ISOLATION, CHARACTERIZATION AND REGULATION OF 1-AMINOCYCLOPROPANE-1-CARBOXYLATE DEAMINASE GENES FROM PLANT GROWTH-PROMOTING RHIZOBACTERIA

by

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ABSTRACT

Enterobacter cloacae UW4 and CAL2 produce an inducible 1-aminocyclopropane-1-carboxylate (ACC) deaminase that hydrolyzes ACC into α-ketobutyrate and ammonia. The genes encoding the ACC deaminases, i.e. *acdS*, were cloned from both species of *E. cloacae* and sequenced. The deduced amino acid sequences of the ACC deaminases are highly homologous to previously reported pseudomonad ACC deaminase sequences (95-99% identity) and the cloned genes can be expressed in *E. coli* cells. When the gene was introduced into other soil bacteria, these strains gained the ability to grow on ACC as a sole source of nitrogen and to promote canola root elongation. It was found that the regulation of the *acdS* gene in strain UW4 requires both the product of an ORF (upstream of *acdS*) and ACC. The ORF was identified as an Lrp-like protein containing a helix-turn-helix motif. The results from *trans* complementation experiments showed that the protein encoded by the ORF behaves in a manner similar to known Lrp proteins to positively regulate the *acdS* promoter. Using *luxAB* reporter genes as promoter-probes, in addition to ACC, L-alanine also induced *acdS* gene expression, albeit at a low level.

To evaluate the role of ACC deaminase in plant growth-promoting rhizobacteria (PGPR), a model has been proposed in which PGPR stimulate plant growth as a consequence of their production of ACC deaminase which causes a lowering of plant ethylene levels

resulting in longer roots. In an attempt to assess the model, an ACC deaminase mutant was created by targeted gene replacement and has been shown to be completely deficient in its ability to use ACC as a nitrogen source, devoid of ACC deaminase activity and in contrast to the wild-type *E. cloacae* UW4, unable to promote canola root elongation in gnotobiotic growth pouches. This result suggests that ACC deaminase plays a direct role in the promotion of plant growth as described in the model.

In addition, a novel gene from strain UW4 with the ability to hydrolyze ACC has been isolated and partially characterized. Comparison studies of the novel gene with other similar genes suggests that this gene may have evolved from the primitive protein common to the amidohydrolase superfamily of proteins, and that this gene encodes a distinct type of ACC deaminase.

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1. INTRODUCTION

1.1 Plant growth-promoting rhizobacteria (PGPR)

1.1.1. What are PGPR

By the late 1800s, beneficial interactions between symbiotic rhizobia and legumes were well characterized. Biological symbiotic nitrogen fixation annually supplies nearly 60% of the earth's fixed nitrogen (Kim and Rees, 1994) and without the potential negative environmental impact that results from the use of chemical fertilizers. However, natural symbiotic nitrogen fixation is limited to legumes, although numerous scientists have devoted much of their research effort to introducing nitrogen-fixing genes into other crops, an effort that has as yet met with little success. The high cost of chemical fertilizers and their danger to animals and humans have driven microbiologists to attempt to develop biological fertilizers as replacements for hazardous chemical fertilizers. Questions have been raised as to whether the same benefits will exist with nonleguminous plants' interactions with other soil bacteria and whether is it possible to at least partly replace chemical fertilizers with biological fertilizers. Furthermore, researchers have inquired into the mechanisms of the effect of bacterial inoculation on plant growth promotion and how biological fertilizers can be manipulated to allow for a more effective promotion of crop production.

Pioneering work on nonrhizobial systems was done in Russia with the bacterization of seeds, work that began as early as 1895 using *Bacillus* spp. and in 1909 using *Azotobacter chroococcun* to improve plant growth (Mishustin and

Naumova, 1962). In the 1930s, the Soviet Institute of Agricultural Microbiology recommended the widespread agricultural use of preparations of *B. megaterium* and *A. chroococcum* (Cooper, 1959), and by 1962, industrial production of these preparations in Russia and the Ukraine was sufficient to treat 35 million ha (Mishustin, and Naumova, 1962). Numerous studies with these inoculants were reported during the 1960s, and concluded that bacterization generally resulted in yield increases of up to 10% for cereal crops and 15 to 50% with various vegetables (Kloepper, 1994).

The term *"Rhizosphere"* was first used by Hiltner (1904) to describe the stimulation of microbial biomass and activity in the soil surrounding the plant root. The rhizosphere is a volume of soil influenced by root activity. As a root develops it takes up water and mineral nutrients from the rhizosphere. The rhizosphere is also a zone of enhanced biological activity in which population densities of microorganisms are greater than those in bulk soil (Newsman, 1985) due to the exudation of high levels of metabolite substrates including many soluble forms of nutrients such as sugars, hormones, vitamins and amino acids. These exuded substrates can be used efficiently by soil microorganisms as carbon and nitrogen sources, and signals (O'Sullivan et al., 1991; Fisher and Long 1992). From the term rhizosphere came "Rhizobacteria", which are those members of the total rhizospheric bacterial population that are able to colonize plant roots (Schroth and Hancock, 1982).

Early studies had demonstrated that some free-living nitrogen-fixing bacteria, such as *Azotobacter*, had beneficial effects on plant growth, as yield increases of up to 25% were sometimes obtained (Schmidt, 1974). However, *Azotobacter* strains are not normal inhabitants of the rhizosphere and have a

low rhizosphere competence compared with native rhizosphere bacteria. In the late 1970s, researchers at the University of California in Berkeley discovered that some pseudomonads could efficiently compete and persist in the rhizosphere in the presence of other soil microorganisms and colonize roots throughout the growing season (Kloepper et al., 1980). This observation was followed by an increase in the amount of work in this research area with the realization that rhizobacteria comprise a group of free-living soil bacteria that possess the ability to move toward and bind to roots, preferentially inhabit the rhizosphere and multiply on root surfaces (Suslow and Schroth, 1982; Weller, 1983). Therefore, it is believed that rhizobacteria do have some associative interactions with plant roots and descriptions of these interactions have been reported over the past 40 years.

Rhizobacteria may exert one of three kinds of effects on the inoculated host plant: deleterious, neutral, or beneficial (Schroth and Hancock, 1982). Those rhizobacteria with a beneficial effect on plant growth are termed plant growth-promoting rhizobacteria (PGPR) (Kloepper and Schroth, 1978). Moreover, the mechanisms of the beneficial effects of PGPR on inoculated plants have been proposed to fall into two broad categories, direct or indirect, i.e. plant growth promotion or plant disease suppression (Kloepper et al., 1989).

1.1.2. Plant growth promotion

Plant growth promotion by PGPR is evidenced by increases in seedling emergence, root system development, vegetative vigor, root and shoot biomass, plant height, nutrient content of plant tissues, and yield (Kloepper et

al., 1988; Chanway et al., 1988; Turner and Backman, 1991). Soil bacteria that have been reported to be PGPR include: (1) gram-negative bacteria: *Azospirillum, Alcaligenes, Acetobacter, Arthrobacter, Burkholeria,* pseudomonads, *Enterobacter agglomerates, Enterobacter cloacae, Erwinia herbicola, Kluyuera ascorbata, Serratia liquefaciens* and *Serratia marcescens* (Brown, 1974; Elmerich, 1984; Yuen and Schroth, 1986; Kloepper et al., 1988; 1994; Nelson, 1988; Bashan and Levanony, 1990; Glick, 1995; Burd et al., 1998; Glick et al., 1999); (2) Gram-positive bacteria: *Bacillus cereus, B. circulans,* and *B. subtilis* (Merriman et al., 1974; Chanway et al., 1989; 1991). Hence, the ability to promote plant growth or to provide biological disease control seems to be widely distributed within rhizospheric bacteria.

There are a number of proposed mechanisms that have been used to explain the direct promotion of plant growth. These mechanisms include the (1) fixation of atmospheric nitrogen; (2) solubilization of iron from the soil; (3) solubilization and mineralization of phosphorus; (4) production of phytohormones such as auxins, cytokinins and gibberellins; (5) synergistic promotion by mixed inoculants (Glick, 1995; Glick and Bashan, 1997; Glick et al., 1999); and (6) the secretion of organic acids, such as succinic and lactic acids, from the PGPR (Yoshikawa, 1993).

(1) Nitrogen fixation. Unlike rhizobia that fix atmospheric nitrogen through symbiotic relations with plants by producing nodules in the roots, many PGPR are diazotrophs, and thus fix nitrogen directly from the air independently. As rhizobacteria, these diazotrophs are able to attach to the root surface establishing an associative, non-obligate interaction with the plants for non-legume associative nitrogen fixation (Malik et al., 1980). In the

1950s studies first began on the nitrogen-fixing association of rhizobacteria with sugar cane, rice, and some other grasses (Dobereiner and Alvahydi, 1959). Several different nitrogen-fixing bacteria have since been isolated from the rhizosphere, including *Klebsiella, Beijerinckia, Azospirillum, Alcaligenes Bacillus*, and others (Zafar et al., 1986; Li and You, 1992). These diazotrophs were expected to be beneficial to plant growth and supply nitrogen to economically important crops like cereals and forage grasses (Brown, 1974; Okon, 1985). Although many of these nitrogen-fixing bacteria were able to promote plant growth, with some species providing a significant advantage, nitrogen fixation is not considered to be a primary mechanism of PGPR promotion of plant growth. Since the nitrogen supplied by these diazotrophs was found in limited amounts (Hong, et al., 1991a; James and Olivares, 1997) it was deemed unlikely to be of benefit to the plant when working in conjunction with nitrogen fertilizer added to the soil (Glick et al., 1999). However, fixed nitrogen provided by associated rhizobacteria can be still important when the nitrogen supply from the soil is limiting, and some recent results show that agronomically significant quantities of nitrogen can be derived from associated nitrogen fixation in the case of certain forage grasses, wetland rice and sugar cane (Boddey and Dobereiner, 1994).

(2) Siderophore production. The first mechanism proposed for the promotion of plant growth by PGPR was the production of siderophores. Free available iron in aerobic environments is present at a concentration of approximately 10^{-18} M at pH 7.0 (Biedermann and Schindler, 1957) and the solubility of the ferric oxyhydroxy complexes is approximately 10^{-38} M. To combat this low solubility, most soil bacteria and fungal species have evolved

a mechanism to produce low-molecular-weight molecules, called siderophores, that have a high affinity for ferric iron (Leong, 1986). Siderophores secreted by a PGPR can bind iron in the soil, and a membrane transport system recognizes and takes in the iron-siderophore complex (Neilands et al., 1984). The solubilization of iron by bacterial siderophores makes it readily available for plant growth. In addition, a number of plants can bind the bacterial iron-siderophore complex and reductively release the iron from the complex (Bar-Ness et al., 1991; Wang et al., 1993). The role of siderophores in plant growth promotion is supported by the fact that siderophore-defective mutants of *Pseudomonas putida* WCS358 lost plant growth-promoting activity (Bakker et al., 1987). In light of the proposed mechanisms, the effects of siderophores on plant growth are believed to be more important for competitive growth in the biocontrol of pathogens in the rhizosphere (section 1.1.3.) rather than plant growth promotion.

(3) Solubilization of phosphorus. Another mechanism suggested for plant growth promotion by PGPR is the mineralization of organic phosphorus compounds or the solubilization of unavailable inorganic phosphates. Some PGPR have been found to have phosphate-solubilizing activity that can increase the availability of phosphorus to plants (Subba Rao, 1982). This observation was confirmed by Lifshitz et al. (1987) when they showed that the PGPR *Pseudomonas putida* GR12-2 stimulated an increase in uptake of ^{32}P-labeled phosphate in canola seedlings. Inoculation of seeds with the pseudomonad PGPR resulted in significant increases in phosphates levels in the roots and shoots of plants, and is linearly correlated with root and shoot lengths.

