAGCGTATTCTGATGAGCCACTGCCGCGACGA 1250
AGAGACGTTTGAGAAGACCGTCACCATGGTGAACGACTTCAAACAGTACT 1300
TCATCGCCCACAATCAGCCGATTTACGAGAACCCGTCGCCGGGCAACAAG 1350
GCGGGCGGGATCACCACGCTGGAAGAGAAATCCCTCGGCTGCACCCAGAA 1400
AGCGGgCGCGAGCCAGGTGGTGGACGTGCTGCGCTACGGCGAGCGCCTGA 1450
AAACCCACGGTCTGAACCTGCTGAGCGCGCCGGGCAATGACGCCGTCGCA 1500
CCAGCGCGCTGGCGGGTGCTGGCTGTCATATGGTGCTGTCAGCACCGGTC 1550
GCGGTACGCCGTACGGCGGTTTTGTGCCGACGGTGAAAATCGCCACCAAC 1600
AGCGAGCTGGCGACGAAGAAAAAACACTGGATTGATTTCGATGCGGGTCA 1650
GCTGATCCACGGCAAAGCGATGCCGCAGCTGCTGACGGAGTTTGTGGACA 1700
CTATCGTGGAATTTGCGAACGGTAAGCAGACCTGTAACGAGAAGAATGAC 1750
TTcCGGGAGCTGGCGATTTTTAAGAGCGGtGTGACGCTTTAATCGGTGCG 1800
GGCTGGTGCCCTCACCCTGACCCTCTCCCACTGTACGGTCCGGGGACATG 1850
GTAGACAGGGTTCGGGGACATGGtGAACACTTTTTAACATCCTTTACCCA 1900
TGGTGATCGACTTTTTCTTcAGGTCGATCACCCCCACTTTCGTGCTGTAC 1950
CACCACACCTCGTAGCTGCCGTCTTcCTGCATCTCCTTCAGCCCGACCCT 2000
TTcTCCCCTGAACGCCTTGCCTGCGCTCAACTTACCCCTTTCACGCTCAG 2050
TTTCCGCTGATATCCACTTTTCTGACCATCACCCCTTCATCGTATTCCGG 2100
GGGCGTTGTGTTGCCGCTGTACTGCCGCGCTGACGGCTGATACCGCGAGC 2150
CCGGTACCGCCATATCCAGCGCCTCGTGCGGGCGTTCAAGGTTATAGACC 2200
GTCCGCCAGTGGTCGAAGGCGCGCTGCAGTTCACCGCTGTCTGCGAACCA 2250
TTTTCCCTGCAGCACTTCCGCCTTCAGGCTGCGGTGAAAACGCTCCAGCT 2300
TGCCCTGCGTCTGCGGATGATAAGGCCGGGAGTGCCCCACCCGGATACCC 2350
AGGCGCATCAGCCACAGCTCAAGTGCCGTCCAGGTGCCGGTGGTGTCTCC 2400
CCACGGTGAGCCGTTGTCCATCGTCATCCGGTCCGGCAGGCCGTAACGCT 2450
CAAACACGCTGACCAGCTGCTGCTGCACGGTCTCGCGCCGTTCATCGGTA 2500

CAGTGCGCCAGGCACAGGGAAAAACGGGAGTGGTCGTCCAGCAGGGTGAG 2550
CGGATGGCAGCGGCCACCGCCAAAGGGAAAATGGCCCTTAAAATCCATCT 2600
GCCAGAGGCGGTTCGGCGCGTCGTGTTCGAACCGGCCCGTGGCGGGAATG 2650
CCCGGTGAAGCGCCCGGCAGCAGGCCATGGCGGGCCATCAGGTTATGGAC 2700
GGTGCTGAAGGCGGGCATGATGTGCCCCTGGTCTTCGAGCCAGCGCTTTA 2750
TCTTGCGGGCTCCCCAGCGTTCATGGCGGTCATGTGCCATACCTCAGCAG 2800
GATCAGTAATGTAGTCTGACAATCGATTGATGGTGCAATGACGGGAGTTG 2850
AAGATGTCGCACTTCCTTACCAGCCGCAGCTTGAGGTGAGTGAGCCAAAG 2900
TGTACAGAAGAAGGATGTCCCCGTCTGCAAGGACAACACCGGCGTAATGC 2950
ATGGTATCTCTCGCATCCCAGGGCATAAGCGACTCCATACGGTTCTATGC 3000
CTTAGTTGTAAGTGTCTACCATGTCCCCCGGACCGTACACCCACGGGGAGA 3050
GGGTACAAACACTAAAAACGGCAACCTCAGGGTTGCCGTTTTGCTGTTAA 3100
CCTCGAAGTGCATTCTTCGCTAAACGCGCGTCTTCCGCCTGACAGGCCGC 3150
TGCGGTAAACAGCACGTCGGTAGAGGAGTTCAGCGCGGTTTCGCAGGAGT 3200
CCTGCAGTACGCCGATGATAAAGCCGACGGCCACAACCTGCATTGCGATC 3250
TCGTTCGGGATACCGAACATATTACAGGCCAGCGGGATCAGCAGCAGCGA 3300
ACCGCCCGCCACGCCGGATGCGCCGCAGGCACACAGCGATGCCACTACGC 3350
TTAGCAGCAGCGCCGTAAGCAGGTCCACCGGAACGCCCAGCGTATGCACC 3400
GCCGCCAGCGTCAGCACGGTAATAGTGATTGCTGCGCCCGCCATGTTCAC 3450
GGTTGCACCCAGCGGGATCGACACGGAATAGGTGTCGCGGTCCAGGTTCA 3500
GCTTCTCCGCCAGCGCCATGTTAACCGGAATGTTCGCCGCAGAGCTGCGG 3550
GTTGAAAGAAAGGCGTACACGCCGCTCTCGCGCAGGCAGGTCAGCACCAG 3600
CGGATACGGGTTGCGGCGGATCTGCCAGAACACCAGCAGTGGGTTGATCA 3650
CCAGCGCCACCAGCAGCATACAGCCGACCAGCACAACCAGCAGCTGCGCG 3700
TAGCCCCACAGCGCGTCAAAACCGGTGGTTGCCAGCGTCGAAGAAACCAG 3750
CCCAAAGATCCCGATAGGCGCAAAGCGAATCACCAGCTTCACCATAAAGG 3800
TGACTGCGTTCGACATATCGTTCACCAGGTTTTTGGTGGTGTCGTTACCG 3850
TGGCGCAGCGCGAAGCCGAGTCCAATCGCCCAGACCAGAATGCCAATGTA 3900
GTTCGCATTCATCAGCGCGGTGATGGGGTTAGAGACCATGCTCATCAGCA 3950
GACCGCGGAGCACTTCGACAATGCCGGACGGCGGGGTAATATCACCGGCC 4000
GCGCTGGTCAGGTGCAGCATGGACGGGAACAGGAAGCTGAAAACAACGGC 4050
CGTTAAGGCCGCAGCGAAGGTTCCCAGAAGATACAGGAACAGAATAGGGC 4100
GAATGTTGGTTTTTTGTCCGTGCTGGTGGTTGGCAATCGACGCCATCACC 4150
AGCATCAGAACCAGTACCGGTGCGACGGCCTTCAGCGCGCCAACGAAAAG 4200
GGTGCCGAGCAGTCCCGTGGCTTCCGCAGCAGGTTTTGACACCATCGCCA 4250
GCAGAATACCCAGTACCAACccGATCAAGAAATTTGTTTTACAAGACTGC 4300
CCTGCGCCAGGCGCGCGAACAGACCGCTTGATTGTGTGCTCATACGTTAT 4350
TCCTGAGTGAAATTGCGTTCCATCCTGGATGTGCTCTAAGTCACATTTAT 4400
GTCCGGATGTAAGTATTTGTTTGCCTGGTTGAGTATCAGGGAAAGCAGCG 4450
AAAGGCGGAAGCGTAAAATGCTGGATTTTACGTGTTGCATCATATTTTTT 4500
AACTGTTGAGTTACAAAAGTGTGTTCACCCTCTCCCTGTGGGAGAGGGTC 4550
GGGGTGAGGGCATCAGGCCGCACCGAACGTTTTACTCTACCTGCTGCTTC 4600
TTATCGTGCTGGCGGTTAACCCAGGTATTGATAATCAGCGTCACGATCAG 4650
AATGCCAAACACCACGCCGAGCGAAATGGCGATTGGGATGTGGTAGAAAT 4700
CGACGATCAGCATCTTGATACCGATAAACACCAGGATCACCGACAGGCCG 4750
TACTTCAGCATTGAGAAGCGCTCCGCCGCGCCTGCCAGCAGGAAGTACAT 4800
GGCACGCAGGCCGAGAATGGCGAACAGGTTAGACGTCAGCACGATGAACG 4850
GGTCGGTGGTGACGGCAAAGATTGCCGGAATGCTGTCAACCGCGAAAATC 4900
ACGTCGCTCAGCTCGACCAGAATCAGCACCAGCAGCAGCGGGGTGGCAAA 4950
CAGCAGGCCGTTCTTGCGAACGAAGAAGTGCTCGCTCTCGATCCCCGGGT 5000

Fig. 50. The open reading frame in the first 2.1 kb of pUC327, that corresponds to *Escherichia coli* altronate hydrolase (1485 bp), is shown in bold face type.

```
1/1                                                 31/11
CTC GCT GCC TGC AGG TCG ACT CTA GAG GAT CAG AAA GAC GGC ATG GAG CGT CAA ATG ACG
L   A   A   C   R   S   T   L   E   D   Q   K   D   G   M   E   R   Q   M   T
 S   L   P   A   G   R   L   *   R   I   R   K   T   A   W   S   V   K   *   R
  R   C   L   Q   V   D   S   R   G   S   E   R   R   H   G   A   S   N   D   A
61/21                                               91/31
CAG CTG GCG CAG CTT GGC CTG CTG AGT CGC TTT GTC GGT ATG CTG ACC GAC AGC CGC AGC
Q   L   A   Q   L   G   L   L   S   R   F   V   G   M   L   T   D   S   R   S
 S   W   R   S   L   A   C   *   V   A   L   S   V   C   *   P   T   A   A   A
  A   G   A   A   W   P   A   E   S   L   C   R   Y   A   D   R   Q   P   Q   L
121/41                                              151/51
TTC CTG TCC TAT ACC CGC CAT GAA TAC TTC CGC CGC ATT CTG TGC CAG ATG ATT GGC CGC
F   L   S   Y   T   R   H   E   Y   F   R   R   I   L   C   Q   M   I   G   R
 S   C   P   I   P   A   M   N   T   S   A   A   F   C   A   R   *   L   A   A
  P   V   L   Y   P   P   *   I   L   P   P   H   S   V   P   D   D   W   P   L
181/61                                              211/71
TGG GTG CAC GCG GGC GAA GCG CCA GCG GAT ATC CAG CTG CTG GGC GAA ATG GTG AAA AAC
W   V   H   A   G   E   A   P   A   D   I   Q   L   L   G   E   M   V   K   N
 G   C   T   R   A   K   R   Q   R   I   S   S   C   W   A   K   W   *   K   T
  G   A   R   G   R   S   A   S   G   Y   P   A   A   G   R   N   G   E   K   H
241/81                                              271/91
ATC TGC TTT AAT AAT GCG CGT GAC TAC TTC GCC ATT GAA CTG AAC TAA GGC CGT CTG AGG
I   C   F   N   N   A   R   D   Y   F   A   I   E   L   N   *   G   R   L   R
 S   A   L   I   M   R   V   T   T   S   P   L   N   *   T   K   A   V   *   G
  L   L   *   *   C   A   *   L   L   R   H   *   T   E   L   R   P   S   E   V
301/101                                             331/111
TTG ATA TGC AAT ACA TCA AAA TCC ATT CGC TGG ATA ACG TGG CCG TCG CGC TGG CTG ATT
L   I   C   N   T   S   K   S   I   R   W   I   T   W   P   S   R   W   L   I
 *   Y   A   I   H   Q   N   P   F   A   G   *   R   G   R   R   A   G   *   F
  D   M   Q   Y   I   K   I   H   S   L   D   M   V   A   V   A   L   A   D   L
361/121                                             391/131
TAA CCG AAG GAA CGG ACG TCA CGT TTG ACG GTC AGT CGG TCA CGT TGC GCC AGG CCA TTG
*   P   K   E   R   T   S   R   L   T   V   S   R   S   R   C   A   R   P   L
 N   R   R   N   G   R   H   V   *   R   S   V   G   H   V   A   P   G   H   W
  T   E   G   T   D   V   T   F   D   G   Q   S   V   T   L   R   Q   A   I   G
421/141                                             451/151
GGC GTG GGC ACA AGT TTG CCC TGC TCC CCA TTG CGA AAG GGG AGA ACG TGG TGA AGT ACG
G   V   G   T   S   L   P   C   S   P   L   R   K   G   R   T   W   *   S   T
 A   W   A   Q   V   C   P   A   P   H   C   E   R   G   E   R   G   E   V   R
  R   G   H   K   F   A   L   L   P   I   A   K   G   E   M   V   V   K   Y   G
481/161                                             511/171
GTT TGC CCA TCG GTC ACG CGC TGG CGG ATA TTG CGC CGG GTG AAT ATA TTC ACT CCC ACA
V   C   P   S   V   T   R   W   R   I   L   R   R   V   N   I   F   T   P   T
 F   A   H   R   S   R   A   G   G   Y   C   A   G   *   I   Y   S   L   P   Q
  L   P   I   G   H   A   L   A   D   I   A   P   G   E   Y   I   H   S   H   M
541/181                                             571/191
ATA CCC GCA CCA ATC TGA GCG ATC TGG ACG AGT ACA GCT ATC AAC CTG ATT TCC AGG CGG
I   P   A   P   I   *   A   I   W   T   S   T   A   I   N   L   I   S   R   R
 Y   P   H   Q   S   E   R   S   G   R   V   Q   L   S   T   *   F   P   G   G
  T   R   T   M   L   S   D   L   D   E   Y   S   Y   Q   P   D   F   Q   A   E
601/201                                             631/211
AGG CCG GAC AGG CAG CGG ATC GTG AAG TGC AGA TCT ACC GTC GCG CCA GCG GCG AGG TGG
R   P   D   R   Q   R   I   V   K   C   R   S   T   V   A   P   A   A   R   W
 G   R   T   G   S   G   S   *   S   A   D   L   P   S   R   Q   R   R   G   G
  A   G   Q   A   A   D   R   E   V   Q   I   Y   R   R   A   S   G   E   V   G
661/221                                             691/231
GGA TCC GCA ACG AAC TGT GGA TCC TCC CGA CCG TCG GCT GCG TGA ATG GGA TTG CGC GTC
G   S   A   T   N   C   G   S   S   R   P   S   A   A   *   M   G   L   R   V
 D   P   Q   R   T   V   D   P   P   D   R   R   L   R   E   W   D   C   A   S
  I   R   M   E   L   W   I   L   P   T   V   G   C   V   M   G   I   A   R   Q
```

721/241 751/251

AGA TCC AGA CGC GTT TCC TGA AAG aGA CCC GCG ACG CCG AAC GCA CTG ACG GCG TGC ATC

R S R R V S * K R P A T P N A L T A C I

D P D A F P E R D P R R R T H * R R A S

I Q T R F L K E T R D A E R T D G V H L

781/261 811/271

TGT TCA GCC ATA CCT ATG GCT GCT CGC AGC TCG GCG ACG ACC ACA TCA ATA CCC GCA CCA

C S A I P M A A R S S A T T T S I P A P

V Q P Y L W L L A A R R R P H Q Y P H H

F S H T Y G C S Q L G D D H I M T R T M

841/281 871/291

TGC TGC AAA ACA TGG TGC GCC ACC CGA ACG CGG GCG CGG TGC TGG TGA TTG GCC TCG GCT

C C K T W C A T R T R A R C W * L A S A

A A K H G A P P E R G R G A G D W P R L

L Q M M V R H P M A G A V L V I G L G C

901/301 931/311

GCG AGA ACA ATC AGG TTG ACG CTT TCC GCG ACA CGC TGG GCG AGT TCG ATC CTG AGC GCG

A R T I R L T L S A T R W A S S I L S A

R E Q S G * R F P R H A G R V R S * A R

E M M Q V D A F R D T L G E P D P E R V

961/321 991/331

TGC ATT TTA TGG TGT GTC AGC ATC AGG ATG ACG AAG TGG AAG CGG GCG TTG AGC AGC TTC

C I L W C V S I R M T K W K R A L S S F

A F Y G V S A S G * R S G S G R * A A S

H F M V C Q H Q D D E V E A G V E Q L H

1021/341 1051/351

ATC AGT TGT ATG ACG TCA TGC GCC ATG ACA AGC GCG AGC CGG GCA AGC TGA GCG AGC TGA

I S C M T S C A M T S A S R A S * A S *

S V V * R H A P * Q A R A G Q A E R A E

Q L Y D V M R H D K R E P G K L S E L K

1081/361 1111/371

AGT TTG GTC TCG AGT GTG GCG GAT CTG ATG GCC TTT CCG GCA TTA CCG CTA ACC CGA TGC

S L V S S V A D L M A F P A L P L T R C

V W S R V W R I * W P F R H Y R * P D A

F G L E C G G S D G L S G I T A N P M L

1141/381 1171/391

TCG GCC GTT TCT CCG ACT ACG TTA TCG CTA ACG GCG GCA CCA CGG TGC TGA CCG AAG TGC

S A V S P T T L S L T A A P R C * P K C

R P F L R L R Y R * R R H H G A D R S A

G R F S D Y V I A M G G T T V L T E V P

1201/401 1231/411

CGG AAA TGT TCG GCG CGG AGC GTA TTC TGA TGA GCC ACT GCC GCG ACG AAG AGA CGT TTG

R K C S A R S V F * * A T A A T K R R L

G N V R R G A Y S D E P L P R R R D V *

E M F G A E R I L M S H C R D E E T F E

1261/421 1291/431

AGA AGA CCG TCA CCA TGG TGA ACG ACT TCA AAC AGT ACT TCA TCG CCC ACA ATC AGC CGA

R R P S P W * T T S N S T S S P T I S R

E D R H H G E R L Q T V L H R P Q S A D

K T V T M V M D F K Q Y F I A H M Q P I

1321/441 1351/451

TTT ACG AGA ACC CGT CGC CGG GCA ACA AGG CGG GCG GGA TCA CCA CGC TGG AAG AGA AAT

F T R T R R R A T R R A G S P R W K R N

L R E P V A G Q Q G G R D H H A G R E I

Y E M P S P G M K A G G I T T L E E K S

1381/461 1411/471

CCC TCG GCT GCA CCC AGA AAG CGG gCG CGA GCC AGG TGG TGG ACG TGC TGC GCT ACG GCG

P S A A P R K R A R A R W W T C C A T A

P R L H P E S G R E P G G G R A A L R R

L G C T Q K A G A S Q V V D V L R Y G E

```
1441/481                                                    1471/491
AGC GCC TGA AAA CCC ACG GTC TGA ACC TGC TGA GCG CGC CGG GCA ATG ACG CCG TCG CAA
S   A   *   K   P   T   V   *   T   C   *   A   R   R   A   M   T   P   S   Q
 A   P   E   N   P   R   S   E   P   A   E   R   A   G   Q   *   R   R   R   N
  R   L   K   T   H   G   L   M   L   L   S   A   P   G   M   D   A   V   A   T
1501/501                                                    1531/511
CCA GCG CGC TGG CGG GTG CTG GCT GTC ATA TGG TGC TGG TCA GCA CCG GTC GCG GTA CGC
P   A   R   W   R   V   L   A   V   I   W   C   W   S   A   P   V   A   V   R
 Q   R   A   G   G   C   W   L   S   Y   G   A   G   Q   H   R   S   R   Y   A
  S   A   L   A   G   A   G   C   H   M   V   L   V   S   T   G   R   G   T   P
1561/521                                                    1591/531
CGT ACG GCG GTT TTG TGC CGA CGG TGA AAA TCG CCA CCA ACA GCG AGC TGG CGA CGA AGA
R   T   A   V   L   C   R   R   *   K   S   P   P   T   A   S   W   R   R   R
 V   R   R   F   C   A   D   G   E   N   R   H   Q   Q   R   A   G   D   E   E
  Y   G   G   F   V   P   T   V   K   I   A   T   M   S   E   L   A   T   K   K
1621/541                                                    1651/551
AAA AAC ACT GGA TTG ATT TCG ATG CGG GTC AGC TGA TCC ACG GCA AAG CGA TGC CGC AGC
K   N   T   G   L   I   S   M   R   V   S   *   S   T   A   K   R   C   R   S
 K   T   L   D   *   F   R   C   G   S   A   D   P   R   Q   S   D   A   A   A
  K   H   W   I   D   F   D   A   G   Q   L   I   H   G   K   A   M   P   Q   L
1681/561                                                    1711/571
TGC TGA CGG AGT TTG TGG ACA CTA TCG TGG AAT TTG CGA ACG GTA AGC AGA CCT GTA ACG
C   *   R   S   L   W   T   L   S   W   N   L   R   T   V   S   R   P   V   T
 A   D   G   V   C   G   H   Y   R   G   I   C   E   R   *   A   D   L   *   R
  L   T   E   F   V   D   T   I   V   E   F   A   M   G   K   Q   T   C   M   E
1741/581                                                    1771/591
AGA AGA ATG ACT TCC GGG AGC TGG CGA TTT TTA AGA GCG GtG TGA CGC TTT AAT CGG TGC
R   R   M   T   S   G   S   W   R   F   L   R   A   V   *   R   F   N   R   C
 E   E   *   L   P   G   A   G   D   F   *   E   R   C   D   A   L   I   G   A
  K   N   D   F   R   E   L   A   I   F   K   S   G   V   T   L   *   S   V   R
1801/601                                                    1831/611
GGG CTG GTG CCC TCA CCC TGA CCC TCT CCC ACT GTA CGG TCC GGG GAC ATG GTA GAC AGG
G   L   V   P   S   P   *   P   S   P   T   V   R   S   G   D   M   V   D   R
 G   W   C   P   H   P   D   P   L   P   L   Y   G   P   G   T   W   *   T   G
  A   G   A   L   T   L   T   L   S   H   C   T   V   R   G   H   G   R   Q   G
1861/621                                                    1891/631
GTT CGG GGA CAT GGt GAA CAC TTT TTA ACA TCC TTT ACC CAT GGT GAT CGA CTT TTT CTT
V   R   G   H   G   E   H   F   L   T   S   F   T   H   G   D   R   L   F   L
 F   G   D   M   V   N   T   F   *   H   P   L   P   M   V   I   D   F   F   F
  S   G   T   W   *   T   L   F   N   I   L   Y   P   W   *   S   T   F   S   S
1921/641                                                    1951/651
cAG GTC GAT CAC CCC CAC TTT CGT GCT GTA CCA CCA CAC CTC GTA GCT GCC GTC TTc CTG
Q   V   D   H   P   H   F   R   A   V   P   P   H   L   V   A   A   V   F   L
 R   S   I   T   P   T   F   V   L   Y   H   H   T   S   *   L   P   S   S   C
  G   R   S   P   P   L   S   C   C   T   T   T   P   R   S   C   R   L   P   A
1981/661                                                    2011/671
CAT CTC CTT CAG CCC GAC CCT TTc TCC CCT GAA CGC CTT GCC TGC GCT CAA CTT ACC CCT
H   L   L   Q   P   D   P   F   S   P   E   R   L   A   C   A   Q   L   T   P
 I   S   F   S   P   T   L   S   P   L   N   A   L   P   A   L   N   L   P   L
  S   P   S   A   R   P   F   L   P   *   T   P   C   L   R   S   T   Y   P   F
2041/681                                                    2071/691
TTC ACG CTC AGT TTC CGC TGA TAT CCA CTT TTC TGA CCA TCA CCC CTT CAT CGT ATT CCG
F   T   L   S   F   R   *   Y   P   L   F   *   P   S   P   L   H   R   I   P
 S   R   S   V   S   A   D   I   H   F   S   D   H   H   P   F   I   V   F   R
  H   A   Q   F   P   L   I   S   T   F   L   T   I   T   P   S   S   Y   S   G
```

Fig. 51. Alignment of the deduced amino acid sequence from pUC327 with the sequence of *Escherichia coli* altronate hydrolase obtained from GenBank. The + represents a derived amino that is similar but not identical to the amino acid in the subject amino acid sequence and that is not essential for the proper protein structure. The numbers beside the sequences indicate the nucleotide numbers in each sequence.