(4) Production of phytohormones. The mechanism that has been most often invoked to explain the various effects of PGPR on plants is the production of phytohormones with most of the attention focused on indole-3-acetic acid (IAA) (Brown, 1974; Tien et al., 1979; Patten and Glick, 1996). Since IAA can be synthesized and released from its ester-conjugate in plant roots at certain developmental stages, application of external IAA may change the level and metabolism of endogenous IAA in the root. It was found depending on the plant that application of a low concentration of IAA (10^{-9} M) enhances root growth, whereas a higher concentration of IAA (10^{-6} M) inhibits root elongation (Pilet and Saugy, 1987), but stimulates the initiation of lateral and adventitious roots (Evans, 1985). When various plants are treated with constitutively IAA-producing strains of *Azospirillum* the effects were found to be dependent upon inoculum size. For example, *Azospirillum* at a concentration of 10^5 to 10^7 cfu (colony-forming units) per plant enhanced cell division in the roots of wheat (Levanony and Bashan, 1989), and the diameter and length of the lateral roots in maize seedlings were increased as observed by Hartmann et al. (1983). At a concentration of 10^8 cfu per plant, the production of root hairs in tomato seedlings was enhanced (Hadas and Okon, 1987). The isolation of mutant strains that overproduce IAA has been reported (Hartmann et al., 1983), however, it has been difficult to obtain mutants defective in IAA synthesis, suggesting that bacteria may possess more than one IAA biosynthetic pathway (Patten and Glick, 1996). Mutants that synthesize low levels of IAA no longer promote the formation of lateral roots of wheat seedlings compared to wild-type PGPR strains (Barbieri et al., 1986; Barbieri and Galli, 1993), while the response of plants treated with an

IAA-overproducing mutant vary between plant species, an observation that may depend upon the existing auxin levels within the treated plant (Dubeikovsky et al., 1993).

(5) Promotion of *Rhizobium* nodulation of legumes. As early as the 1940s certain rhizosphere bacteria, termed "activating bacteria" were reported to enhance the rate and extent of nodulation and nitrogen fixation by *Rhizobium trifoli* (Krasil'nikov, 1944). Some pseudomonad strains were also reported to markedly increase *Rhizobium* nodulation (Grimes and Mount, 1987). Polonenko et al., (1987) tested and selected PGPR strains that enhanced *Bradyrhizobium japonicum* nodulation and soybean plant growth in field soil and termed these strains as nodulation-promoting rhizobacteria (NPR). Recently, using PCR to examine the population fluctuation of rhizobacteria, plants inoculated with *Rhizobium etli* and the NPR *Bacillus polymyxa* showed an increase in the *Rhizobium etli* population and extent of nodulation in the rhizosphere of *Phaseolus vulgaris*, while also demonstrating an increase in lateral root formation and nodule numbers (Petersen et al., 1996). Clover plants inoculated with a mixture of *Azospirillum brasilense* Sp7 and *Rhizobium*, showed an increased extent of acetylene reduction, nodulation, and shoot dry weight when compared with *Rhizobium* alone (Oliveira et al., 1997).

(6) Synergistic effects. Combinations of different strains of PGPR were found to have significant synergistic activity (Kloepper, 1989). Early studies documented beneficial effects on plant growth from mixtures of rhizobacteria (Rovira and Davey, 1974). Park et al. (1988) validated the concept that mixed inoculants may be superior to individual treatments when using mixtures where each component strain had a different known basic mechanism. For

example, multicomponent inoculants for legumes, consisting of *Rhizobium*, mycorrhizae and a free-living bacteria has been used to inoculate plants as both single components or a mixture. The results showed that plant biomass was increased by each component individually, although the greatest response occurred when all three components were present (Meyer and Linderman, 1986). Combined inoculation of *A. brasilense* and the phosphate solubilizing bacteria *Pseudomonas striata* or *Bacillus polymyxa* significantly increased grain yield and nitrogen and phosphorus uptake by sorghum (Alagawadi and Gaur, 1992). Recent studies have shown that in a mixed culture, species of *Staphylococcus* release compounds that promote the nitrogen-fixing activity of *Azospirillum* (Holguin and Bashan, 1997). Kumar (1996) reported that an antibiotic producing *Bacillus* strain SR2 mixed with conventional seed bacterization showed not only enhanced seed germination, shoot height, root length, fresh and dry weights but also reduced a number of wilted chick pea plants in wilt-sick soil. In addition, coinoculation of PGPR with vesicular-arbuscular mycorrhizae creates a synergistic interaction resulting in significant increases in many growth parameters as compared to single inoculations (Toro et al., 1997).

1.1.3. Plant disease suppression

In addition to the mechanisms that have been proposed to promote plant growth directly, it is generally believed that plant growth promotion, induced by PGPR, often occurs as a result of suppression of the native deleterious rhizosphere microflora. For example, plant growth promotion often does not occur when plants are grown in soil-less media or sterilized

soil (Suslow, 1982). Although there were some reports to show that some pseudomonad PGPR strains induce significant increases in root length, root dry weight, shoot length, and shoot dry weight directly under soil-free gnotobiotic conditions (Lifshitz et al., 1987), most field research work with bacterial inocula has focused more on biological control of plant diseases than on growth promotion (Kloepper, 1989).

During the past decade PGPR have been used successfully as seed inoculants to control soil-borne plant pathogens (Weller, 1988) and several PGPR in use as biocontrol agents have been well-studied (Glick et al., 1999). *Pseudomonas putida* WCS358, isolated from potato, is not only an efficient root colonizer, it can also induce plant systemic resistance; *Pseudomonas fluorescens* 2-79, isolated from wheat, has been found to protect against take-all disease caused by *Gaeumannomyces graminis* var *tritici* (Rovira et al., 1990); *P. fluorescens* strain CHA0, isolated from Switzerland tobacco soil, can reduce black root rot of tobacco caused by *Thielaviopsis basicola* and also demonstrated biological control against diverse pathogens, including *T. basicola* on tobacco, cotton, cherry and *G. graminis* var. *tritici* on wheat, *Aphanomyces euteiches* on pea, *Fusarium oxysporum* f. sp. *lycopersici* on tomato, *F. oxysporum* f. sp. *lini* on flax, and *Rhizoctonia solani* on cotton (Défago et al., 1990). *Enterobacter cloacae* is a model biocontrol agent and has been used to antagonize *Pythium* spp., *Fusarium* spp., and *Rhizopus* spp. (Wilson et al., 1987). A number of different mechanisms have been proposed to explain PGPR-mediated phytopathogen protection. These mechanisms include: outcompeting the phytopathogen, the synthesis of antibiotics, the synthesis of a variety of small molecules that can inhibit phytopathogen growth, the production of enzymes that can hydrolyze

components of the phytopathogen and the stimulation of the systemic resistance of the plant (Glick and Bashan, 1997).

(1) Antibiotic production. Fluorescent pseudomonads have been found to produce a number of antibiotics (Lesinger and Margraff, 1979; Glick and Bashan, 1997). Some PGPR are also reported to produce a wide range of low molecular weight metabolites with anti-fungal activity (Dowling and O'Gara, 1994). The production of antibiotics by PGPR has been put forth as a mechanism for biological control of plant diseases since these antibiotics may be active against pathogenic soil microorganisms, reducing the extent of damage to the plant (Weller, 1988). For example, *P. fluorescens* strain CHA0 can produce several extracellular metabolites, including HCN, siderophores, salicylic acid, and three antibiotics (2,4-diacetylphloroglucinol, pyoluteorin, and monoacetylphloroglucinol) that reduce black root rot of tobacco (Défago et al., 1990). Evidence for the direct involvement of antibiotic production in biocontrol PGPR-mediated disease-suppression comes from several non-antibiotic-producing mutants that either were no longer able to protect plants from phytopathogens or protect them to a much lesser extent than the wild-type bacterium (Thomashow and Weller, 1988; Hamdan et al., 1991; Keel et al., 1992; Hill et al., 1994). Moreover, overproduction of the antibiotics pyoluteorin and 2,4-diacetylphloroglucinol by mutant strains of *Pseudomonas fluorescens* protected cucumber plants against disease caused by *Pythium ultimun* more effectively than the wild-type strain (Maurhofer et al., 1992; Schnider et al., 1994). The production of antimicrobial compounds by *Pseudomonas fluorescens* biocontrol strains has been found to be regulated by environmental factors, such as the ratio of carbon source levels to nutrient

concentration (Duffy and Defago, 1999; Nielsen et al., 1998). Recently, a gene necessary for pyrrolnitrin (an antibiotic produced by *Pseudomonas fluorescens*) production was shown to be regulated by *rpoS*, which encodes the stationary-phase sigma (required for the transcription of genes in stationary-phase cells of *P. fluorescens*). Further results indicated that the *rpoS* sigma factor influences not only antibiotic production, but also biological control activity and the survival of *P. fluorescens* on plant surfaces (Sarniguet et al., 1995). It is believed that antibiotic production could account for 60 to 75% of the disease suppression activity of the wild-type strain (Kloepper et al., 1988).

(2) Depletion of iron from the rhizosphere. PGPR produce siderophores that chelate the ferric iron in the rhizosphere, allowing the PGPR to out-compete other microorganisms that have less affinity for iron (Kloepper et al., 1988). It is known that siderophores synthesized by fungal phytopathogens have a lower affinity for iron than do the siderophores produced by PGPR (Schippers et al., 1987). Mutant strains of pseudomonads that either overproduce siderophores or are deficient in their production were shown to be either more effective or ineffective in controlling a pathogenic strain of *Fusarium oxysporum* to tomatoes, respectively, when compared with the wild-type bacterium (Vandenburgh and Gonzalez, 1984; Buysens et al., 1994). However, early studies indicated that the critical level of Fe (III) necessary for the suppression of a fungal pathogen by siderophore-producing pseudomonads was lower than 10^{-19}M (Simeoni et al., 1987).

(3) Enzymes hydrolyzing fungal cells. Pathogenesis related (PR) proteins including chitinase, β-1,3-glucanase, protease and lipase that can lyse fungal cells (Chet and Inbar, 1994) have been found to be secreted by some

PGPR (Lim et al., 1991). For example, three strains of the recently isolated biocontrol organism *Enterobacter agglomerans* can produce a complex of four separate enzymes responsible for the chitinolytic activity. These bacteria are antagonistic to fungal pathogens and significantly decrease the damage to cotton plants infected with *Rhizoctonia solani* (Chernin et al., 1995). Other studies showed that a biocontrol strain of *Enterobacter cloacae* could partially control pea and cucumber seedling rot caused by *Pythium* spp. by forming a sheath around the hyphae and lysing them (Hadar et al., 1983). Interestingly, enzymes that are responsible for biocontrol activity tend to be encoded by a single operon in bacteria, thus it should be relatively straightforward to manipulate these genes as a means to genetically engineer PGPR (Glick and Bashan, 1997).

(4) Induced systemic resistance (ISR) in plants. Another proposed mechanism involved in biocontrol by PGPR was the apparent stimulation of the synthesis of some anti-pathogen enzymes within the plant (Kessmann et al., 1994). The resistance is believed to be dependent on the endogenous accumulation of salicylic acid (SA) and is characterized by the activation of genes encoding pathogenesis-related (PR) proteins (Pieterse et al., 1996). Seed inoculation might offer the possibility of immunizing whole plants against bacterial foliar disease, as well as fungal disease (Van Peer et al., 1991). When Van Wees et al. (1997) elucidated ISR using an *Arabidopsis*-based model system, they found that rhizospheric pseudomonads were differentially active in inducing ISR in related plant species. ISR-inducing bacteria could produce more than one single factor that triggered ISR in *Arabidopsis* and one system

was independent of salicylic acid (SA) accumulation and pathogenesis-related (PR) gene expression.