Query: pUC327 from *Enterobacter cloacae* CAL3

Sbjct: Altronate hydrolase from Escherichia coli, P42604 (Blattner et al., 1997)

```
Query: 306  MQYIKIHSLDNVAVALADLTEGTDVTFDGQSVTLRQAIGRGHKFALLPIAKGENVVKYGL 485
            MQYIKIH+LDNVAVALADL EGT+V+ D Q+VTLRQ + RGHKFAL  IAKG NV+KYGL
Sbjct: 1    MQYIKIHALDNVAVALADLAEGTEVSVDNQTVTLRQDVARGHKFALTDIAKGANVIKYGL 60

Query: 486  PIGHALADIAPGEYIHSHNTRTNLSDLDEYSYQPDFQAEAGQAADREVQIYRRASGEVGI 665
            PIG+ALADIA G ++H+HNTRTNLSDLD+Y YQPDFQ    QAADREVQIYRRA+G+VG+
Sbjct: 61   PIGYALADIAAGVHVHAHNTRTNLSDLDQYRYQPDFQDLPAQAADREVQIYRRANGDVGV 120

Query: 666  RNELWILPTVGCVNGIARQIQTRFLKETRDAERTDGVHLFSHTYGCSQLGDDHINTRTML 845
            RNELWILPTVGCVNGIARQIQ RFLKET +AE TDGV LFSHTYGCSQLGDDHINTRTML
Sbjct: 121  RNELWILPTVGCVNGIARQIQNRFLKETNNAEGTDGVFLFSHTYGCSQLGDDHINTRTML 180

Query: 846  QNMVRHPNAGAVLVIGLGCENNQVDAFRDTLGEFDPERVHFMVCQHQDDEVEAGVEQLHQ 1025
            QNMVRHPNAGAVLVIGLGCENNQV AFR+TLG+ DPERVHFM+CQ QDDE+EAG+E LHQ
Sbjct: 181  QNMVRHPNAGAVLVIGLGCENNQVAAFRETLGDIDPERVHFMICQQQDDEIEAGIEHLHQ 240

Query: 1026 LYDVMRHDKREPGKLSELKFGLECGGSDGLSGITANPMLGRFSDYVIANGGTTVLTEVPE 1205
            LY+VMR+DKREPGKLSELKFGLECGGSDGLSGITANPMLGRFSDYVIANGGTTVLTEVPE
Sbjct: 241  LYNVMRNDKREPGKLSELKFGLECGGSDGLSGITANPMLGRFSDYVIANGGTTVLTEVPE 300

Query: 1206 MFGAERILMSHCRDEETFEKTVTMVNDFKQYFIAHNQPIYENPSPGNKAGGITTLEEKSL 1385
            MFGAE++LM HCRDE TFEK VTMVNDFKQYFIAH+QPIYENPSPGNKAGGITTLE+KSL
Sbjct: 301  MFGAEQLLMDHCRDEATFEKLVTMVNDFKQYFIAHDQPIYENPSPGNKAGGITTLEDKSL 360

Query: 1386 GCTQKAGASQVVDVLRYGERLKTHGLNLLSAPGNDAVATSALAGAGCHMVLVSTGRGTPY 1565
            GCTQKAG+S VVDVLRYGERLKT GLNLLSAPGNDAVATSALAGAGCHMVL STGRGTPY
Sbjct: 361  GCTQKAGSSVVVDVLRYGERLKTPGLNLLSAPGNDAVATSALAGAGCHMVLFSTGRGTPY 420

Query: 1566 GGFVPTVKIATNSELATKKKHWIDFDAGQLIHGKAMPQLLTEFVDTIVEFANGKQTCNEK 1745
            GGFVPTVKIATNSELA KKKHWIDFDAGQLIHGKAMPQLL EF+DTIVEFANGKQTCNE+
Sbjct: 421  GGFVPTVKIATNSELAAKKKHWIDFDAGQLIHGKAMPQLLEEFIDTIVEFANGKQTCNER 480

Query: 1746 NDFRELAIFKSGVTL 1790
            NDFRELAIFKSGVTL
Sbjct: 481  NDFRELAIFKSGVTL 495
```

Fig. 52. The open reading frame in the last 2.1 kb of pUC327, that corresponds to a 43.5 kD hypothetical protein (1214 bp) in the egbC-exuT intergenic region of *Escherichia coli* (strain K-12), is shown in underlined bold face type.

```
1/1                                                                 31/11
CTG ATC GAa tTC GAC TCG GTA CCC GGG GAT CGA GAG CGA GCA CTT CTT CGT TCG CAA GAA
L   I   E   F   D   S   V   P   G   D   R   E   R   A   L   L   R   S   Q   E
 *   S   N   S   T   R   Y   P   G   I   E   S   E   H   F   F   V   R   K   N
  D   R   I   R   L   G   T   R   G   S   R   A   S   T   S   S   F   A   R   T
61/21                                                               91/31
CGG CCT GCT GTT TGC CAC CCC GCT GCT GCT GGT GCT GAT TCT GGT CGA GCT GAG CGA CGT
R   P   A   V   C   H   P   A   A   A   G   A   D   S   G   R   A   E   R   R
 G   L   L   F   A   T   P   L   L   L   V   L   I   L   V   E   L   S   D   V
  A   C   C   L   P   P   R   C   C   W   C   *   F   W   S   S   *   A   T   *
121/41                                                              151/51
GAT TTT CGC GGT TGA CAG CAT TCC GGC AAT CTT TGC CGT CAC CAC CGA CCC GTT CAT CGT
D   F   R   G   *   Q   H   S   G   N   L   C   R   H   H   R   P   V   H   R
 I   F   A   V   D   S   I   P   A   I   F   A   V   T   T   D   P   F   I   V
  F   S   R   L   T   A   F   R   Q   S   L   P   S   P   P   T   R   S   S   C
181/61                                                              211/71
GCT GAC GTC TAA CCT GTT CGC CAT TCT CGG CCT GCG TGC CAT GTA CTT CCT GCT GGC AGG
A   D   V   *   P   V   R   H   S   R   P   A   C   H   V   L   P   A   G   R
 L   T   S   N   L   F   A   I   L   G   L   R   A   M   Y   F   L   L   A   G
  *   R   L   T   C   S   P   F   S   A   C   V   P   C   T   S   C   W   Q   A
241/81                                                              271/91
CGC GGC GGA GCG CTT CTC AAT GCT GAA GTA CGG CCT GTC GGT GAT CCT GGT GTT TAT CGG
R   G   G   A   L   L   N   A   E   V   R   P   V   G   D   P   G   V   Y   R
 A   A   E   R   F   S   M   L   K   Y   G   L   S   V   I   L   V   F   I   G
  R   R   S   A   S   Q   C   *   S   T   A   C   R   *   S   W   C   L   S   V
301/101                                                             331/111
TAT CAA GAT GCT GAT CGT CGA TTT CTA CCA CAT CCC AAT CGC CAT TTC GCT CGG CGT GGT
Y   Q   D   A   D   R   R   F   L   P   H   P   N   R   H   F   A   R   R   G
 I   K   M   L   I   V   D   F   Y   H   I   P   I   A   I   S   L   G   V   V
  S   R   C   *   S   S   I   S   T   T   S   Q   S   P   F   R   S   A   W   C
361/121                                                             391/131
GTT TGG CAT TCT GAT CGT GAC GCT GAT TAT CAA TAC CTG GGT TAA CCG CCA GCA CGA TAA
V   W   H   S   D   R   D   A   D   Y   Q   Y   L   G   *   P   P   A   R   *
 F   G   I   L   I   V   T   L   I   I   N   T   W   V   N   R   Q   H   D   K
  L   A   F   *   S   *   R   *   L   S   I   P   G   L   T   A   S   T   I   R
421/141                                                             451/151
GAA GCA GCA GGT AGA GTA AAA CGT TCG GTG CGG CCT GAT GCC CTC ACC CCG ACC CTC TCC
E   A   A   G   R   V   K   R   S   V   R   P   D   A   L   T   P   T   L   S
 K   Q   Q   V   E   *   N   V   R   C   G   L   M   P   S   P   R   P   S   P
  S   S   R   *   S   K   T   F   G   A   A   *   C   P   H   P   D   P   L   P
481/161                                                             511/171
CAC AGG GAG AGG GTG AAC ACA CTT TTG TAA CTC AAC AGT TAA AAA ATA TGA TGC AAC ACG
H   R   E   R   V   N   T   L   L   *   L   N   S   *   K   I   *   C   N   T
 T   G   R   G   *   T   H   F   C   N   S   T   V   K   K   Y   D   A   T   R
  Q   G   E   G   E   H   T   F   V   T   Q   Q   L   K   N   M   M   Q   H   V
541/181                                                             571/191
TAA AAT CCA GCA TTT TAC GCT TCC GCC TTT CGC TGC TTT CCC TGA TAC TCA ACC AGG CAA
*   N   P   A   F   Y   A   S   A   F   R   C   F   P   *   Y   S   T   R   Q
 K   I   Q   H   F   T   L   P   P   F   A   A   F   P   D   T   Q   P   G   K
  K   S   S   I   L   R   F   R   L   S   L   L   S   L   I   L   N   Q   A   N
601/201                                                             631/211
ACA AAT ACT TAC ATC CGG ACA TAA ATG TGA CTT AGA GCA CAT CCA GGA TGG AAC GCA ATT
T   N   T   Y   I   R   T   *   M   *   L   R   A   H   P   G   W   N   A   I
 Q   I   L   T   S   G   H   K   C   D   L   E   H   I   Q   D   G   T   Q   F
  K   Y   L   H   P   D   I   N   V   T   *   S   T   S   R   M   E   R   N   F
661/221                                                             691/231
TCA CTC AGG AAT AAC GTA TGA GCA CAC AAT CAA GCG GTC TGT TCG CGC GCC TGG CGC AGG
S   L   R   N   N   V   *   A   H   N   Q   A   V   C   S   R   A   W   R   R
 H   S   G   I   T   Y   E   H   T   I   K   R   S   V   R   A   P   G   A   G
  T   Q   E   *   R   M   S   T   Q   S   S   G   L   F   A   R   L   A   Q   G
```

721/241 751/251
GCA GTC TTG TAA AAC AAA TTT CTT GAT Cgg GTT GGT ACT GGG TAT TCT GCT GGC GAT GGT
A V L * N K F L D R V G T G Y S A G D G
Q S C K T N F L I G L V L G I L L A M V
S L V K Q I S * S G W Y W V F C W R W C
781/261 811/271
GTC AAA ACC TGC TGC GGA AGC CAC GGG ACT GCT CGG CAC CCT TTT CGT TGG CGC GCT GAA
V K T C C G S H G T A R H P F R W R A E
S K P A A E A T G L L G T L F V G A L K
Q N L L R K P R D C S A P F S L A R * R
841/281 871/291
GGC CGT CGC ACC GGT ACT GGT TCT GAT GCT GGT GAT GGC GTC GAT TGC CAA CCA CCA GCA
G R R T G T G S D A G D G V D C Q P P A
A V A P V L V L M L V M A S I A N H Q H
P S H R Y W F * C W * W R R L P T T S T
901/301 931/311
CGG ACA AAA AAC CAA CAT TCG CCC TAT TCT GTT CCT GTA TCT TCT GGG AAC CTT CGC TGC
R T K N Q H S P Y S V P V S S G N L R C
G Q K T N I R P I L F L Y L L G T F A A
D K K P T F A L F C S C I F W E P S L R
961/321 991/331
GGC CTT AAC GGC CGT TGT TTT CAG CTT CCT GTT CCC GTC CAT GCT GCA CCT GAC CAG CGC
G L N G R C F Q L P V P V H A A P D Q R
A L T A V V F S F L F P S N L H L T S A
P * R P L F S A S C S R P C C T * P A R
1021/341 1051/351
GGC CGG TGA TAT TAC CCC GCC GTC CGG CAT TGT CGA AGT GCT CCG CGG TCT GCT GAT GAG
G R * Y Y P A V R H C R S A P R S A D E
A G D I T P P S G I V E V L R G L L M S
P V I L P R R P A L S K C S A V C * * A
1081/361 1111/371
CAT GGT CTC TAA CCC CAT CAC CGC GCT GAT GAA TGC GAA CTA CAT TGG CAT TCT GGT CTG
H G L * P H H R A D E C E L H W H S G L
M V S N P I T A L M N A N Y I G I L V W
W S L T P S P R * * M R T T L A F W S G
1141/381 1171/391
GGC GAT TGG ACT CGG CTT CGC GCT GCG CCA CGG TAA CGA CAC CAC CAA AAA CCT GGT GAA
G D W T R L R A A P R * R H H Q K P G E
A I G L G P A L R H G N D T T K N L V N
R L D S A S R C A T V T T P P K T W * T
1201/401 1231/411
CGA TAT GTC GAA CGC AGT CAC CTT TAT GGT GAA GCT GGT GAT TCG CTT TGC GCC TAT CGG
R Y V E R S H L Y G E A G D S L C A Y R
D M S N A V T F M V K L V I R F A P I G
I C R T Q S P L W * S W * F A L R L S G
1261/421 1291/431
GAT CTT TGG GCT GGT TTC TTC GAC GCT GGC AAC CAC CGG TTT TGA CGC GCT GTG GGG CTA
D L W A G F F D A G N H R F * R A V G L
I F G L V S S T L A T T G F D A L W G Y
S L G W F L R R W Q P P V L T R C G A T
1321/441 1351/451
CGC GCA GCT GCT GGT TGT GCT GGT CGG CTG TAT GCT GCT GGT GGC GCT GGT GAT CAA CCC
R A A A G C A G R L Y A A G G A G D Q P
A Q L L V V L V G C M L L V A L V I N P
R S C W L C W S A V C C W W R W * S T H
1381/461 1411/471
ACT GCT GGT GTT CTG GCA GAT CCG CCG CAA CCC GTA TCC GCT GGT GCT GAC CTG CCT GCG
T A G V L A D P P Q P V S A G A D L P A
L L V F N Q I R R N P Y P L V L T C L R
C W C S G R S A A T R I R W C * P A C A

1441/481 1471/491

CGA GAG CGG CGT GTA CGC CTT TCT TTC AAC CCG CAG CTC TGC GGC GAA CAT TCC GGT TAA

R E R R V R L S F N P Q L C G E H S G *

E S G V Y A F L S T R S S A A M I P V M

R A A C T P F F Q P A A L R R T F R L T

1501/501 1531/511

CAT GGC GCT GGC GGA GAA GCT GAA CCT GGA CCG CGA CAC CTA TTC CGT GTC GAT CCC GCT

H G A G G E A E P G P R H L F R V D P A

M A L A E K L M L D R D T Y S V S I P L

W R W R R S * T W T A T P I P C R S R W

1561/521 1591/531

GGG TGC AAC CGT GAA CAT GGC GGG CGC AGC AAT CAC TAT TAC CGT GCT GAC GCT GGC GGC

G C N R E H G G R S N H Y Y R A D A G G

G A T V M M A G A A I T I T V L T L A A

V Q P * T W R A Q Q S L L P C * R W R R

1621/541 1651/551

GGT GCA TAC GCT GGG CGT TCC GGT GGA CCT GCT TAC GGC GCT GCT GCT AAG CGT AGT GGC

G A Y A G R S G G P A Y G A A A K R S G

V H T L G V P V D L L T A L L L S V V A

C I R W A F R W T C L R R C C * A * W H

1681/561 1711/571

ATC GCT GTG TGC CTG CGG CGC ATC CGG CGT GGC GGG CGG TTC GCT GCT GCT GAT CCC GCT

I A V C L R R I R R G G R F A A A D P A

S L C A C G A S G V A G G S L L L I P L

R C V P A A H P A W R A V R C C * S R W

1741/581 1771/591

GGC CTG TAA TAT GTT CGG TAT CCC GAA CGA GAT CGC AAT GCA GGT TGT GGC CGT CGG CTT

G L * Y V R Y P E R D R N A G C G R R L

A C M M F G I P M E I A M Q V V A V G P

P V I C S V S R T R S Q C R L W P S A L

1801/601 1831/611

TAT CAT CGG CGT ACT GCA GGA CTC CTG CGA AAC CGC GCT GAA CTC CTC TAC CGA CGT GCT

Y H R R T A G L L R N R A E L L Y R R A

I I G V L Q D S C E T A L M S S T D V L

S S A Y C R T P A K P R * T P L P T C C

1861/621 1891/631

GTT TAC CGC AGC GGC CTG TCA GGC GGA AGA CGC GCG TTT AGC GAA GAA TGC ACT TCG AGG

V Y R S G L S G G R R A F S E E C T S R

F T A A A C Q A E D A R L A K M A L R G

L P Q R P V R R K T R V * R R M H F E V

1921/641 1951/651

TTA ACA GCA AAA CGG CAA CCC TGA GGT TGC CGT TTT TAG TGT TTG TAC CCT CTC CCC GTG

L T A K R Q P * G C R F * C L Y P L P V

* Q Q N G N P E V A V F S V C T L S P W

N S K T A T L R L P F L V F V P S P R G

1981/661 2011/671

GGT GTA CGG TCC GGG GAC ATA GTG ACA CTT CTC CGA CAT GGT GCC TTC ACT AAG CAT AAA

G V R S G D I V T L L R H G A F T K H K

V Y G P G T * * H F S D M V P S L S I K

C T V R G H S D T S P T W C L H * A * N

2041/681 2071/691

ACC CTT CTT TGG ACT TTT TTC CCG GTA TGC AAC AAT CCA TTG TTT TTC CCC CCG AGT

T L L W T F F P V C N N P L F F P P S

P F F G L F S R Y A T I H C F S P R

P S L D F F P G M Q Q S I V F P P E

Fig. 53. Alignment of the deduced amino acid sequence from pUC327 with the sequence of a hypothetical 43.5 kD protein in the egbC-exuT intergenic region of *Escherichia coli* (strain K-12), obtained from GenBank. The X's represent stretches of derived amino acids where the BLAST program finds no consensus with the amino acid sequences of the subject; the + represents a deduced amino acid that is similar but not identical to the amino acid in the subject sequence and that is not essential for the proper protein structure. The numbers beside the sequences indicate the nucleotide numbers in each sequence.

Query: pUC327 from *Enterobacter cloacae* CAL3

Sbjct: *Escherichia coli* hypothetical 43.5 kD protein, P42602. (Blattner et al., 1997).

```
Query: 704  RAPG-----AGQSCKTNFLIGLVLGILLAMVSKPAAEATGLLGTLFVGXXXXXXXXXXXXX 868
             R+PG     A  S     L+GLVLGILLA +SKPAAEA GLLGTLFVG
Sbjct: 5    RSPGLFRRLAHGSLVKQILVGLVLGILLAWISKPAAEAVGLLGTLFVGALKAVAPILVLM 64

Query: 869  XXXXSIANHQHGQKTNIRPILFLYLLGTFAAALTAVVFSFLFPSMLHLTSAAGDITPPSG 1048
                 SIANHQHGQKTNIRPILFLYLLGTF+AAL AVVFSF FPS LHL+S+AGDI+PPSG
Sbjct: 65   LVMASIANHQHGQKTNIRPILFLYLLGTFSAALAAVVFSFAFPSTLHLSSSAGDISPPSG 124

Query: 1049 IVEVLRGLLMSMVSNPITALMNANYIGILVWAIGLGFALRHGNDTTKNLVNDMSNAVTFM 1228
             IVEV+RGL+MSMVSNPI AL+  NYIGILVWAIGLGFALRHGN+TTKNLVNDMSNAVTFM
Sbjct: 125  IVEVMRGLVMSMVSNPIDALLKGNYIGILVWAIGLGFALRHGNETTKNLVNDMSNAVTFM 184

Query: 1229 VKLVIRFAPIGIFGLVSSTLATTGFDALWGYAQXXXXXXXXXXXXXXXXINPLLVFWQIRR 1408
             VKLVIRFAPIGIFGLVSSTLATTGF  LWGYAQ                +NPLLV+W+IRR
Sbjct: 185  VKLVIRFAPIGIFGLVSSTLATTGFSTLWGYAQLLVVLVGCMLLVALVVNPLLVWWKIRR 244

Query: 1409 NPYPLVLTCLRESGVYAPLSTRSSAANIPVNMALAEKLNLDRDTYSVSIPLGATVNMAGX 1588
             NP+PLVL CLRESGVYAF  TRSSAANIPVNMAL EKLNLDRDTYSVSIPLGAT+NMAG
Sbjct: 245  NPFPLVLLCLRESGVYAFF-TRSSAANIPVNMALCEKLNLDRDTYSVSIPLGATINMAGA 303

Query: 1589 XXXXXXXXXXXXHTLGVPVDXXXXXXXXXXXXXXXXXXXXXXXXXXXXIPLACNMFGIPN 1768
                         +TLG+PVD                                IPLACNMFGI N
Sbjct: 304  AITITVLTLAAVNTLGIPVDLPTALLLSVVASLCACGASGVAGGSLLLIPLACNMFGISN 363

Query: 1769 EIAMQVVAVGFIIGVLQDSCETALNSSTDVLFTAAACQAEDARLAKNALR 1918
             +IAMQVVAVGFIIGVLQDSCETALNSSTDVLFTAAACQAED RLA +ALR
Sbjct: 364  DIAMQVVAVGFIIGVLQDSCETALNSSTDVLFTAAACQAEDDRLANSALR 413
```

4. Discussion

Soil contains a diverse collection of microorganisms that can utilize unusual carbon or nitrogen sources. The capability of a certain microorganism to survive on an uncommon substrate, such as opine or naphthalene, may provide the microorganism with a competitive advantage in some soil environments. The enzyme, ACC deaminase, has been found in a variety of soil microorganisms that can hydrolyze ACC into α-ketobutyrate and ammonia (Glick et al., 1999b). In nature, ACC is produced primarily by plants as a precursor to ethylene (Adams and Yang, 1979). Although the role of ACC deaminase in microorganisms has not been fully explicated, it has been suggested that ACC deaminase may function within plant-microbe interactions, as one of the mechanisms used by soil bacteria to enhance the growth of plants (Glick et al., 1995; 1997; 1999b) (see section 1.4.5.).