1.1.4. Biological interactions in the rhizosphere

Soil contains many different types of microorganisms including bacteria, fungi, actinomycetes, protozoa and algae (Paul and Clark, 1989). Among them bacteria are by far the most common in the rhizosphere as they can grow rapidly and have the ability to use a wide range of different substances as either carbon or nitrogen sources (Glick, 1995a). Within the interactions between plant roots and rhizobacteria, root exudates are considered an important facet since most biological processes in the rhizosphere are influenced by plant-root exudates (Martin, 1977). The exudates of plant roots create a "rhizosphere effect" that manifests itself in intense microbial activity (O'Connell et al., 1996). Several important properties involved in the interactions among PGPR and root exudates have been reported:

1. PGPR possess an ability to metabolize the constituents of seed and root exudates, that often contain unique or unusual carbon and nitrogen sources, more efficiently than other soil microorganisms. It was found that soil bacteria can be enriched by a factor of 100 or more in the rhizosphere as compared to their numbers in the rest of the soil because plant roots continuously exude nutrients that serve as food for these bacteria (Weller, 1988). However, not all soil bacteria are rhizobacteria. Only those soil bacteria that have the ability to compete for nutrients available in seed and root exudates and those that are motile through chemotactic attraction to

exudate constituents have the potential to enter and survive in the rhizosphere (Scher et al., 1985; Glick, 1995).

2. The composition of the associated rhizobacteria depends mostly on the quality and quantity of the root exudates and is often shown to be plant species-dependent (Stephens et al., 1993). Exudation from plant roots is influenced by different factors such as the age of the plant, temperature, light intensity, bacteria population, and soil texture and humidity (Kipe-Nolt et al., 1985). Waschütza (1993) found higher exudation rates in rice seedlings when compared to older rice plants. Organic acids such as citrate, pyruvate, succinate, and acetate were excreted in higher amounts than sugars, and lysine in the exudates was immediately used by the bacteria. Since diverse carbon substrates are exuded by different plants, soil bacteria communities show a host-specific interaction in that the ability of soil bacteria to utilize carbon substrates varies based on the plant. For example, malic acid is the principal acid exuded by different plant species that can attract and be efficiently metabolized by *Azospirillum* (Okon et al., 1980). *Pseudomonas* spp. have been reported to require a higher carbon content in the environment compared to *Azospirillum*, thus in an environment with a low carbon content, *Azospirillum*, will outcompete *Pseudomonas* spp. (Del Gallo and Fabbri, 1990). In recent studies Conn (1997) investigated the growth promotion of potatoes among different cultivars by a nonfluorescent *Pseudomonas* sp. strain and found that the colonization of the strain varied with different cultivars.

3. Chemotaxis and aerotaxis of rhizobacteria toward plant roots is an active process and it may involve a signal transduction system. For example, *Pseudomonas putida* GR12-2 shows a chemotactic response to asparagine

secreted from canola roots (Lifshitz et al., 1986). *Azospirillum* is attracted by many organic substances including sucrose; however, sucrose cannot be metabolized by *Azospirillum* (Heinrich and Hess, 1985). The patterns of chemoattractants differ among strains and may be one of the factors determining host-specificity (Reinhold et al., 1985). In gnotobiotic experiments, a motile chemotaxis and aerotaxis negative mutant of *Azospirillum* spp. still colonized the roots of both kallar grass and rice in a manner similar to the wild-type. In a competition study, however, the wild-type strains significantly outcompeted the mutant on kallar grass roots up to 150-fold and on rice roots up to 40-fold (Kimmel, et al., 1990). In addition to the chemotactic response to organic substances, rhizobacteria also show aerotactic movement with a preference for zones containing low levels of dissolved oxygen (Barak et al., 1983). Most rhizobacteria are microaerobes or facultative anaerobes and when they move aerotactically to root surfaces they create gradients of low dissolved oxygen (an optimal redox potential) and form aerotactic bands in capillaries in a few seconds. Thus, in the microaerobic conditions of the root surface, bacteria with aerotactic properties will be the first to arrive at the colonization sites (Okon et al., 1980).

4. Adsorption and colonization of rhizobacteria on plant roots. It was proposed that bacterial cell surface proteins or proteins secreted by the bacteria are likely to be involved in the plant-microbe interaction as signal transducers or as structural elements (e.g. flagella or adherins) (de Weger et al, 1987; Buell and Anderson, 1992). Many cellular components on the outer membrane and exocellular surface of bacteria have been proposed to be involved in adsorption. Examples of cellular components involved in

adsorption include LPS (lipopolysaccharide), CPS (capsular-polysaccharides), EPS (exopolysaccharides), glycoproteins as well as many others (Bashan, and Levanony, 1990). Mutants of *Pseudomonas* biocontrol bacteria that lack flagella or the O-antigen of lipopolysaccharide (LPS) were impaired in potato rhizosphere colonization in field soil systems and showed a reduced colonization ability in gnotobiotic systems. Achouak et al. (1998) isolated an outer membrane protein from the nitrogen-fixing enterobacterium *Rahnella* and suggested that the role of this protein may be as a porin and as a root adhesin. Studies with colonization-defective mutants revealed that the gene involved in root colonization encoded a protein that was similar to the lambda integrate family of site-specific recombinase, suggesting that genetic rearrangements may be involved in the generation of different phenotypes in colonization that allows a bacterial population to occupy various habitats (Dekkers et al., 1998). However, cells which are adsorbed to surfaces are not readily available for translocation, thus preventing effective vertical dispersion within the root system.

5. The interactions between PGPR and plants may be partially governed by signal transduction. The exudate constituents of plant seeds and roots may contain some compounds that can act as signals for PGPR (O'Sullivan et al., 1991). Bastelaere et al. (1993) demonstrated that the expression of a 40 kDa protein in *Azospirillum brasilense* was induced by exudates from wheat, maize bean and alfalfa. Increasing evidence indicates that communication among soil microorganisms is important for their survival in the diverse ecology of the rhizosphere. For example, Fedi et al. (1997) found that the fungus *Pythium ultimum* could produce a signal that

repressed the expression of a particular gene in *Pseudomonas fluorescens* F113, that resulted in a decrease in its ability to colonize the sugar beet root. Recently, a two-component system ColR/ColS has been characterized from *Pseudomonas fluorescens* strain WCS365 and has been proposed to play an important role in root colonization in which an environmental stimulus, acting in conjunction with the system, activates an unknown trait crucial for colonization (Dekkers et al., 1998).

So far, there is no doubt that bacterial inocula can increase the yield of various crops significantly, but, not surprisingly, performance has generally been inconsistent. Bacterial effects on plant growth result largely from multiple interactions between the introduced bacteria, the associated crops, the soil microflora and the soil conditions (Kloepper, 1989). From the numerous studies concerning the mechanisms of PGPR on plant growth, it has become apparent that a particular bacterium may affect plant growth by any one or more of these mechanisms and that two different PGPR strains on the same crop may use different mechanisms. Furthermore, since many PGPR 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 (Glick et al., 1999). It is also likely that the impact of all of the mechanisms used by PGPR varies considerably depending upon the soil composition. For example, numerous researchers have observed that PGPR are more effective at stimulating plant growth when the plants are cultivated in nutrient-poor soil than nutrient-rich soil (Glick et al., 1999). In today's world, where conventional, intensive agricultural practices are being challenged for both economic and environmental reasons, a more integrative

and quantitative approach of rhizospheric processes is indeed required. This knowledge is a fundamental prerequisite to managing plant growth and nutrition in agricultural, forested, and natural environments including bioremediation (Hinsinger, 1998).

1.2. ACC deaminase

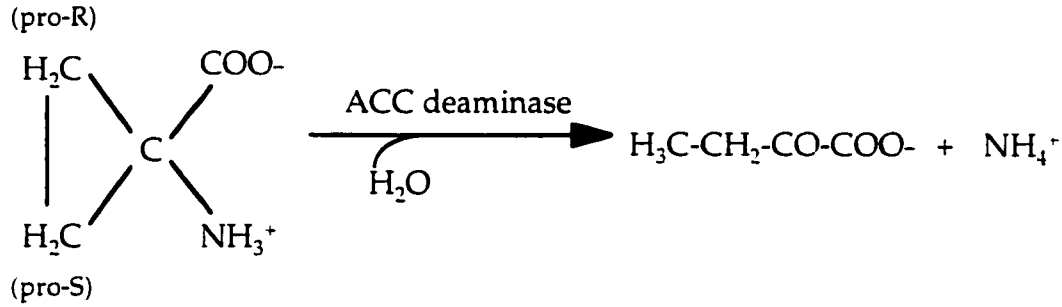
1.2.1. Biochemical properties

In 1978, Honma observed that several soil microorganisms could produce an enzyme that allowed them to grow in a medium containing 1-aminocyclopropane-1-carboxylic acid (ACC) as the sole source of nitrogen. This enzyme was found to catalyze the deamination of ACC to form α-ketobutyrate and ammonia (Fig. 1) and has since been named ACC deaminase (E.C.4.1.99.4; Honma and Shimomura, 1978).

At the time Honma report, however, the importance of ACC deaminase was limited to fermentation of several fruits, since ACC was found in apple, pear and cow berry and as a major component in fermented ciderapple and perry pear juices (Burroughs, 1957). ACC is quite different from normal amino acids, the structure of an α-dialkyl-α-amino acid with a cyclopropane ring. Interestingly, ACC cannot be assimilated by enzymes that participate in the metabolism of α-dialkyl-α-amino acids, such as dialkyl-amino-acid decarboxylase (E.C.4.1.1.64; Aaslestad and Larson, 1964) and α-methylserine hydroxymethyltransferase (E.C.2.1.2.7; Wilson and Snell, 1962). Thus, the metabolism of ACC may be dependent on a unique reaction and it was important to further study those microorganisms that were capable of hydrolyzing ACC.

Fig.1. The reaction catalyzed by ACC deaminase.

ACC: 1-Aminocyclopropane-1-carboxylic acid.



ACC α–Ketobutyrate ammonia

Realizing the importance of ACC deaminase, Honma's group isolated and purified ACC deaminase enzymes from *Pseudomonas* sp. strain ACP (Honma 1978, 1993b), a yeast *Hansenula saturnus* (Honma, 1978, Minami et al., 1998) and *Penicillium citrinum* (Jia et al., 1999). The three enzymes were characterized and ACC deaminase from *Pseudomonas* sp. ACP was found to have a molecular weight of 104,000 daltons, and a Km of 1.5 mM for ACC. The enzyme from *Hansenula saturnus* has a molecular weight of 69,000 daltons with a Km for ACC of 2.6 mM, and *Penicillium citrinum* produced an ACC deaminase that showed one band in SDS-polyacrylamide gel electrophoresis with a molecular weight of 41,000 daltons with a Km for ACC of 4.8 mM. Biochemical studies further revealed that ACC deaminase from *Pseudomonas* sp. strain ACP was a trimeric enzyme made up of identical 36,500 dalton subunits (Honma, 1985). Honma and his associates also demonstrated that the activity of ACC deaminase could be assayed by monitoring the production of either α-ketobutyrate or ammonia, and determined that ACC deaminase activity was optimal at 30°C and pH 8.5 (Honma and Shimomura, 1978; Jacobson et al., 1994).

1.2.2. Catalytic mechanisms

In order to understand the catalytic mechanism of ACC deaminase, Honma (1979, 1993c) and Walsh et al. (1981) incubated bacterial ACC deaminase with various analogous of ACC as possible substrates. The results showed that in addition to ACC, a number of its analogs, such as (1S, 2S)-2-ethyl-ACC, (1S, 2S)-2-methyl-ACC, (1S)-2,2-dimethyl-ACC, (1S, 2R)-2-methyl-ACC and 2-vinyl-ACC could also be fragmentated by ACC deaminase.