Previous studies have elucidated the biochemical properties of ACC deaminase, and have shown that the metabolism of ACC, an α-dialkyl-α-amino acid containing a cyclopropane ring, depends on a unique regiospecific ring-opening reaction (Honma and Shimomura, 1978; Honma 1985, 1986, 1993). More recently, a number of ACC deaminase genes have been isolated from soil bacteria (Glick et al., 1999b) and several of these genes have been introduced into plants, including tomato (Klee and Kishore, 1992; Yang and Hiatt, 1991; Lund et

al., 1998; Robison et al., submitted for publication) tobacco and Arabidopsis (Romano et al., 1993). The ethylene levels in these transgenic plants were substantially lowered. There was also a noticeable decline in the levels of stress ethylene produced by plants grown in the presence of ACC-deaminase containing strains of plant growth–promoting bacteria—canola seedlings grown in the presence of high levels of nickel and cucumber plants exposed to phytopathogens (Burd et al., 1998; Wang et al., submitted for publication).

The focus of this study was to investigate the role of ACC deaminase in plant growth-promoting bacteria. As a first step, the level of ACC was measured in the tissues of canola seedlings, and the tissues and exudates of germinating canola seeds, and these were compared with the levels of ACC in the tissues and exudates of germinating canola seeds treated with the plant growth-promoting bacterium, *Enterobacter cloacae* CAL3. This work included the development of a method for measuring ACC in plant tissues and exudates, and the quantification of amino acids related to ACC in the same plant materials. Following this, an attempt was made to isolate the ACC deaminase gene from *Enterobacter cloacae* CAL3 and characterize the strains of *Escherichia coli*, containing the putative ACC deaminase genes, viz-a-viz their growth-promoting capabilities.

4.1. Development of a method for measuring ACC in plant tissues and exudates

4.1.1. Comparison with other methods of measuring ACC

A number of procedures have been developed for the measurement of ACC in plant tissues. ACC is most frequently quantified by the method of Lizada and Yang (1979) in which ACC is chemically converted to ethylene and measured by gas chromatography. This technique, although rapid and relatively simple, is susceptible to interference by a number of compounds including phenolics. In addition, this procedure measures ACC indirectly and lacks sensitivity (at least one ng of ACC is required for detection) (Sitrit et al., 1998; McGaw et al., 1985). The other method of indirect ACC measurement involves the conversion of ACC to α-ketobutyrate by the enzyme, ACC deaminase. However, this procedure depends on a source of ACC deaminase and requires a rather high level for detection—at least one ng of ACC (Honma, 1983).

A range of methods have been developed to measure ACC directly and all depend on the derivatization of ACC prior to its measurement. Various ACC derivatives—a 2,4-dintrophenol derivative of ACC, a phthalimido methyl ester of ACC and an N-benzoyl-n-propyl derivative of ACC—have been measured by a combination of gas chromatography-mass spectrometry (Savidge et al., 1983; McGaw et al., 1985; Hall et al., 1989). Although more sensitive than the indirect

methods—10 pg of ACC can be measured accurately—this procedure requires a lengthy purification process that involves three HPLC purification steps and the purchase of expensive equipment. As well, a capillary column gas chromatograph fitted with a standard nitrogen phosphorus detector has been used to quantify ACC as an N-benzoyl-n-propyl derivative of ACC, one of the ACC derivatives also measured by a gas chromatograph-mass spectrometer method (Hall et al., 1993). When compared with the gas chromatograph-mass spectrometer procedure, the cost of equipment required for this method was lower; the sensitivity of the measurement was similar but the detection of ACC was much less specific.

A highly specific method of ACC detection and quantification was developed by Chauvaux et al. (1993; 1997). ACC is measured as a phenylisothiocyanate derivative by selected ion monitoring and liquid chromatography connected to a mass spectrometer equipped with a thermospray interface. By using the same equipment with a thermospray tandem mass spectrometer instead of a single thermospray mass spectrometer, the detection limit of ACC is lowered from 10 to 0.1 pmoles of ACC and both free and conjugated ACC can be quantified. However, the equipment required for both of these techniques is extremely expensive.

An ion-exchange amino acid analyzer has been used to measure ACC. Unfortunately, quantification of ACC in biological samples by

this method, is problematic because the ACC peak is not well resolved. It co-elutes with the threonine peak in the midst of several other compounds making the separation of ACC from threonine and other amino acids, very difficult (Lanneluc-Sanson et al., 1986). A variety of ACC derivatives have also been used to measure ACC by reversed phase HPLC. An orthophthalaldehyde derivative of ACC was quantified by reversed phase HPLC and fluoresence detection. Although this procedure is rapid, sensitive—one pmole of ACC can be detected—and does not require expensive equipment, the orthophthalaldehyde derivative of ACC degrades quickly prior to injection. The procedure is complicated by the requirement for accurate timing of the derivatization reaction and column injection to avoid ACC degradation (Bushey et al., 1987). Phenylthiocarbamyl derivatives of ACC, formed by the coupling of ACC with phenylisothiocyanate, are detected and measured by reversed phase HPLC and a UV monitor (Lanneluc-Sanson et al., 1986). This method is rapid and relatively inexpensive but not very sensitive—10-20 pmoles of ACC are detected. In addition, the samples require vacuum drying after derivatization to remove reaction byproducts, a process that may result in the loss of ACC.

4.1.2. The Waters AccQ•Tag™ method of amino acid analysis

The method of ACC quantification used in this study is a modification of the Waters AccQ•Tag™ method of amino acid analysis, which is based on the work of Cohen and Michaud (1993). This technique is both simple and sensitive, and avoids many of the problems associated with the other methods. The AccQ•Fluor reagent, AMQ (6-aminoquinolyl-N-hydroxysuccini-midyl carbamate), is used to make stable derivatives of primary and secondary amines in a simple reaction; the AccQ•Tag labelled-derivatives of ACC are resolved by reversed phase HPLC on a C18 column and measured by a fluoresence detector. The only major fluorescent by-product of the derivatization reaction, AMQ (6-aminoquinoline), does not interfere with the detection of the amino acid derivatives and does not have to be removed before the sample is injected onto the column. The Waters AccQ•Tag™ method of amino acid analysis has the advantages of the other pre-column derivatization procedures that measure amino acids by reversed phase chromatography: the procedure is fast, relatively inexpensive and sensitive. The amino acids are derivatized during a one-minute, one-step reaction. The expense of the AccQ•Tag column, reagents, HPLC equipment and fluorescence detector is less than that required by a gas chromatograph-mass spectrometer method of measuring ACC.

4.1.2.1. Quantification of ACC by the Waters AccQ•Tag™ method of amino acid analysis

The sensitivity of the Waters AccQ•Tag™ method of amino acid analysis is very similar to that of the orthophthalaldehyde pre-column derivatization procedure which detects one pmole (or less) of ACC. ACC was quantified in fluoresence units by using a freshly prepared standard curve which is linear between 1 and 25 pmoles. However, the peak areas of one pmole of ACC measured by the Waters AccQ•Tag™ method showed some variability. Cohen and Michaud (1993) have suggested that the detection of very low levels of amino acids by this method may not be possible because of background levels of amino acids in the 0.1 to 1.0 pmole range in the hydrolyzed blank controls. These background peaks were visible in the chromatograms of very dilute samples. Indeed, one of these background peaks occurred very near the ACC peak in dilute samples of seed exudates and seed extracts.

4.1.3. Modification of the Waters AccQ•Tag™ method of amino acid analysis

Although ACC was resolved in samples of canola seed extracts or exudates by the Waters AccQ•Tag™ method of amino acid analysis, in this study the gradient table recommended for the Waters AccQ•Tag System was modified to enhance the separation of ACC from other amino acids and to avoid the background peaks seen in very dilute

samples of seed extracts or exudates. The amino acids normally eluted in the first 21 minutes were compressed into 6.5 minutes; the column washing and conditioning at the end of run, was reduced from 19 to 9 minutes, and the elution of amino acids including ACC, α-, β-, and γ-aminobutyric acids, tyrosine, cysteine, valine and methionine, was spread out over 7 instead of 5 minutes, during the middle portion of the run. As a result of the gradient modification, the separation of ACC from the surrounding amino acid peaks was improved and the length of time for each run was reduced from 45 to 23 minutes, making it possible to analyze more samples during each day.

4.1.4. Stability of ACC

ACC is a rather unstable compound in solution and degrades quickly. Lanneluc-Sanson et al. (1986) noted a decrease of 3.3% per day when refrigerated. A similar reduction, of approximately 5%, was seen in the peak area of a 32 pmole sample of ACC, stored at 4°C overnight. The peak area decreased further after two nights at 4°C, and following three nights at this temperature, the chromatograms of the sample showed several new peaks suggesting that ACC was degrading, even when refrigerated as a fairly concentrated solution.

When stored at –20°C, ACC was relatively stable for long periods of time: the peak area of a 32 pmole sample of ACC decreased by approximately 2% after one night in the freezer and between 5 and 10%

after 2 months at –20°C. Following three freeze-thaw cycles, there was a reduction of approximately 2.25% in the peak area of this sample for each freeze-thaw cycle. However, the chromatograms of the samples following three freeze-thaw cycles, showed well-resolved peaks of ACC which indicated that there was very little degradation of ACC at –20°C. Throughout this study, in order to minimize the hydrolysis of ACC, standard and sample solutions containing ACC, were stored frozen at either –20°C or –80°C and not subjected to more than one freeze-thaw cycle.

4.1.5. Stability of AccQ•Tag-labelled ACC

The Waters AccQ•Tag™ method of amino acid analysis depends on the coupling of the AccQ•Fluor reagent, AQC, with primary and secondary amines to yield fluorescent derivatives, which are stable for seven days at room temperature. The stability of AccQ•Tag-labelled ACC was monitored over 26 days at room temperature. The ACC derivatives were stable for four days and then began to degrade; by six days the degradation was very noticeable—only 70% of the original amount of ACC was still present. Thus, in order to avoid the loss of ACC by degradation, all standards and samples used in this study, were analyzed within one day of being derivatized.

4.1.6. Recovery of ACC from amino acid standard mixtures, canola seed exudate and canola seed exudate

A mixture of 17 hydrolyzed amino acids (tryptophan, glutamine, and asparagine not included) which contains 25 pmoles of each amino acids (with the exception of cysteine which is 12.5 pmoles) was used as a calibration standard. The gradient was modified to extend the elution of the amino acids: cysteine, tyrosine, valine and methionine, which appeared as well-resolved, measurable peaks. When four different concentrations of ACC, between 2 and 16 pmoles, were added separately to the standard mixture, ACC was eluted along with amino acid hydrolysates, also as a well-resolved peak. At all four ACC concentrations, at least 92% of the ACC added to the mixture, was recovered.

Several concentrations of ACC, between 5 and 18.75 pmoles, were added separately to exudate samples collected from canola seeds treated with $MgSO_4$ five hours after seed treatment. Well-resolved peaks of ACC were observed in the chromatograms of the undiluted exudates and in the chromatograms of the diluted exudates plus various concentrations of ACC. The recovery of ACC from the exudate samples hovered around 80% when the concentrations of added ACC were between 5 and 12.5 pmoles; above 12.5 pmoles, at least 100% of the added ACC was recovered. Normally, the exudate samples were not diluted prior to analysis because the amount of ACC was low. In this

case, the exudate was diluted between five- and ten-fold before the addition of the ACC. A small difference in the ACC measurement, between the undiluted and diluted sample, would be magnified after the dilution was taken into account. This may explain the lower-than-expected recovery of ACC.

The recovery of ACC, added to extracts of canola seeds treated with *Enterobacter cloacae* CAL3, was consistently around 100% for all three concentrations of ACC added to it. Aside from the recovery of the lower amounts of ACC added to the canola seed exudate, it appeared that ACC, added to a variety of samples including amino acid standards, canola seed exudates, and canola seed extracts, can be fully recovered and that the lower amount of ACC recovered from exudate may be caused by the preparation of the sample i.e., dilution, and not the measurement of ACC.

4.2. Assessment of plant growth-promoting bacteria

4.2.1. Root elongation

An assessment of the plant growth-promoting capabilities of the bacterium, *Enterobacter cloacae* CAL3, was based on enhanced root lengths of 4.5-day old plants grown under gnotobiotic conditions from canola seeds inoculated with this bacterium. In comparison to the root lengths of the control plants—those grown from seeds treated with $MgSO_4$—the roots of the *Enterobacter cloacae* CAL3-treated seeds were

significantly longer. Shorter roots were also seen in seedlings from seeds treated with *Pseudomonas putida* pRK415, a *Pseudomonas* strain that contains a broad host range plasmid but does not carry an ACC deaminase gene. However, the longest roots were observed in plants from seeds treated with a *Pseudomonas* strain transformed with a broad host range plasmid carrying a gene for ACC deaminase, *Pseudomonas putida* pRKACC.

4.2.2. Presence of ACC in canola seedlings

It is well known that ethylene is important during the early phase of plant growth. Ethylene is required for seed germination by many plants species and the rate of ethylene production increases during germination and seedling growth (Abeles et al., 1992). Although low levels of ethylene appear to enhance root initiation and growth, and promote root extension, high levels of ethylene produced by fast growing roots can lead to inhibition of root elongation (Mattoo and Suttle, 1991; Ma et al., 1998). We have speculated that the longer roots seen in plants from seeds treated with the plant growth-promoting bacterium, *Enterobacter cloacae* CAL3, or the *Pseudomonas* strain, *Pseudomonas putida* pRKACC, that carries an ACC deaminase gene on a broad host range plasmid, are the result of a reduced ethylene concentration. Following germination, the level of ACC and hence ethylene, is lowered in or around the roots by the presence of the

enzyme, ACC deaminase, which hydrolyzes the ACC exuded by the seeds and roots, to α-ketobutyrate and ammonia. It was the speculation about the levels of ACC, in and around the roots of seedlings, and seeds and seed exudates, that became the focus of this study.

We used canola seedlings, grown under gnotobiotic conditions for the root elongation assay, as a source of root and shoot tissue for the ACC measurements in seedlings. With the instability of ACC in mind, the degradation of ACC was minimized during the preparation of seedling tissue extracts. The roots and shoots were excised from the seedlings, and immediately frozen in liquid nitrogen. The ACC in the extracts was most stable when the frozen powdered roots were suspended in a very small volume of buffer, briefly centrifuged to remove cell debris, clarified by high speed centrifugation and the resultant supernatant collected in glass vials and frozen at –80°C.

As predicted, the quantity of ACC in root tissues from seeds treated with a bacterium containing an ACC deaminase gene, was lower than in the control plants. Lower levels of ACC were present in the roots from seeds treated with the plant growth-promoting strain, *Enterobacter cloacae* CAL3, and the *Pseudomonas* strain containing ACC deaminase on the plasmid, pRKACC, than in the roots from seeds treated with $MgSO_4$ or the *Pseudomonas* strain that lacks the ACC deaminase gene. Roots from canola seeds treated with the ethylene inhibitor, AVG, also showed a reduced level of ACC although the

reduction was less than that observed in the roots from the seeds treated with ACC deaminase-containing bacteria (results not shown).

There were small, but insignificant, differences in the quantities of ACC in the shoots of the seedlings from seeds exposed to the various treatments. However, the levels of ACC in canola shoots were consistently lower than those found in roots for all the seed treatments. A similar effect was seen in four day-old sunflower seedlings fed high levels of ACC through their roots. Although ACC was rapidly transported from the roots to the shoots after feeding, the ACC levels in the shoots remained lower than those found in the roots, and the amount of ACC was consistently less than the amount of M-ACC (N-malonyl-ACC) (Finlayson et al., 1991). It is possible in our study, that some of the ACC present in the shoots existed in a conjugated form such as M-ACC, and was not measured by our method. Seedlings, particularly those with high levels of ACC in the roots—those grown from seeds treated with $MgSO_4$ or the non-ACC deaminase containing *Pseudomonas* strain, may have had high levels of M-ACC.

4.2.3. Relationship of root elongation, root ACC content and ACC deaminase activity of bacterial inoculants

Previously, we have assumed that enhanced root elongation, and low levels of ACC in roots are due to high levels of ACC deaminase activity in the bacterial seed inoculants. In this work, the relationship

between ACC deaminase activity and the plant growth-promoting capabilities of the bacterial inoculants, *Enterobacter cloacae* CAL3, and *Pseudomonas putida* pRKACC, the *Pseudomonas* strain containing an ACC deaminase gene, were examined. Each of these two bacterial strains and $MgSO_4$ were used as treatments for canola seeds in two root elongation assays. In the first assay, seeds were treated with either $MgSO_4$ or inoculated with strains of bacteria containing relatively high levels of ACC deaminase activity and in the second assay, seeds were treated with either $MgSO_4$ or strains of bacteria containing low levels of ACC deaminase activity. Following 4.5 days of growth under gnotobiotic conditions, the root lengths were assessed and the levels of ACC in the roots were measured. When the ACC deaminase activities of the inoculants were high, the root lengths of the bacteria-treated seedlings were longer than those of the control plants and the levels of ACC in the roots from the bacteria-treated seeds were lower than those from the control seeds. However, very little root elongation and very high levels of root ACC were observed in the bacteria-treated plants when the bacterial inoculants contained low levels of ACC deaminase activity. These results indicated that a fairly high level of ACC deaminase activity was required for successful plant growth-promotion by these bacteria.

4.2.4. Presence of ACC in the exudates and extracts of canola seeds

A procedure was designed to monitor the presence of ACC in both the tissues and the exudates of germinating canola seeds. Although some amino acids had been detected and quantified in canola seed exudate by Bayliss et al., (1993; 1997), there was no information about the measurement of ACC in canola seeds or seed exudate. In the present study, samples of canola seeds and seed exudates were removed at specific time intervals until 50 hours after seed treatment. After 50 hours, most of the seeds had lost their seed coats; the radicals had grown 7 to 10 mm and the second set of leaves had emerged. The seeds were too crowded to grow properly and were also absorbing liquid, rapidly. The seeds and seed exudates were harvested at specified times with the instability of ACC in mind. The seeds were removed from the sieves, immediately placed in liquid nitrogen and stored frozen at –80°C. The seed extracts were prepared in the same fashion as the root extracts (see section 4.2.1). The seed exudates were removed from the petri dishes and filtered through sterile 0.2 μm syringe filters to remove any contaminating microorganisms and immediately frozen at –80°C.

The pattern of ACC exudation was similar for both the $MgSO_4$-treated seeds and the *Enterobacter cloacae* CAL3-inoculated seeds: two peaks within the first 24 hours after seed treatment followed by a drop in the level of ACC. Prior to the first peak that occurred within the first hour of seed treatment, there was a high level of ACC in the exudate of

the *Enterobacter cloacae* CAL3-treated seeds. The treatment of the seeds with a bacterium may act as a stress and the seeds respond by producing a small ethylene peak. However, the later peaks of ACC in the exudates from the bacterium-treated seeds, were both much smaller than those from the control seeds, and then rapidly dropped to a very low level. This suggests, that by two hours after seed treatment, the ACC exuded by these seeds, was being sequestered and hydrolyzed by the bacterial ACC deaminase. As a result, there was a dramatically reduced level of ACC in the exudate of the bacterium-treated seeds.

Initially, low levels of ACC were observed in the extracts of both the bacterium-inoculated and $MgSO_4$-treated seeds. Two peaks of ACC were observed in the bacterium-treated seed extract: a small early peak and a second larger peak at approximately 30 hours. The extract from the control seeds also showed a small early peak; this was followed by an increasing level of ACC up until 50 hours. A similar pattern has been observed in the production of ethylene in plants, following infection with phytopathogens: a small early peak of ethylene and a much larger peak two to three days later (Abeles et al., 1992). The first peak acts as a signal and the second larger peak is part of the plant defense response (Glick et al., 1999b). Both ACC peaks in the *Enterobacter cloacae* CAL3 seed extracts occurred earlier and were smaller than those in the control seed extracts. It is likely that the burst of ethylene necessary for germination of the seeds was smaller in the bacterium-treated seeds

than the control seeds because the ACC levels were lower in these seeds. More ACC was being exuded from the bacterium-treated seeds in order to maintain the equilibrium between the levels of ACC outside and inside the seeds.

4.3. Presence of other amino acids in the extracts of canola seedlings, and in the exudates and extracts of canola seeds

Although the purpose of this study was to monitor ACC in plant tissues and exudates, it was possible to use our method of ACC quantification to detect and measure other amino acids in the same plant materials. As a result of the modification of the Waters AccQ•Tag™ method of amino acid analysis, several other amino acids, including ACC, α-, β-, and γ-aminobutyric acid, tyrosine, cysteine, valine and methionine, were eluted in well-resolved peaks which could be easily and accurately quantified. Of these amino acids, α- and γ-aminobutyric acid, tyrosine, valine and methionine, were quantified in canola seed extracts and exudates, and in canola seedlings; cysteine and β-aminobutyric acid were detected but the levels were too low to measure.

4.3.1. Presence of α-aminobutyric acid in the extracts and exudates of canola seeds

α-Aminobutyric acid is a four carbon non-protein amino acid which has been shown to promote ethylene production and

accumulation of pathogenesis-related proteins in tomato plants (Cohen et al., 1994). The presence of this amino acid in the canola seed exudate samples, during the first 50 hours following seed treatment, was almost identical for both seed treatments. There was a large peak within the first ten hours after seed treatment and a smaller peak, about 40% of the size of the larger peak, at 44 hours. The highest levels of α-aminobutyric acid in the exudates of both the bacterium-treated and $MgSO_4$-treated canola seeds were larger than the levels of ACC attained in canola seed exudates from either treatment. α-Aminobutyric acid is known to function as an inducer of ACC deaminase activity—to almost the same extent as ACC—but not as a substrate, with the *Pseudomonas* sp. strain ACP (Honma, 1983). It is possible that α-aminobutyric acid may act in this capacity with *Enterobacter cloacae* CAL3. There were quantities of α-aminobutyric acid (comparable to those of ACC) in the exudate of the control seeds that could induce the ACC deaminase activity. The high levels of α-aminobutyric acid in the exudate from *Enterobacter cloacae* CAL3-treated seeds indicate that α-aminobutyric acid was not hydrolyzed and therefore, not used as a substrate by the enzyme, ACC deaminase. However, the initial α-aminobutyric acid peak occurred several hours earlier in the exudate of the bacterium-treated seeds than the control seeds. Frequently, the presence of a bacterium can increase exudation from seeds and roots (Glick et al., 1999) which may result in

the early appearance of compounds, such as amino acids, in seed and root exudate.

Very low levels of α-aminobutyric acid were observed in the extracts of canola seeds: the highest quantities of α-aminobutyric acid in the extracts from both seed treatments, were seven- to eight-fold lower than those of ACC in the same extracts. The presence of α-aminobutyric acid in the extracts of the MgSO4-treated seeds followed the same curve, albeit at a much lower level, as that of ACC in the same extract: a small peak around 18 hours followed by an increase in the level of α-aminobutyric acid up to 50 hours. The amount of α-aminobutyric acid increased in the extract of the bacterium-treated seeds between 7 and 44 hours after seed treatment and then reached a plateau level by 50 hours. The level of α-aminobutyric acid in the bacterium-treated seed extract at 50 hours was lower than the control seed extract at 50 hours which may reduce the ethylene production by these seeds.