Among the four possible stereoisomers of each analog (1S, 2S, 1R and 2R), only the 1S, 2S isomer was a suitable substrate for ACC deaminase. Furthermore, when the two hydrogen atoms in the *pro-S* group of the ACC molecule were substituted with two methyl groups, the reaction still occurred (Honma, 1993c). Further examination of the stereoselectivity of binding between ACC and ACC deaminase was done by studying the binding of either L- or D-amino acids. ACC deaminase was found to be ineffective at cleaving any L-amino acids while some D-amino acids, such as D-serine, 3-chloro-D-alanine, D-alanine and O-acetyl-D-serine, were weak substrates for the enzyme (Honma et al., 1979, 1986, 1993a and 1993b). Interestingly, although L-amino acids are not substrates for ACC deaminase, they do act as inhibitors (Honma et al., 1979, 1993a). These findings suggest that ACC deaminase catalyzes a unique regiospecific ring-opening reaction and the stereospecific cleavage of the bond to the α-carbon in the cyclopropane ring is similar between ACC and D-form amino acids. D-form stereospecific cleavage was also reported in a protein superfamily that contains bacterial alanine racemase (Wang and Walsh, 1978), bacterial D-amino acid transaminse (Soper et al., 1977), and liver serine transhydroxymethylase (Wang et al., 1981).

Studies on ACC deaminase showed that pure ACC deaminase is tightly bound to pyridoxal 5'-phosphate (PLP) (Honma and Shimomura, 1978). PLP is a prosthetic group that acts as a carrier for amino groups removed from amino acids and is involved in transamination reactions as well as the enzymatic decarboxylation of α-amino acids, dehydration of serine, and removal of sulfur from cysteine (Lehninger, 1970). In deamination

reactions, PLP-dependent enzymes are known to catalyze the cleavage of groups bound to the α-carbon of amino acids (Braunstein, 1963). The α-carbon (C_α) of amino acids is bound to four chemical groups, i.e. an amino (NH_2) group, a carboxyl (COOH) group, a hydrogen (H) atom, and one variable side chain or R group. Among the four α-carbon groups, the cleavage reaction by PLP-dependent enzymes generally occurs at the α-hydrogen bond. However, ACC, being an α-dialkyl-α-amino acid, does not contain an α-hydrogen bond but a cyclopropane ring. According to the structure of the ACC molecule, Honma proposed that cleavage of ACC by ACC deaminase involved a special site-specific binding that cleaves the bond between the α-carbon and *pro-S* methylene carbon (C_α-C_β) by forming the intermediates with PLP (Walsh et al., 1981). Although cleavage of the C_α-C_β bond also occurs in other PLP-dependent enzymes, such as serine transhydroxymethylase or δ-aminolevulinate synthetase, reactions catalyzed by such enzymes proceed through a retro-aldol type cleavage, a mechanism that is not available for the deamination of ACC (Walsh et al., 1981).

Two alternative routes for opening of the cyclopropane ring have been proposed by Walsh et al. (1981), a nucleophilic addition/elimination route and a β-proton abstraction fragmentation route. In the first route, the intermediate complex between ACC and the PLP-enzyme complex may be the β, γ-olefinic PLP-ρ-quinoid adduct. Proof of the existence of such an intermediate has been provided in experiments in which D-vinylglycine was incubated with ACC deaminase yielding the intermediate vinylglycine-PLP-ρ-quinoid adduct. This route was consistent with the mechanism of amino acid transamination proposed later by Michael and Kirsch (1989) who worked

on aspartate aminotransferase. As with the proposed aspartate transamination mechanism, reactions catalyzed by PLP-dependent enzymes are believed to involve several steps, (1) formation of an internal aldimine, which is the PLP-enzyme complex forming through a Schiff's base, (2) conversion into an external aldimine in which the PLP-enzyme complex binds to the substrate, (3) passage through a quinonoid intermediate, where the enzyme removes the amino group from the substrate leaving the PLP-substrate intermediate, (4) the intermediate is transformed into ketimine, releasing an α–keto acid and ammonia. Evidence in support of the formation of the intermediate during ACC deamination was provided by Honma et al. (1993a) who demonstrated that incubation of ACC deaminase with D-alanine caused the appearance of a new absorbance band at 510 nm, suggesting the formation of a quinoidal intermediate of PLP with D-alanine.

Of interest in understanding the mechanism of ACC deaminase was whether or not solvent hydrogen was incorporated into α-ketobutyrate. Walsh et al. (1981) incubated ACC deaminase with ACC in D_2O and obtained deuterium α-ketobutyrate, with one deuterium atom at the C-4 methyl group and one at the C-3 methylene group. Further investigation with stereochemical analysis and the kinetic reaction suggested that enzymatic turnover introduced one solvent deuterium onto the C-4 methyl group while the methylene proton at C-3 was exchanged nonenzymatically and the hydrogen atom replaced with one of the solvent was from the *proR* methylene of ACC (Walsh et al., 1981). The proton NMR spectrum of 1 M D-alanine in D_2O normally has an α-proton signal that, upon incubation with ACC deaminase, disappears. The spectrum of L-alanine, on the other hand, does

not change under the same conditions. The disappearance of the NMR signal indicates the α-proton exchange between D-alanine and a solvent deuterium by ACC deaminase (Honma et al., 1993).

ACC deaminase purified from *Pseudomonas* sp. strain ACP has 18 cysteine residues. As sulfhydryl groups in proteins tend to be nucleophiles, the role of cysteine as a nucleophile in ACC deaminase was investigated (Honma, 1985, 1993a). In the presence of sulfhydryl-modifying reagents, such as tetrathionate, ρ-chloromercuribenzoate and 5,5'-dithiobis (2-nitrobenzonic acid) (DTNB), ACC deaminase was inhibited. Enzyme activity was inhibited 98.8% with the addition of 2 mM DTNB, however, the activity was restored to 96.2% with dithiothreitol, a thiol compound that regenerated the critical enzyme sulfhydryl groups. On the other hand, the sensitive sulfhydryl groups of ACC deaminase were protected from modification by sulfhydryl-modifying reagents in the presence of a L-serine, a competitive inhibitor of ACC deaminase. Therefore, the sulfhydryl groups seemed to be located in one of the active sites of ACC deaminase, or in close proximity to one.

With the help of stereospecific substrate labeling with deuterium and tritium, insight into the mechanism of the ring fragmentation of ACC was obtained by Liu et al. (1984). Their elaborate experiments showed that ACC deaminase has at least two and probably three active-site basic groups involved in proton transfer from substrates to intermediates and nascent products, as well as catalyzing a partial internal exchange of substrate hydrogen to the product.

1.2.3. Active sites

Nearly all organisms can use proteins and amino acids as carbon sources and the metabolism of amino acids occurs through a series of reactions catalyzed by many different enzymes. Most of these enzymes, such as transaminases, deaminases, aminotransferases, decarboxylases, racemases, trytophan synthases, and hydroxymethyltransferases, belong to the class of PLP-dependent enzymes. These enzymes have been isolated from many organisms ranging from bacteria to mammals and highlight an important class of convergent evolution resulting in a variety of different proteins that catalyze identical reactions, but have different functions (Vaaler and Snell, 1989). Overall, PLP can bind tightly to enzymes either by noncovalent interactions or by covalent linkage with a lysine residue at the active site through an internal aldimine (Schiff base). Lysine, having a ε-NH_2 group, is capable of attaching to PLP to form an internal aldimine, which mediates the transfer of PLP to the substrate, such as an amino acid, by a transaldimine reaction to form the external aldimine. Next, the ε-NH_2 group of the lysine residue abstracts the α-proton from the amino acid substrate to generate a corresponding α-keto acid after passing through several intermediates (Yoshimura, 1992). Based on the analysis of PLP-dependent enzymes, it has been proposed that the lysine residue serves one or more major roles in the enzymatic activity of these enzymes, including cofactor binding, formation of enzyme-substrate intermediates, catalysis and product release.

An important mechanism in the action of PLP is the aldehyde group, which can reversibly form a Schiff's base with the ε-amino group of a lysine residue in the enzyme (Lehninger, 1970). Since ACC deaminase was

discovered to bind tightly with PLP (Honma and Shimomura, 1978), the active sites for the binding and its possible function in catalysis have been studied. Using the absorbance spectrum at 420 nm, a characteristic for Schiff base formation, Honma and Shimomura (1978) observed that the purified enzyme had a maximum absorbance at 420 nm and that the absorbance rapidly decreased when ACC deaminase was inactivated with β-chloro-D-alanine (Honma, 1986). The product from ACC deaminase inactivation was the same as the product from the aldol condensation of PLP and pyruvate, an adduct formed by the attack of the substrate on the Schiff base of PLP with a lysine residue (Likos et al., 1982).

ACC deaminase from *Pseudomonas* sp. strain ACP has 11 lysine residues. When the amino acid sequences of ACC deaminase and the β-subunit of tryptophan synthase (one of PLP-dependent enzyme) were compared, there was only 24% homology, however, the sequence from 34 to 52 in ACC deaminase that contained Lys-51 was 53% homologous with sequence 73-88, containing Lys-87, in the β-subunit of tryptophan synthase (Honma, 1993a). It is known that the β-subunit of tryptophan synthase catalyzes trytophan synthesis from indole and L-serine and deamination of L-serine. Interestingly, the latter reaction is the same type as deamination of D-serine by ACC deaminase.

Lys-51 was further analyzed for its function as an active site in the enzyme using site-directed mutagenesis (Murakami, 1997). The K51A mutant, with alanine in the position of Lys-51, had less than 0.04% of the activity of the wild-type enzyme but its absorbance pattern did not show a loss of PLP (Murakami, 1997). Thus, the Lys-51 seemed to be involved not

only in internal aldimine formation, but also in enzymatic catalysis (Murakami, 1997).

The possible roles of lysine residues in other PLP enzymes have also been investigated. In aspartate aminotransferase studies, the ε-NH_2 moiety of Lysine-258 (Kirsch, 1984) was identified as the base responsible for the proton transfer that is central to the transamination mechanisms. When Lys-258 from aspartate aminotransferase was replaced with alanine (K258A) (Malcolm and Kirsch, 1985; Toney and Kirsch, 1989) or arginine (K258N) (Kuramitsu et al., 1987), PLP binding was not prevented at all, but the mutants were devoid of enzyme activity (less than 10^{-6} of the wild-type activity). Some exogenous amines could partially replace the function of the Lys-258 in aspartate aminotransferase (Toney and Kirsch, 1989). However, while studying lysine-145 in bacterial D-amino acid transaminase, Manning (1992) reported that using the mutated enzymes K145Q and K145N (in which Lys-145 was replaced by glutamine and asparagine residues, respectively) (Futaki et al., 1990; Yoshimura et al., 1992), exogenous amines, to a certain extent, were able to form an external aldimine with PLP, but had no effect on the abstraction of the α-proton from the substrate. Thus, it was suggested that another lysine residue, Lys-267, in the mutant enzyme may substitute for Lys-145 of the wild-type enzyme and provide an alternate ε-NH_2 group that can catalyze transamination, although with a reduced efficiency. Interestingly, Shostak and Schirch (1988) have also proposed that a two-base model present in serine transhydroxymethylase to explain its racemase activity.

Lys-87, an essential catalytic residue in the β-subunit of tryptophan synthase, which is considered to be a member of a second class of PLP-

dependent enzymes, based on its different 3-D structure, has been also investigated for a possible role in PLP-binding activity. By analyzing the rates of formation of enzyme-substrate intermediates with different Lys-87 mutants, Lu et al. (1993) demonstrated that Lys-87 catalyzes the removal of the α-proton during internal aldimine formation. Lys-87 also facilitates the formation of external aldimines as it was observed that the rates of external aldimine formation were slow when lysine-87 was replaced by threonine. Furthermore, lysine-87 is important for substrate and product release, the dissociation of complexes from the mutant occurred very slowly.

In addition to the lysine residue, a conserved sequence consisting of Ser-X-His-(phosphopyridoxylidene)Lys was found in 37 PLP-dependent enzymes, and most notably in decarboxylase enzymes, from different organisms including *E. coli, P. putida, Drosophila,* and tissues of pig, chicken, rabbit, and human (Vaaler and Snell, 1989). The Ser-X-His-(P-Pxd)Lys sequence was completely conserved in 6 different decarboxylases, the lysine (K) was found at the terminal position of the conserved sequence in all 37 aligned sequences, histidine (H) was present in 11 decarboxylases and 8 additional enzymes, and serine (S) was found in 6 decarboxylases as well as 13 other enzymes (Vaaler and Snell, 1989). To investigate the roles of each of the conserved residues in the enzyme activity, histidine decarboxylase from *Morganella morganii* was cloned into *E. coli* and fourteen site-directed mutants were constructed within the Ser^{229}-X^{230}-His^{231}- (P-Pxd)-Lys^{232} (Vaaler and Snell, 1989). As before when Lys was replaced with alanine, the mutant protein was virtually inactive, but still retained the ability to bind both PLP and the substrate. It was suggested that in the PLP-dependent histidine

decarboxylase, Lys-232 plays an essential role, perhaps as a proton donor in the catalysis of decarboxylation. Histidine, on the other hand, is not essential for catalysis, however, it may provide a hydrogen bond or a salt bridge to bind with the substrate or PLP. The Ser-229 residue is also not essential for catalysis, but it is necessary for optimal structure and function of the enzyme through its contribution to its conformation.

1.2.4. Gene isolation

When ACC was recognized as the immediate precursor of ethylene in plants (section 1.3), ACC deaminase received more attention and research began to focus on the isolation of ACC deaminase genes. Sheehy et al. (1991) cloned the gene that codes for ACC deaminase from *Pseudomonas* sp. strain ACP. At the same time, Klee et al. (1991) screened 600 microorganisms for ACC degrading ability and characterized two pseudomonads with ACC deaminase ability. Seven soil microorganisms including *Enterobacter cloacae* and *Pseudomonas* sp. strains from Californian and Canadian soil samples were selected based on plant growth-promoting activity and growth on ACC medium (Glick, 1995). Campbell (1996) screened 103 soil microorganisms isolated from various regions in South Africa and 81 of the microorganisms displayed varying degrees of ACC degrading ability. Among them, seven bacteria displayed the most vigorous growth on ACC medium and were identified as fluorescent pseudomonads. Recently, 130 strains from Antarctica were screened for ACC deaminase, and eight strains were able to grow on ACC agar (H. Kawahara, personal communication). Another recent report is that twenty-two strains isolated from rhizoplane of pea, saperda

mustard and carex in five different soil and sewage sludge have ACC deaminase activity (Belimov et al., submitted). Detailed information on all ACC deaminase-containing microorganisms was summarized by Penrose and Glick (1997) and Glick et al. (1999).

So far, the genes encoding ACC deaminases have been isolated by (1) Sheehy et al. (1991) from *Pseudomonas* sp. strain ACP, (2) Klee et al. (1991) from *Pseudomonas chloroaphis* 6G5 and *P.* sp. strain 3F2, (3) Campbell and Thomson (1996) from *Pseudomonas fluorescens* F17 (4) Shah et al. (1998) from *Enterobacter cloacae* UW4 and CAL2, and (5) Minami (1998) from the yeast *Hansenula saturunus*. When the amino acid sequences derived from the DNA sequences were compared all of the genes were found to be highly homologous (96%-99% identity) except those from *Pseudomonas* sp. strain ACP (81%-82% identity) and from the yeast (60-63% identity). The DNA sequences of bacterial ACC deaminase genes contain an open reading frame of 1017 bp with a predicted molecular weight of 36,800 daltons, which was similar to the molecular mass of 36,500 daltons predicted by Honma (1985) and Jacobson et al., (1994) from SDS-PAGE analysis. Compared with bacterial ACC deaminases, the enzyme from yeast was slightly larger with 441 amino acid residues. The open reading frame for the yeast enzyme has been cloned and expressed in *E. coli* as an active enzyme.

1.3. The role of ACC in plants

1.3.1. Ethylene biosynthesis in plants

When ACC deaminase was first isolated from soil microorganisms (Honma and Shimomura, 1978), its only known function was to break down

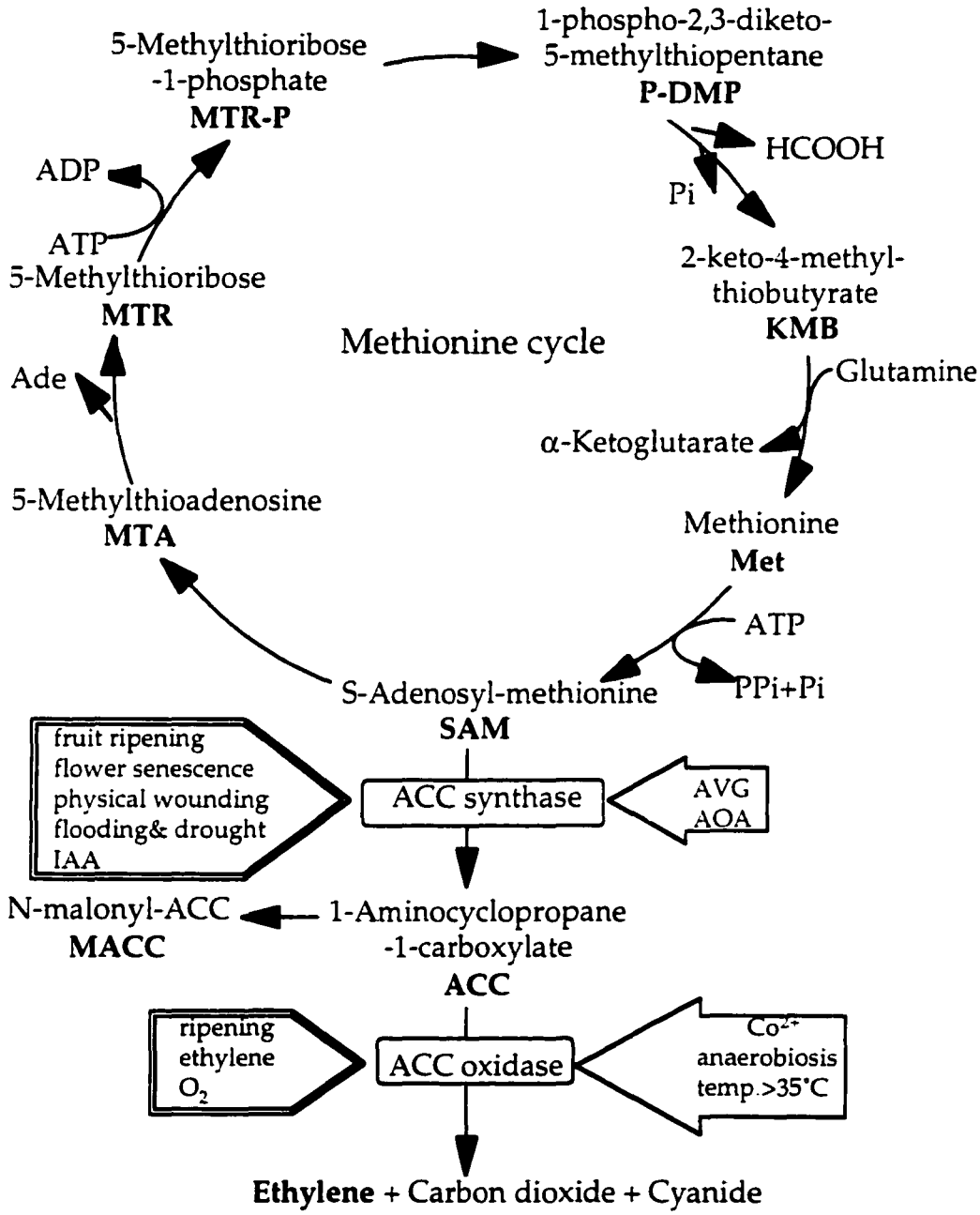
ACC. Later, the significance of ACC was realized when Adams and Yang (1979) discovered that ACC was a key intermediate in the pathway of ethylene biosynthesis in higher plants.

Early studies of ethylene biosynthesis in plants lead to the realization that ethylene was derived from carbons 3 and 4 of methionine (Lieberman and Mapson, 1964). Since the level of methionine in apple tissue was found to be too low to sustain a normal rate of ethylene production, Yang et al., (1984) demonstrated that the sulfur of methionine was recycled back to methionine so as to maintain a steady rate of ethylene production. This cycle, named the Methionine Cycle or Yang cycle, as well as the pathway of ethylene biosynthesis, were established by Yang and Hoffmann (1984) (Fig. 2) and, moreover, it was shown that the capacity to convert ACC to ethylene is present in most plant tissues.

While working on the ethylene biosynthesis pathway, Adams and Yang (1979) found that when ACC was applied to various plant organs, an increase in ethylene production was obtained. From their observations, ACC, as a key intermediate that linked the methionine cycle and ethylene biosynthesis, was deemed to be the direct precursor of ethylene production with its levels directly controlling ethylene synthesis in plants (Yang and Hoffman, 1984).

Ethylene biosynthesis consists of three steps: (1) L-methionine is converted to S-adenosyl L-methionine (AdoMet), a reaction catalyzed by methionine S-adenosyl transferase. AdoMet is also utilized in other cellular reactions such as ethylation and polyamine synthesis; (2) The conversion of

Fig. 2. The pathway of ethylene biosynthesis from the methionine cycle in higher plants. Key: the factors in the symbols  are inducers, while the factors in the symbols  are inhibitors for both ACC synthase and ACC oxidase.


5-Methylthioribose
-1-phosphate
MTR-P
1-phospho-2,3-diketo-
5-methylthiopentane
P-DMP
HCOOH
Pi
ADP
ATP
5-Methylthioribose
MTR
2-keto-4-methyl-
thiobutyrate
KMB
Methionine cycle
Glutamine
Ade
α-Ketoglutarate
5-Methylthioadenosine
MTA
Methionine
Met
ATP
PPi+Pi
S-Adenosyl-methionine
SAM
fruit ripening
flower senescence
physical wounding
flooding& drought
IAA
ACC synthase
AVG
AOA
N-malonyl-ACC
MACC
1-Aminocyclopropane
-1-carboxylate
ACC
ripening
ethylene
O_2
ACC oxidase
Co^{2+}
anaerobiosis
temp.>35°C
Ethylene + Carbon dioxide + Cyanide

AdoMet to ACC which is catalyzed by ACC synthase. The ACC synthase step is considered to be the rate-limiting step in the pathway. ACC synthase is a key regulatory enzyme in that it is only synthesized during particular stages of plant development or induced by selected external factors (Beyer et al., 1984); (3) ACC is further metabolized to ethylene, carbon dioxide, and cyanide by ACC oxidase, previously known as the ethylene forming enzyme. It is present in low levels in most tissues then induced when it is needed (Yang and Hoffman, 1984) (Fig. 2).

1.3.2. Ethylene production in plants

Most plant tissues have the ability to produce ethylene in amounts ranging from 0.1 nL to 500 nL/g plant tissue/per hour. Ethylene is biologically active in amounts as little as 10-100 nL/L of air (Burg, 1962). Ethylene production is induced: (1) during certain stages of growth, such as seed germination, fruit ripening, leaf abscission, and flower senescence, (2) by particular external factors ranging from mechanical wounding, viral infection and insect damage to auxin treatment, and (3) under stress-inducing environmental conditions, e.g. flooding, cold temperature, drought and radiation (Abeles, 1973; Yang and Hoffman, 1984).