4.3.2. Presence of γ-aminobutyric acid in the exudates and extracts of canola seeds

γ-Aminobutyric acid, another nonprotein amino acid, is accumulated in response to environmental stimuli, is a significant component of the free amino acid pool and stimulates ethylene production by acting on ACC synthase (Kathiresan et al., 1997). The levels of γ-aminobutyric acid in canola seed exudates were very high

compared with those of ACC: the highest level of γ-aminobutyric acid was approximately 5- and 20-fold greater in the exudates from the control seeds and bacterium-treated seeds, respectively. However, the levels of γ-aminobutyric acid in canola seed exudates followed a pattern similar to that of ACC: early peaks, followed by a drop in the level of γ-aminobutyric acid. The peaks in the exudates from the bacterium-treated seeds occurred earlier and were consistently lower than those from the $MgSO_4$-treated seeds. However, by 50 hours, while the quantity of γ-aminobutyric acid was still dropping in the exudates of the control seeds, the level of γ-aminobutyric acid had begun to increase in exudates from the bacterium-treated seeds. Perhaps the bacterium, *Enterobacter cloacae* CAL3, can metabolize γ-aminobutyric acid; structurally, it is a flexible molecule that can assume several conformations in solution, including a cyclic structure similar to proline, and possibly ACC (Shelp et al., 1999). It is also probable that γ-aminobutyric acid and other amino acids are synthesized, and exuded, by the seeds in cycles during germination. Hence, one would expect an increase in the level of γ-aminobutyric acid in the exudates of the control seeds at time, somewhat later than 50 hours.

When compared with ACC, the pattern of γ-aminobutyric acid presence in canola seed extracts from both seed treatments was quite similar although the levels of γ-aminobutyric acid were about twice as much as the amount of ACC in the same extracts. There was a modest

basal level of γ-aminobutyric acid in both extracts—a large peak in the bacterium-treated seed extracts at approximately 30 hours, and an increasing level of γ-aminobutyric acid in control seed extracts by 50 hours. As with the exudation of γ-aminobutyric acid, it is quite likely that this amino acid was being synthesized in cycles and would peak in the control seed extracts several hours later, possibly at a level greater than that in the bacterium-treated seeds. High levels of γ-aminobutyric acid are known to inhibit stem elongation (Shelp et al., 1999). Thus, the seedling benefits from the presence of the plant growth-promoting bacteria in the seed treatment, if the amount of γ-aminobutyric acid in the seed is lowered and prevents inhibition of stem elongation.

4.3.3. Presence of valine and tyrosine in the extracts of canola seedlings and in the exudates and extracts of canola seeds

The synthesis of valine, isoleucine and leucine, three amino acids which possess branched aliphatic side chains, is similar. The ACC deaminase gene promoter in *Enterobacter cloacae* UW4 is under the transcriptional control of a leucine-reponsive regulatory protein that is regulated by ACC (Grichko and Glick, submitted for publication). Valine was detected in the exudates and extracts of canola seeds, and in 4.5-day old seedlings. However, in the exudate samples, valine appeared at levels higher than ACC—approximately 5- and 8-fold greater than ACC in exudates from $MgSO_4$-treated and bacterium-

treated seeds, respectively. The pattern of valine content over 50 hours was very similar to that of ACC and some other amino acids: a small peak followed by a large peak and then a drop to zero by 20 hours. The valine peaks in the exudates of the bacterium-treated seeds were much smaller, especially the second peak, than the peaks in the control seed exudate that may indicate hydrolysis of the amino acid by the bacterium. It is also possible that the high levels of valine in the seed exudate may be involved in the regulation of the ACC deaminase gene.

The amounts of valine measured at the specified time intervals over 50 hours were remarkably similar to the quantities of ACC in the extracts of canola seeds treated with MgSO4 or *Enterobacter cloacae* CAL3 seed. By 4.5 days the quantities in the root and shoot extracts from all the seed treatments were less than 50% of those for ACC. The highest levels of valine were seen in the roots of the $MgSO_4$-treated seeds and lower, almost identical levels were observed in the roots of all the bacteria-treated seeds (those treated with *Enterobacter cloacae* CAL3, *Pseudomonas putida* pRK415, or *Pseudomonas putida* pRKACC). As with ACC, there was very little difference in the amounts of valine in canola shoots from seeds exposed to different treatments although the levels were lower than ACC levels in shoots. It appears that valine was being synthesized in the seedlings—at lower levels than ACC—and that some of the valine was exuded from the bacterium-treated seeds and seedlings, and then metabolized by the bacterium.

Tyrosine was present in the exudate of canola seeds at levels similar to those of ACC but dropped to zero by 24 hours after seed treatment. The same profile of tyrosine content was observed in the exudates of the both the $MgSO_4$-treated and *Enterobacter cloacae* CAL3 treated-seeds although, as with ACC, the peaks were lower and occurred earlier in the exudates of the bacterium-treated seeds. It is likely that the tyrosine exuded by the *Enterobacter cloacae* CAL3-treated seeds was being metabolized by the bacterium.

The amount of tyrosine detected over 50 hours following seed treatment was approximately 50% of that seen with ACC in canola seed extracts but the profile was almost identical. A small tyrosine peak was followed by a larger peak in the extract of the bacterium-treated seeds. The pattern of tyrosine content in the control seed extracts indicated that this amino acid was being synthesized in cycles in the seeds and would peak several hours later than the bacterium-treated seeds, and possibly at a higher level.

4.3.4. Presence of methionine in the exudates and extracts of canola seeds

In higher plants ethylene is synthesized from L-methionine via the intermediates SAM, and ACC which is accompanied by recycling of the methylthio moiety (Yang and Hoffman, 1984). The presence of this salvage pathway in most plants, ensures maximum availability of

methionine, which exists in low levels in higher plants, and SAM to the plant (Fluhr and Matoo, 1996).

As expected, low levels of methionine were observed in the exudate of canola seeds—less than 50% of those of ACC. The pattern of methionine content was similar to the ones seen with other amino acids: two peaks in the first 20 hours followed by a drop to zero. The peaks in the exudates from the bacterium-treated seeds were much lower than those from the control seeds. The second peak in the exudate of the $MgSO_4$-treated seeds was very large compared to similar peaks of other amino acids. Perhaps there was a relatively large quantity of methionine stored in the seeds which was exuded very soon after seed treatment.

The profile of methionine content in the canola seed extracts was similar to that of ACC and other amino acids although the overall levels of methionine were about five-fold lower than ACC levels in the same extracts. This was not unexpected because methionine is maintained at low levels in plant tissue (Ravanel et al., 1998). It appeared that methionine was being produced in cycles in both the bacterium-treated seeds and the control seeds. The first cycle in the seeds treated with $MgSO_4$ was around 20 hours after treatment, and the second was sometime after 50 hours. As was seen with the other amino acids including ACC, methionine seemed to cycle at an earlier time in the bacterium-treated seeds.

4.3.5. Application of the method of ACC quantification

The Waters AccQ•Tag™ method of amino acid analysis proved to be very useful for the measurement of ACC in canola seeds and seedlings, when the gradient was modified to enhance the separation of ACC from other amino acids. In fact, the procedure was sensitive enough to detect and quantify ACC in canola seed exudates that contain very low levels of ACC. By using this technique, it was possible to show that ACC was present in canola seed exudates and that the levels of ACC were lower in the exudates of seeds treated with the plant growth-promoting bacterium, *Enterobacter cloacae* CAL3 than those from the seeds treated with $MgSO_4$. It appeared that the ACC in the exudates from the *Enterobacter cloacae* CAL3-treated seeds was being hydolyzed by the bacterium, probably by the activity of ACC deaminase.

Other amino acids were also measured. These amino acids eluted in the portion of the gradient that was expanded by us to increase the resolution of ACC and these amino acids, as well as ACC, were eluted as well-resolved peaks. The Waters AccQ•Tag™ method of amino acid analysis, which is a simple and rapid technique, could be further modified to enhance the separation of most other amino acids.

4.4.1. Isolation and characterization of putative ACC deaminase genes from *Enterobacter cloacae* CAL3

In this study, an attempt was made to isolate an ACC deaminase gene from the plant growth-promoting strain, *Enterobacter cloacae* CAL3. Previously in our laboratory, ACC deaminase genes had been isolated from two strains of *Enterobacter cloacae*, UW4 and CAL2. DNA sequencing and comparison of the deduced amino acid sequences revealed that the ACC deaminase genes isolated from these strains were highly homologous with the reported pseudomonad ACC deaminase genes (95-99% identity). The K_m values for ACC of crude cell-free extracts from these strains were also similar to those of the pseudomonads, which bind ACC relatively poorly. The ACC deaminase genes from both *Enterobacter cloacae* strains were expressed in *Escherichia coli* cells with their native promoters (Shah et al., 1998).

The seven growth-promoting bacterial strains previously isolated in our lab shared a number of traits in common; they were able to grow on ACC as a sole source of nitrogen; had ACC deaminase activity and promoted root elongation of canola seedlings (Glick et al., 1995; Shah et al., 1997). However, observations based on Southern hybridization analysis using PCR-generated ACC deaminase gene probes, showed that only the two strains, *Enterobacter cloacae* UW4 and *Enterobacter cloacae* CAL2, contained genes for the known ACC deaminase (Shah et al., 1998). This implied the existence of more than one type of ACC

deaminase gene. Indeed, Li et al. (submitted for publication) and Campbell and Thomson (1996) both isolated clones, based on their growth on minimal medium containing ACC as a sole source of nitrogen, that did not contain ACC deaminase genes. With this in mind, we fully expected that the ACC deaminase gene from *Enterobacter cloacae* CAL3 would be different than those isolated from the other two *Enterobacter cloacae* strains. As well, *Enterobacter cloacae* CAL3 was unlike the other six plant growth-promoting strains: it secreted a high level of siderophores and was also capable of producing large amounts of IAA when grown in the presence of high levels of tryptophan (Shah et al., 1997). Such high levels of IAA are often associated with phytopathogens rather than plant growth-promoting bacteria. Microbial IAA can be synthesized by several different pathways, some of which are induced by tryptophan and some of which are constitutive (Patten and Glick, 1996). Despite its potential to synthesize large amounts of IAA, under more natural conditions—in the absence of tryptophan added to the growth medium—*Enterobacter cloacae* CAL3 produced very little IAA. We were very interested in the ability of *Enterobacter cloacae* CAL3 to synthesize high levels of IAA. The production of IAA by plant growth–promoting bacteria and the uptake and metabolism of it by plants may affect bacterial ACC deaminase activity. IAA is known to stimulate the transcription of ACC synthase (Yip et al., 1992), one of the three key enzymes in the ethylene biosynthetic pathway; ACC synthase

catalyzes the conversion of SAM to ACC and 5'-methylthioadenosine (Yang and Hoffman, 1984). Ethylene production is induced by the *de novo* synthesis of this enzyme (Kende, 1989).

Following the screening of the approximately 7000 clones in the *Enterobacter cloacae* CAL3 clone bank, twelve positive clones were isolated, based on their growth on M9 mininal medium containing ACC as a sole nitrogen source. The growth of these clones on M9 minimal medium, in liquid cultures and on plates, was measured at 30°C and 37°C. The level of ACC deaminase activity was measured and the size of the DNA insert was determined for each of the clones. There was variability in the growth of the clones at 30°C and 37°C: some clones grew well at both temperatures; some grew well at one temperature only, and some grew slowly at both temperatures. Only one clone, pUC324, grew quickly at both temperatures.