The rate of ethylene production in a plant varies with both tissue type and stage of development (Beyer et al., 1984). For instance, in germinating seeds, meristematic tissue and nodal regions are the most vigorous sites of ethylene synthesis. Increases in ethylene production normally occur when the radical starts to penetrate the seed coat and during the period when the seedling forces its way through the soil. In flowers, maximum rates of

ethylene production appear at the initial stages of fading (Biale and Young, 1981), and during the aging of bean leaf, the rate of ethylene production was found to increase 30-fold from pre- to post-abscission (Jackson and Osborne, 1970).

Williamson (1950) was the first to detect increases in ethylene production by injured cells. Since then, stress induced ethylene production has been proven to be universal and the term "stress ethylene" has been used to explain the phenomenon (Beyer et al., 1984). Flooding, a well-studied class of stress, creates an anaerobic environment in the area surrounding roots that causes a rise in internal ethylene levels in the shoots of many plants (Jackson and Campbell, 1976). When tomato roots were treated anaerobically for 12 h, ACC contents were found to increase 11-fold (greater than 3 μM in flooded xylem exudate) (Bradford and Yang, 1984). However, air drying of detached bean and cotton leaves also seemed to stimulate ethylene production (Morgan et al., 1990).

Although ethylene has been found to be present in most plant tissues, translocation of ACC still plays an important role in ethylene production. The conversion of ACC to ethylene by ACC oxidase is an oxygen dependent reaction. During flooding, the ACC content in roots is increased markedly and the ACC is then transported through the xylem from the anaerobic roots to the shoots where it is converted to ethylene, aerobically (Jackson and Campbell, 1976; Bradford and Dilley, 1978). Translocation of ACC has also been observed within flowers where ACC is suggested to move from a stigma and style to an ovary, and finally to the petals, where ACC is converted to ethylene (Nichols et al., 1983).

1.3.3. Physiological effects of Ethylene on plants

Although it was clear by the mid-nineteenth century that the presence of gaseous materials in the air could modify the growth of plants, it was not until the beginning of the century that Russian botanist Neljubow identified ethylene as an active compound of illuminating gas that caused the triple response in dark-grown pea seedlings. However, the exact role of ethylene in plants remained uncertain until the use of gas chromatography for ethylene analysis by Burg in 1959. Since then, research on ethylene rapidly developed and eventually ethylene obtained recognition as a plant hormone (Beyer et al., 1984).

It is now known that ethylene (C_2H_4), one of the five principle plant hormones, regulates many aspects of plant growth, development and senescence. In different plants and at different times during plant development, ethylene may inhibit or promote the elongation of growing stems, roots and other organs, and these effects are, to a certain extent, dependent on its concentration.

1.3.3.1. Seed germination

Germination of seeds is believed to be under the regulation of ethylene. The threshold effects (1 μL/L ~ 10 μL/L) of ethylene can vary depending on the sensitivity of the particular seed species to ethylene. At high concentrations, ethylene stimulates seed germination, while at lower concentrations it can inhibit germination. During germination, the seed undergoes a series of reactions including the metabolism of storage proteins,

the mobilization of nutrients and cell elongation (Bewley and Black, 1994). The role of ethylene in the promotion of seed germination is thought to occur through several mechanisms, such as the promotion of aerobic respiration, the accumulation of amino acids, increases in the growth potential of seed tissue osmoregulation, generation of the necessary force to break the seed coat (Esashi et al., 1977) and controlling the expression of a cysteine proteinase (Cervantes et al., 1994). The evidence for the obligatory role of ethylene in germination was obtained from experiments with the *Arabidopsis* ethylene-mutant *etr1* (ethylene insensitivity mutant). When compared with wild-type, *etr1* seeds showed poor germination (Bleecker et al., 1988).

1.3.3.2. Root elongation

As with seed germination, the effect of ethylene on root growth and development is generally believed to be dependent on its concentration (Abeles and Morgan, 1992). When the ethylene level in seedling roots is below 1 μL/L, root elongation occurs. However, when the level is increased to over 1 μL/L, root elongation is inhibited. The inhibition of root elongation is accompanied by increases in radial expansion of the root, leading to shorter, fatter roots often producing additional root hairs. The absorptive functions of the shorter root systems is not appreciably affected. The mechanisms of ethylene treatment at the cellular level have been proposed to include a reorientation of the microtubules in the longitudinal direction, thereby facilitating a switch from elongation to radial expansion and causing the roots to be shorter and wider (Shibaoka, 1994). Ethylene may also regulate the expression of cell wall peroxidases involved in the control of cell wall

extensibility and cell growth (Ridge and Osborne, 1971; Schopfer, 1996). However, ethylene is thought to be of functional importance in the process of seedling emergence. The thick hypocotyl allows the seedling to exert more force when growing through the soil, whereas the apical hook is believed to protect the apical meristem against injuries during the emergence process (Liptay and Geier, 1983; Clarke and Moore, 1986).

1.3.3.3. Fruit ripening

In climacteric fruits, the sharp increase in ethylene production at the onset of ripening is thought to trigger changes in the color, aroma, texture, and flavor of fruit. During fruit ripening, ethylene regulates the expression of genes that are responsible for enhancing the rate of respiration, autocatalytic ethylene production, chlorophyll degradation, carotenoid synthesis, conversion of starch to sugars, and increased activity of cell wall degrading enzymes, which lead to changes in the appearance, texture, and taste of the fruit (Gray et al., 1992).

1.3.3.4. Leaf and floral senescence

Senescence is a complex process controlled by many factors, many of which are not yet well understood. Picton et al. (1993) demonstrated that ethylene and senescence were closely related. It has been reported that ethylene and other compounds such as methyl jasmonate and abscisic acid (Zhi-Yi et al., 1988) promote aging and senescence. At the onset of senescence, ethylene levels were observed to increase from 0.4 to 16.0 nL/g. f.wt/h. In leaf and floral senescence, ethylene has been shown to increase the

rate of chlorophyll, RNA and protein degradation (Abeles et al., 1989; Aharoni, 1989) and to stimulate some catabolic enzymes that result in membrane changes, cellular deterioration and cell wall lignification (Faragher et al., 1987). Ethylene has also been shown to increase peroxidase activity, which is thought to play a role in cell wall lignification (Morgens et al., 1990).

While studying the senescence of carnations, Lawton et al. (1990) and Michael et al. (1993) isolated senescence-related (SR) cDNA clones from a senescent carnation petal library and suggested that several mechanisms exist for the regulation of gene expression during flower senescence and at least one class of mRNA is strictly dependent on ethylene production. In addition to ethylene, other plant hormones may affect the rate of aging and senescence. For example, both cytokinins and auxin can retard the ability of ethylene to promote senescence (Gepstein and Thimann, 1981). Stress ethylene may also play a role in senescence (Nichols and Frost, 1985).

1.3.3.5. External stresses

The increased levels of ethylene that follow flooding can stimulate the epinastic growth of petioles (Bradford and Dilley, 1989) and can also account for other symptoms of flooding including leaf yellowing, reduction in plant weight, stem thickening, adventitious root formation, wilting and abscission of leaves (Jackson and Campbell, 1976; Bradford and Yang, 1981). Drought stress ethylene has been suggested to alter the tissue sensitivity to ethylene as well (Abeles, 1992). Mechanical wounding has been found to proceed an increase in the activity of phenylalanine ammonia-lyase (PAL), and it was shown that ethylene plays an intermediary role in the induction of PAL

following excision (Hyodo, 1983). Stress ethylene has also been implicated in inducing increased ribonuclease activity in sliced potato tuber tissue (Isola and Franzoni, 1989). Rubbing the stems of bean plants resulted in a two-fold increase in ethylene production and also promoted the senescence of leaves (Giridhar and Jaffe, 1988).

The rate of ethylene biosynthesis also increases when a plant is infected with phytopathogens (Yang and Hoffman, 1984). Ethylene induces the expression of a series of pathogenesis-related (PR) proteins, such as β-1,3-glucanase, chitinase, proteinase inhibitors and peroxidase (Archer and Hislop, 1975). Stress ethylene production by infected plants has also been reported to precede the formation of lesions, yellowing and rot development (De Laat and van Loon, 1983; Spanu and Boller, 1989). Exogenous ethylene was found to exacerbate the severity of Verticillum wilt when applied to tomato plants (Cronshaw and Pegg, 1976). So far, hundreds of reports have demonstrated that plants infected with fungi produce stress ethylene. Leaf browning, a common source of loss in many plants including lettuce and tobacco, is also induced by ethylene (Spanu and Boller, 1989). Controversy remains as to whether or not the ethylene produced by the infected plant is a signal that acts to amplify cell death or whether is simply correlated with advanced disease symptoms. Recently, Lund et al. (1998) reported that a tomato mutant impaired in ethylene perception-*Never ripe* (*Nr*) caused a significant reduction in disease symptoms in comparison to the wild-type after infections with one eukaryotic and two prokaryotic pathogens. They also provided evidence that the susceptible response to foliar pathogen infection consist of two stages with regard to ethylene action, ethylene-independent primary lesion formation and

ethylene-dependent symptom development. In addition, it was recently shown that transgenic tomato plants expressing a bacterial ACC deaminase gene under the transcriptional control of either the 35S (constitutive expression), RolD (root specific expression) or PRB-1b (stress induced promoter) showed significantly reduced disease symptoms, compared to non-transformed plants, following infection with *Verticillum clahliae* (Robison et al., manuscript in preparation).

1.3.4. Manipulation of ethylene levels in plants

Ethylene can affect the growth of a plant at almost every stage of development and participates in a number physiological processes (Reid, 1987). Ethylene is thought to be a powerful regulator of plant metabolism and its effects on plants, as mentioned earlier, are dramatic and of considerable commercial importance. The technologies to reduce ethylene levels as a means to increase post-harvest storage life have been available for decades, however, they remain hindered by such problems as high cost, side effects, and an inability to shut off ethylene production completely (Ferro, et al., 1995).

Since the introduction of genetic transformation techniques in 1983, there has been a revolution in plant biology. Genetic transformation breaks the species barriers, permitting the transfer of genes among different organisms, including animals and plants. The sensitive techniques of Southern and Northern analysis can demonstrate the presence of a particular DNA or RNA respectively in a plant cell. Vectors containing genetic markers that could be used to transfer fragments of DNA into plant cells have been

developed (Walden, 1988) and some, such as the Ti (tumour inducing) plasmid of *Agrobacterium tumefaciens* (Brown, 1990) are widely used and easily manipulated. Further development in transgenic technology led to the use of antisense RNA for the downregulation of gene expression, a technique that has become a powerful tool for the study of gene function in plants (Delauney, 1988). Using antisense technology, the generation of plants in which ethylene production is down-regulated will provide the opportunity to evaluate the role of ethylene in the regulation of plant developmental processes and perhaps make it possible to improve fruit quality and storage life, delay flower senescence, and increase fungal resistance of plants.

In contrast to other phytohormones, the pathway for ethylene biosynthesis has been established (Yang and Hoffman, 1984) and the genes encoding the biosynthetic enzymes, ACC synthase and ACC oxidase, have been isolated from several plant species. Several strategies to down-regulate endogenous ethylene levels in intact plants have been proposed and employed: (1) ACC synthase or ACC oxidase is inhibited by transformation of the plant with the respective antisense RNA (Oeller et al., 1991; Hamilton et al., 1990; John et al., 1995); (2) Availability of the ethylene precursors, either ACC or S-adenosylmethionine, is reduced respectively, by either overexpressing a bacterial gene encoding ACC deaminase (Klee et al., 1991; Sheehy et al., 1993), or overexpressing a viral S-adenosylmethionine hydrolase (Ferro et al., 1995), or promoting the malonylation of ACC to its inactive form, i.e. malonyl-ACC (Liu et al., 1985).

1.3.4.1. Manipulation of ACC synthase

ACC synthase catalyzes one of the rate-limiting steps in the pathway of ethylene biosynthesis in plants, and it exists in a superfamily containing multiple ACC synthase genes that are differentially regulated by various conditions such as stress or elevations in the levels of auxin or ethylene (Rottmann, et al., 1991). In a number of instances increased ethylene production is correlated with the accumulation of ACC synthase transcripts, indicating that ethylene production is often controlled via the transcriptional activation of ACC synthase genes. However, Oetiker et al. (1997) recently reported that there are two groups of ACC synthase transcripts expressed in tomato, elicitor-induced and constitutive.

So far, ACC synthase genes have been reported in species such as tomato (Oetiker et al., 1997), *Arabidopsis* (Liang et al., 1992; Artecas and Arteca, 1999), mungbean (Botella et al., 1992; Sun et al., 1999), zucchini (Huang et al., 1991), cucumber (Shiomi et al., 1998), winter squash (Nakajima et al., 1990; Nakagawa et al., 1991), wheat (Subramaniam et al., 1996) and rice (Zarembinski and Theologis, 1997). The multiple ACC synthase genes are not only regulated by different factors, but the expression of ACC synthase is also differentially regulated in different plant tissues, for example, one ACC synthase gene isolated from wheat was found to be expressed almost exclusively in the root (Subramaniam et al., 1996). Thus, differential regulation within the ACC synthase gene family in transgenic plants with antisense gene constructs causes either differentially repressed ACC synthase expression under different conditions, or differential behaviour among

tissues, such as the reduction of ethylene synthesis in fruit (Oeller et al., 1991) but not leaves (Klee and Romano, 1994).

The expression of an antisense RNA derived from the cDNA of the LE-ACC2 gene (a member of the ACC synthase multigene family that is induced during fruit ripening) in transgenic tomato plants was obtained by Oeller et al (1991), and resulted in severe inhibition of ethylene production in fruit (below 0.1 nL/g·h; i.e. 99.5% inhibition).

1.3.4.2. Manipulation of ACC oxidase

The conversion of ACC to ethylene is catalyzed by the enzyme ACC oxidase. Hamilton et al. (1990) were the first to lower ethylene synthesis in plants by transforming an antisense construct of a cDNA fragment that encoded ACC oxidase into tomato plants. They found that the production of ethylene was 68% lower in wounded leaves and 87% lower in fruits. When John et al. (1995) examined transgenic tomato plants with ACC oxidase antisense constructs, they observed that chlorophyll levels, photosynthetic capacity and chlorophyll fluorescence were higher in transgenic plants compared with senescing wild-type plants of the same age. Leaf senescence in the transgenic tomato plants was delayed and thought to be due to a reduction in ACC oxidase mRNA that is usually found in pre-senescent wild-type but not transgenic plants.

A study by Picton (1993) with transgenic tomatoes expressing an antisense ACC oxidase gene concluded that fruits that expressed the ACC oxidase antisense gene were much more effective in delaying ripening when the fruits were picked before the onset of color change. Similar results were

also reported by Michael et al., (1993) when they isolated the ACC oxidase gene from a senescing carnation petal library and transformed it into a carnation. The transformants they obtained had a greater than 90% reduction in the levels of ethylene produced by the flowers, and the petals of the transgenic plants did not undergo the inrolling phenomenon characteristic of senescence in control carnations. The vase-life of the flowers of the transformed plants was extended to almost double that of nontransgenic carnation plants and the petals showed a slight decrease in pigmentation.

Using antisense ACC oxidase RNA, transformed mustard plants with enhanced shoot regenerability provided evidence that ethylene is inhibitory to cell differentiation and shoot regeneration (Pua and Chi, 1993). When ethylene production under waterlogged conditions was investigated with ACC oxidase antisense constructs, English et al. (1993) showed a delay of 4 h before epinastic curvature commenced in the transgenic plants and larger quantities of ACC accumulated in the root of transgenic plants under waterlogging conditions. However, only a modest attenuation of ethylene production was observed.

Like ACC synthase, ACC oxidase is a member of a gene family. Three ACC oxidase genes were found in the tomato genome (Holdsworth et al., 1987; Koch et al., 1991), and it appears that expression of each of these genes is influenced by different stimuli.

1.3.4.3. Manipulation of bacterial ACC deaminase

Since ACC synthase and ACC oxidase are members of multigene families in which members can be divergent in DNA sequence, the

effectiveness of an antisense construct varies among different plants and plant tissues as does the degree of ethylene inhibition (Gray et al., 1992). The difficulty in working with multigenic proteins stimulated scientists to find other molecular methods for controlling ethylene production. The level of ACC in plants is thought to be critical for ethylene production as ACC is the direct precursor of ethylene and it was found that the rate of ethylene production in plants is largely dependent on the availability of ACC and the oxygen concentration (Yip et al., 1988). Bacterial ACC deaminase hydrolyzes ACC and to date, it is only present in soil microorganisms. Thus, transforming plants with a bacterial ACC deaminase gene was a possible way to reduce ethylene levels in plants and it was expected that the reduction of ethylene would occur in all tissues where the ACC deaminase gene was expressed (Klee et al., 1991; Sheeny, et al., 1991; Robison, et al., manuscript in preparation).

Klee et al. (1991) introduced the ACC deaminase gene into tomato plants. In the best expressing transgenic line, ACC deaminase under the transcriptional control of the 35S promoter from cauliflower mosaic virus, was expressed to 0.5% of the total leaf protein and ethylene levels were reduced by 90%-97%. The transgenic tomato line with the second-highest ACC deaminase levels produced a 78% reduction in ethylene production. Reduction in ethylene synthesis in transgenic plants resulted in significant delays in ripening, with the mature fruits remaining firm for at least six weeks longer than the nontransgenic control fruit.

Sheehy et al. (1993) have cloned an ACC deaminase gene from *Pseudomonas* sp. ACP and transformed it into tomato plants. The ACC

deaminase gene was effectively expressed under the transcriptional control of the 35S promoter in the leaves of the transgenic plants and detectable at the protein level. An ACC deaminase gene was also expressed in transgenic tobacco and *Arabidopsis* plants (Romano et al., 1993). Transgenic tobacco lines that expressed high levels of ACC deaminase (up to 0.5% the extractable protein) were obtained using duplicated or enhanced 35S promoters and displayed a 50% reduction in ethylene production relative to controls. *Arabidopsis* lines transformed with the bacterial ACC deaminase gene were screened directly for reductions in basal ethylene production. Three independent *Arabidopsis* lines that reduced ethylene production by 75% to 90% relative to wild-type plants were identified. Expression of ACC deaminase in plant tissues has been localized using constructs consisting of the ACC deaminase gene fused with the β-glucuronidase (GUS) reporter gene. It was found that in virtually all cell types, the expression of GUS could be detected, suggesting that ACC deaminase can be expressed throughout the entire plant.

ACC deaminase transgenic lines were valuable not only as a means of confirming the function of the gene, but it also make it possible to monitor the effects of altered endogenous ethylene production on plant growth and development. In addition, by using organ-specific or developmentally regulated promoters to drive antisense ACC oxidase or ACC synthase gene expression or ACC deaminase gene expression, it should be possible to inhibit ethylene production in particular parts of a plant or at particular stages in the life cycle (Gray et al., 1992; Theologis et al., 1993).

1.4. The role of ACC deaminase in PGPR

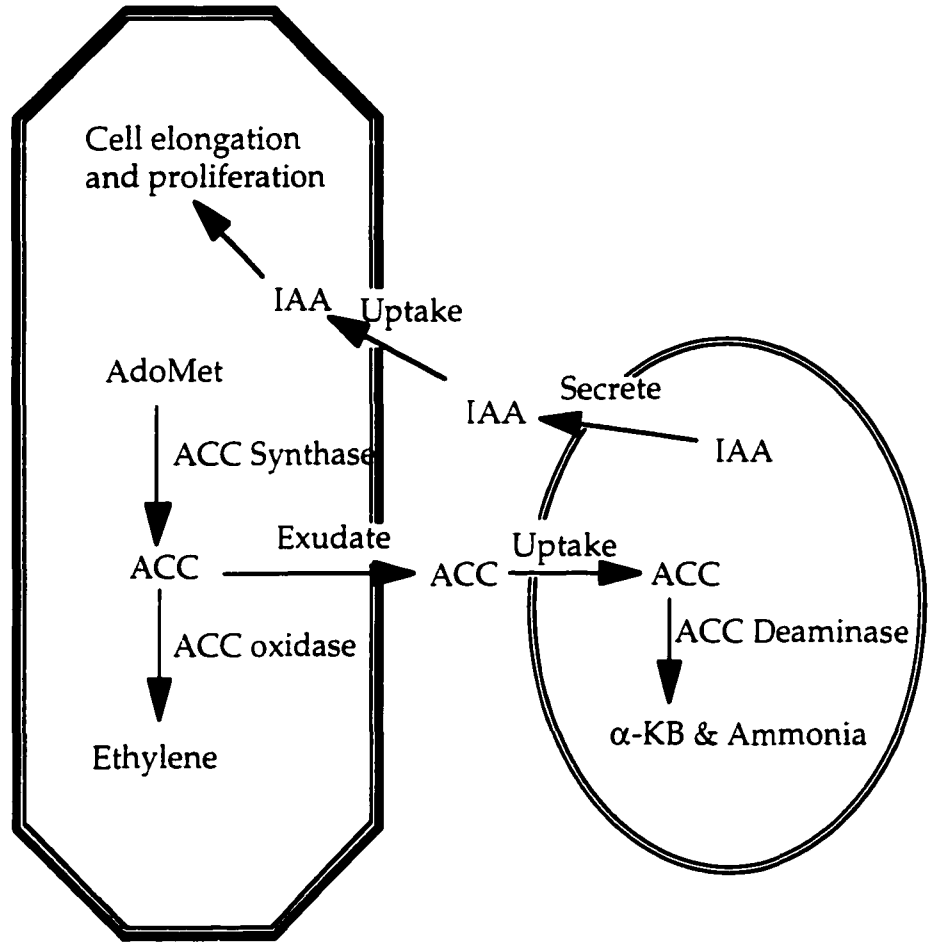
ACC deaminase was first found in soil microorganisms in 1978. At that time, it was shown that ACC deaminase is a pyridoxal-5'-phosphate-dependent enzyme that catalyzes the cleavage of ACC to α-ketobutyrate and ammonia (Honma, 1978). Later, in 1979, Adams and Yang demonstrated that ACC was the direct precursor to ethylene in higher plants. Since then, ACC deaminase has been found in several different soil bacteria and their genes have been isolated. However, ACC deaminase has not yet been found in plants. In a series of monumental experiments, Klee et al., (1991) and Romano et al., (1993) successfully introduced bacterial ACC deaminase genes into several different plants including tomato, tobacco and *Arabidopsis*, resulting in dramatically reduced ethylene levels within the plants. These and other experiments with transgenic plants clearly demonstrated that bacterial ACC deaminase genes could reduce the production of ethylene by hydrolyzing ACC in plants.

In nature, ACC deaminase is usually found in soil bacteria that associate with plant roots in the area known as the rhizosphere. In this association, it was observed that these soil bacteria are able to colonize plant roots and promote root elongation (Glick et al., 1994a, 1994b, 1995; Hall et al., 1996). Questions then arose pertaining to the role of ACC deaminase and which of the apparent functions of ACC deaminase were unique to the bacteria and whether or not they played a role in the interactions between plant and bacteria in the rhizosphere. In other words, was ACC deaminase responsible for conferring plant growth-promoting ability to soil bacteria that enable to lower the level of ethylene in plants?