The ACC deaminase activities of the clones were quite low: they ranged from 1.54 to 3.67 μmoles of α-ketobutyrate formed/h/mg of protein. The highest level of activity was seen in pUC324 at 3.67 μmoles of α-ketobutyrate formed/h/mg of protein. None of the clones had an activity as high as that of *Enterobacter cloacae* CAL3. At 5.86 μmoles of α-ketobutyrate formed/h/mg of protein, the ACC deaminase activity of *Enterobacter cloacae* CAL3 was considered low amongst the activities of the other six plant growth-promoting strains and *Escherichia coli* pRKACC, the *Escherichia coli* strain containing the *Enterobacter cloacae*

UW4 ACC deaminase gene on a broad host range plasmid. Although the ACC deaminase activities of the clones were very low, they nonetheless clearly indicated the presence of enzyme activity. The ACC deaminase activity measured in the *Escherichia coli* strain that contained only the broad host range plasmid, pRK415, (and not an ACC deaminase gene), was routinely less than zero.

The presence of a DNA insert in each clone was confirmed by agarose gel electrophoresis and the insert sizes ranged from less than 1000 bp to approximately 6500 bp. The DNA insert of pUC324 appeared on the gel as a doublet at approximately 2.6 kb in size, the same size as the cloning vector, pUC18. We assumed that the size of this insert was also approximately 2.6 kb. However, the size was actually 318 bp as determined by DNA sequence analysis.

Based on these factors: growth at 30°C and 37°C, ACC deaminase activity and insert size, four clones including pUC319, pUC320, pUC324 and pUC327, were selected for DNA sequencing. pUC324 was the most promising clone: it grew quickly at both temperatures and had the highest level of ACC deaminase activity amongst the clones. The 'supposed' insert size of approximately 2.6 kb seemed likely to contain an entire gene. Two other clones, pUC319 and pUC320, also grew fairly well at both temperatures, and, amongst the clones, had good levels of ACC deaminase activity—2.65 and 2.49 μmoles of α-ketobutyrate formed/h/mg of protein, respectively. The DNA insert size of both

clones appeared to be approximately 2 kb, an appropriate size to carry an entire gene. The *Enterobacter cloacae* UW4 and CAL2 ACC deaminase genes are approximately 1.0 kb in size, not including the promoter region (Shah et al., 1998). The last clone selected, pUC327, grew very well at both temperatures and had an insert size of approximately 6.5 kb, definitely large enough to encode an entire gene. However, this clone had a low level of ACC deaminase activity, 1.54 μmoles of α-ketobutyrate formed/h/mg of protein. The clones: pUC319, pUC320, pUC324, and pUC327, were also assayed for plant growth-promoting capabilities by using the root elongation assay. Only an *Escherichia coli* strain transformed with the plasmid, pUC319, promoted substantial elongation of canola roots; *Escherichia coli* transformed with pUC320 and pUC324, promoted modest increases in canola root lengths and *Escherichia coli* transformed with pUC327 did not enhance canola root growth.

4.4.2. Analysis of putative ACC deaminase genes

The results of DNA sequencing were unexpected and puzzling. The computer program DNA Strider 1.2 was used to derive amino acid sequences from the DNA sequences of each of the clones. Each of the cloned DNA inserts appeared to contain at least one open reading frame. When a BLAST search was used to align the deduced amino acid sequences with similar known sequences, there appeared to be at least

one gene in each clone except pUC320, that was homologous with a known gene. However, none of these genes resembled the sequences for known ACC deaminases. There were no derived amino acid sequences in pUC320 that were homologous with any reported genes. pUC324, the clone that had the highest level of ACC deaminase activity and grew so vigorously, carried a very short piece of DNA, 318 bp in length. The deduced amino acid sequence was highly homologous (94% identity) with a portion of the uracil phosphoribosyltransferase gene from *Escherichia coli*. Uracil phosphoribosyltransferase catalyzes the transfer of a ribosyl phosphate group from the α-D-5-phosphoribosyl-1-pyrophosphate to the N1 nitrogen of uracil (Schumacher et al., 1998). pUC324 contained only the middle section of the gene and not the nucleotides that encoded the first 75 or the last 44 amino acids. However, the cloned segment of the gene contained proline 131, the PRPP (5-phosphoribosyl-α-1 pyrophsophate) binding site, which is critical for the binding of uracil to the enzyme-PRPP complex and for the catalytic activity of the enzyme (Lundegaard and Jensen, 1999). There are a number of reports of gene expression from cloned DNA fragments that carry only a portion of a gene (e.g. Rodrigez et al., 2000). It would appear that expression of the cloned uracil phosphoribosyltransferase provides some growth advantage to the *Escherichia coli* grown in the presence of ACC but it is unclear how these enzyme fragments affect the hydrolysis of ACC.

When a BLAST search was used to align the deduced amino acid sequences of pUC319 with similar known sequences, there were two open reading frames that were homologous (both 85% identity) with a reported flagellar basal body P-ring protein precursor gene and a flagellar protein flgJ from *Salmonella typhimurium*. The first open reading frame of pUC319 appeared to contain the DNA sequence for the entire flagellar basal body P-ring protein precursor gene and the second reading frame appeared to contain 90% of the flagellar protein flgJ gene including the amino terminus. It is likely that the pUC319 DNA insert contains part of the operon that encodes flagellar proteins. The basal body constitutes a major portion of the flagellar organelle and consist of five rings mounted on a central rod: the P-ring is furthest from the central rod and lies in the periplasmic space (Jones et al., 1989). The expression of these flagellar genes may be beneficial for interaction and adherence of the bacterium to the seeds: pUC319 was the only clone capable of stimulating root elongation in canola seedlings. Most plant-associated bacteria are motile in the free-living state. Flagella driven chemotaxis may be important in the early interaction of these bacteria with the host plant, particularly in the rhizosphere (Felix et al., 1999). The expression of flagellar proteins may be related to the uptake and metabolism of ACC.

Clone pUC327, carried a large piece of DNA, approximately 5 kb in length. When a BLAST search was used to align the deduced amino

acid sequences with similar known sequences, there appeared to be two sequences in this clone that were homologous with known genes. There was a high level of homology between the first open reading frame and altronate hydrolase from *Escherichia coli* (87% identity); the sequence for the entire gene appeared to be contained in the pUC327 DNA insert. Altronate hydrolase is involved in the hexauronate degradation pathway (Blanco et al., 1983). The other open reading frame in pUC327 was homologous with a hypothetical protein in the ebgC-exuT intergenic region of *Escherichia coli*—a putative transport protein. There was 71% identity of the derived amino acid sequence in the second open reading frame of pUC327 with a hypothetical 43.5 kd protein. The sequence for the entire gene appeared to be present in the clone. The expression of the genes for altronate hydrolase and the putative transport protein may enhance bacterial growth and possibly ACC uptake. The hypothetical protein is likely an integral membrane protein, most similar to the sodium: dicarboxylate symporter family of proteins (Blattner et al., 1997). Despite the fact that there was a low level of ACC deaminase activity in this clone, vigorous growth was observed at both growth temperatures.

4.4.3. Putative *Enterobacter cloacae* CAL3 ACC deaminase genes—a cautionary tale

Although an ACC deaminase gene was not isolated from *Enterobacter cloacae* CAL3 during this study, eight clones that have still not been sequenced. All of these clones grew on M9 minimal medium with ACC as the sole source of nitrogen and all showed some level of ACC deaminase activity. It is possible that an *Enterobacter cloacae* CAL3 ACC deaminase gene can be found in the DNA insert from one, or more, of these clones. It is also possible that there is no ACC deaminase gene in *Enterobacter cloacae* CAL3. When compared with the other plant growth-promoting strains of bacteria isolated in our lab, *Enterobacter cloacae* CAL3 has a low level of ACC deaminase activity and grows slowly on M9 minimal medium with ACC as a sole source of nitrogen. A situation may exist in *Enterobacter cloacae* CAL3 in which ACC is taken up by the bacterium and hydrolyzed by other enzymes, albeit less efficiently than by ACC deaminase. There is yet another possibility: the genes cloned and sequenced from *Enterobacter cloacae* CAL3 may stimulate the expression of ACC deaminase in *Escherichia coli*. Among the reported ACC deaminase genes, there is a putative gene for ACC deaminase in *Escherichia coli*. There are no reports of ACC deaminase purified from *Escherichia coli* or of ACC deaminase activity in *Escherichia coli*—indeed we saw less than zero activity in *Escherichia coli* that did not

contain an ACC deaminase gene on a plasmid. Nonetheless, if the ACC deaminase gene exists in *Escherichia coli*, it is possible that one or more of the *Enterobacter cloacae* CAL3 genes or partial genes cloned in this study, may induce expression of the ACC deaminase gene in *Escherichia coli*.

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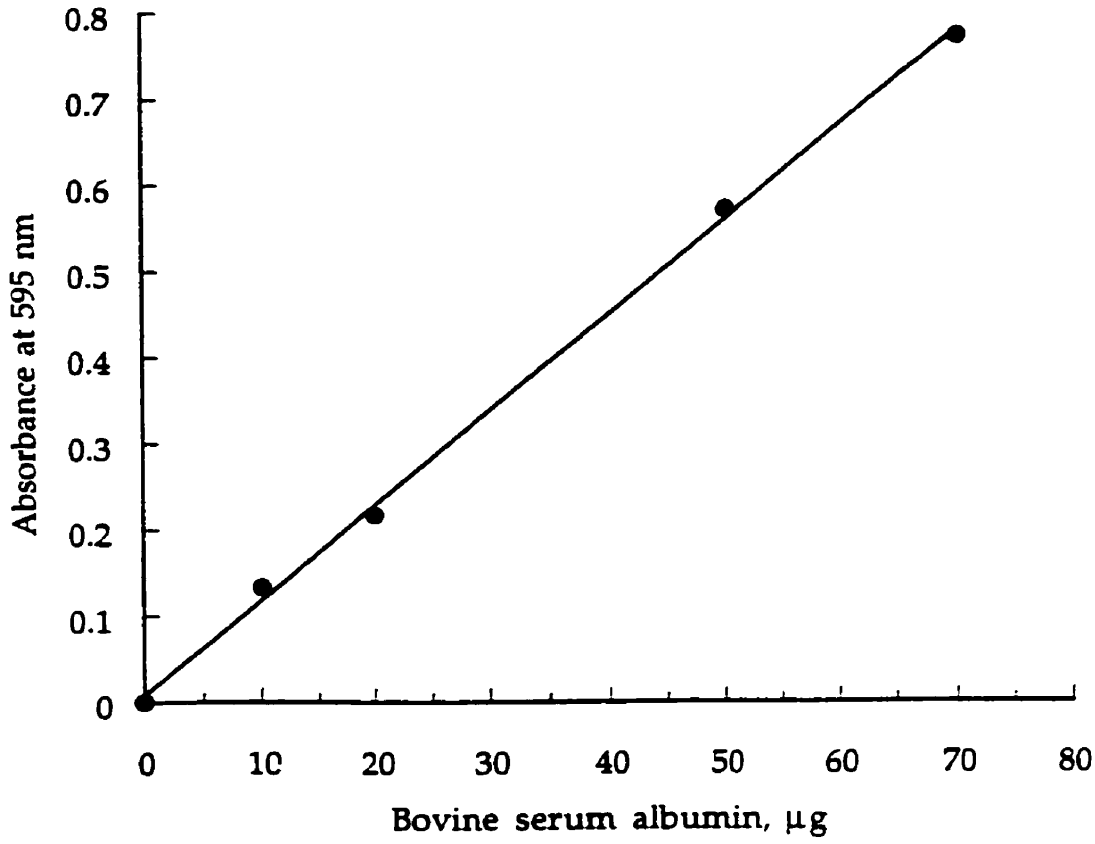
Appendix I. Biorad protein standard curve. Standard curve of bovine serum albumin versus absorbance at 595 nm. Equation: $y = 0.0075706 + 0.011068x$; $R = 0.99926$


0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
Absorbance at 595 nm
0
10
20
30
40
50
60
70
80
Bovine serum albumin, µg