A model proposed by Glick et al. (1995; 1997; 1998) (Fig. 3) describes the potential ability of PGPR, that contain ACC deaminase, to lower plant ethylene levels by binding to the roots and seed coats of the plants hydrolyzing ACC. When comparing the ideas presented in the model with the evidence obtained from transgenic plant experiments, it can be seen that both suggest a reduction in ethylene levels through the hydrolysis of ACC by ACC deaminase. The difference between the model and the transgenic plant experiments is that with the latter, the ACC deaminase is inside the plant and hydrolyzes ACC directly, whereas with the former, ACC must be exuded from the plant into the rhizosphere and then taken up by the bacteria and hydrolyzed. An important question relating to the model is whether or not ACC is exuded from the plants , and if it is, whether or not the ACC can be efficiently taken up by the bacteria and hydrolyzed. Several evidence and previously results obtained from our lab may explain the hypothesis predicted in the model:

(1) It is known that during plant root development, roots exude many soluble nutrients that can be used as carbon and nitrogen sources for microorganisms (O'Sullivan et al., 1991). Since the exudate composition varies between plant species, especially within different organic acids and carbohydrates, soil bacteria colonizing roots tend to be somewhat species specific. ACC, as a small molecule, is believed to be exuded from roots likeother amino acids in what is thought to be a passive process. However, exudation by plant roots can also be stimulated by bacteria colonizing the rhizosphere. Soil bacteria, especially PGPR, have the ability to migrate to plant roots by chemotaxis and colonize the root surface, where they not only

Fig. 3. Schematic representation of the model proposed by Glick (1994, 1995, 1998): a PGPR associates with plant root in the rhizosphere and lowers the ethylene concentration in the root by taking up ACC from the root. Key: IAA, indoleacetic acid; ACC, 1-aminocyclopropane-1-carboxylic acid; AdoMet, S-adenosyl-methionine; α-KB, α-ketobutyrate.


Cell elongation
and proliferation
IAA
Uptake
AdoMet
ACC Synthase
ACC
ACC oxidase
Ethylene
Exudate
IAA
Secrete
IAA
ACC
Uptake
ACC
ACC Deaminase
α-KB & Ammonia
Plant cell
Rhizosphere
PGPR

take up the nutrients from plant exudates but also stimulate root exudation (Lee and Gaskins, 1982; Venkateswarlu and Rao, 1985). Although the mechanisms behind the stimulation of plant exudation by bacteria are not fully understood, a comparison between the inoculated and uninoculated plants has demonstrated that more than 1% of photosynthates were excreted from the inoculated roots as compared to untreated roots (You et al., 1991). Heulin et al. (1987) found that a 36% increase in rice exudate when rice plants were inoculated with strains of *Azospirillum*. Therefore, it is likely that if ACC is exuded from plant roots it may exuded at a higher extent when the roots are inoculated with bacteria. In fact, Penrose et al. (manuscript in preparation) have very recently shown that ACC deaminase-containing PGPR rapidly remove ACC exuded from seeds, stimulate ACC exudation and ultimately lower ACC levels within the seeds.

(2) It has been reported that during apple ripening, ethylene production increases over 1000-fold (0.001 to 2 nmole/g/h) with a concomitant 18-fold increase in the levels of ACC (0.08 to 1.4 nmole/g/h) (Keen, 1984). High rates of ethylene production from growing pea epicotyls showed that the plumule and hook had 3-times the amount of ACC and 10-times the amount of ACC oxidase than basal sections (Schierle et al., 1989). These results indicate that a high rate of ethylene production is proceeded by an increase in the levels of free ACC within the plant. The increased ACC is then converted into ethylene. Thus, the high levels of ACC in plant roots during a certain stage of development, e.g. seed germination, root elongation or flooding, could build up a concentration gradient across the root from inside to outside. Along the concentration gradient, ACC would passively

flow outside of the root and enter the rhizosphere where it would be taken up by bacteria and hydrolyzed by bacterial ACC deaminase. In this way, the bacteria seem to draw ACC out of the roots by creating and maintaining a lower level of the rhizospheric ACC. Moreover, a high rate of ethylene production requires high amounts of ACC, suggesting that the ACC level in the plant is a key factor for ethylene production. It has been observed that, in fruits, ethylene production consumes the total ACC pool every half hour (Knee, 1984). Therefore, even if a small amount of ACC flows out from bacteria-treated roots, there would still be a decrease in ACC levels in the plant resulting to a certain extent in a reduction in ethylene production.

(3) It is shown in the model that IAA in plants can either stimulate plant cell proliferation and/or elongation, or it can stimulate the activity of the enzyme ACC synthase which catalyzes the formation of ACC from S-adenosylmethionine (Kende, 1993). Most PGPR were found to be able to synthesize and secrete IAA when in association with plant roots (Hong et al., 1991; Fallik et al., 1994; Patten and Glick, 1996). Secreted IAA, taken up by the plant root, will cause an increase in the ACC level and thus an increase in ethylene production. In this way, the bacterium causes the plant to synthesize more ACC and stimulates ACC exudation from the plant. If this happens at seed germination, the higher ethylene level from bacterial IAA stimulation may promote seed germination, followed by inhibition of root elongation. However, the ethylene level in these seeds could be reduced if ACC exudation from the seeds could be efficiently driven by the bacteria along the ACC concentration gradient.

(4) It is obvious that there is a competition between the exudation of ACC and its conversion to ethylene by ACC oxidase. Compared with the Km of ACC deaminase for ACC (around 5 mM) (Honma and Shimomura, 1978; Klee and Kishmore, 1991), ACC oxidase has a 100-fold lower Km for ACC (50 μM) (Abeles et al., 1992). However, in transgenic plants it was found when ACC deaminase was overexpressed to approximately 0.5% of the total soluble protein, ethylene production was reduced by as much as 90% in transgenic tomato plants (Klee et al., 1991), and 75% to 90% reduction in transgenic *Arabidopsis* plants (Romano et al., 1993). ACC oxidase in plants has been found to be constitutively present at a low level (Kim and Yang, 1994), while its level is increased during different stages of growth and development (Wang and Woodson, 1992) with the ACC level increased or regulated by factors at the transcriptional level, such as oxygen concentration and ethylene concentration (Dong et al., 1992). Based on these observations it is conceivable that if the bacteria are added to seeds early enough, a certain amount of ACC outflow would occur before the ACC could be converted to ethylene by ACC oxidase. The ACC level in the roots would increase slowly or may not reach levels high enough to induce ACC oxidase at the usual time. Consequences from such a decrease in ACC levels may be a delay or reduction in ethylene production.

(5) Evidence for ACC exudation from seedlings has been recently obtained by Penrose et al. (manuscript in preparation). ACC levels in extracts of canola seedlings as measured by HPLC were lower when the seeds were treated with *P. putida* GR12-2 than in seedlings from untreated seeds. In addition, the ACC levels in root extracts from seedlings in which the seeds

were treated with an ACC deaminase minus mutant of *P. putida* GR12-2 were similar to those from the untreated seedlings (Glick et al., 1998).

1.5. Experimental strategy

The experimental strategy carried in my thesis research is to test parts of the hypothesis predicted in the model described herein. The experimental strategy includes: (1) Elucidating the molecular mechanisms involved in the function and regulation of ACC deaminase; (2) Creating various derivatives of soil bacteria that either have or lack ACC deaminase activity and comparing the behaviour of these derivatives to wild type strains in root elongation tests.

1.5.1. Isolation and characterization of ACC deaminase genes

Two strains from the existing lab collection of seven PGPR strains (*P. putida* UW1, UW3, *P. fluorescens* CAL1 and *E. cloacae* UW2, UW4, CAL2, CAL3) were chosen for the study. The choice of strains was based on their ability to grow on minimal medium with ACC as a sole source of nitrogen, ACC deaminase assays and SDS-PAGE analysis. The ACC deaminase genes of the two selected strains were then isolated by constructing clone banks using the vector pUC18 and screening the clone banks using either a minimal medium containing ACC as the sole source of nitrogen or a PCR-generated ACC deaminase probe. Positive clones were identified by plasmid isolation followed by DNA hybridization with the same probe. Primers were designed to facilitate the complete DNA sequencing of the ACC deaminase genes as well as any upstream and/or downstream regions. The DNA sequences of

the isolated ACC deaminase genes were compared with the sequences of known ACC deaminase genes.

1.5.2. Manipulation of an ACC deaminase promoter

In order to understand the expression and regulation of ACC deaminase in bacteria, the 5' upstream region of ACC deaminase gene was cloned into promoter-probe vector, pQF70, which carries the promoterless luciferase genes (*luxAB*). Monitoring the expression of the reporter gene helped identify promoter and other regulatory sequences in the upstream regions of the gene. Vital regulatory regions were identified using a series of deletion constructs that clone the different upstream regions of ACC deaminase into the promoterless pQF70. To compare the strength of the putative promoter in different bacteria, the promoter-reporter gene construct was subcloned into a broad-host-range vector and introduced into *E. coli* and selected PGPR and expression of the reporter gene was measured. The effect of various amino acids and ACC analogs on the induction of the ACC deaminase promoter were assessed with the construct.

1.5.3. Assessment of the model with different soil bacteria

Using a broad-host-range vector, the ACC deaminase gene was transformed into various soil bacteria, including those that do not normally have ACC deaminase activity. Canola seeds were inoculated with strains that either have or do not have ACC deaminase activity, in order to evaluate whether PGPR containing ACC deaminase could promote root elongation.

1.5.4. Creation of an ACC deaminase defective mutant

In an effort to assess the importance of ACC deaminase in PGPR, a mutant strain of *E. cloacae* UW4 was constructed that lacks a functional ACC deaminase gene. The mutant was created by targeted gene replacement (gene knockout). After transfer of an inactivated ACC deaminase gene into *E. cloacae* UW4, the ACC deaminase activity was measured, and the ability to promote root elongation in canola seedlings was assessed.

1.5.5. A possible evolution of ACC deaminase

Since the ACC deaminase gene is widely distributed in nature, it is possible that the gene was transferred from diverged soil organisms. To analyze ACC deaminase gene arrangement in genomic DNA, it is necessary to localize the gene on either chromosomal or plasmid DNA, since most PGPR contain large plasmids. Whether or not large plasmid(s) present in both *E. cloacae* UW4 and CAL2 were investigated. On the other hand, it appears that some bacteria contain more than one ACC deaminase-like enzyme which allows the bacteria to grow on minimal medium containing ACC as a sole source of nitrogen. All of clones from a genomic library grown on ACC plates were tested for ACC deaminase activity and the insert DNA fragments were sequenced. After comparison with the known ACC deaminase DNA sequences, at least one clone contained a different sequence suggesting that ACC deaminase activity in bacteria may have evolved from more than one precursor gene.

2. MATERIALS AND METHODS

2.1. Bacterial strains, plasmids and culture conditions

The bacteria strains, vectors and plasmids used in this study are described in Table 1.

Escherichia coli DH5α (Hanahan, 1983) was used as the host cell for the construction and maintenance recombinant plasmids in this study. It is a recombinant-deficient suppressing strain that allows α-complementation with the amino terminus of β-galactosidase encoded in pUC vectors. *E. coli* S17-1 (Simon, 1983), a efficient mobilizing strain, was kindly provided by Dr. T. Charles (University of Waterloo, Canada). All *E. coli* strains were cultivated aerobically in Luria-Bertani (LB) broth (Difco laboratories, Detroit, Mich.) at 37°C. Where required, the media were supplemented with appropriate antibiotics at the final concentrations: ampicillin 100 $\mu g \cdot ml^{-1}$; tetracycline 15 $\mu g \cdot ml^{-1}$; kanamycin 25 $\mu g \cdot ml^{-1}$.

Based on the ability to utilize ACC as a sole source of nitrogen, seven strains from soil samples had been isolated and identified as: *Enterobacter cloacae* UW2, UW4, CAL2, CAL3, *Pseudomonas putida* UW1, UW3, and *Pseudomonas fluorescens* CAL1 (Glick et al., 1995; Shah et al., 1998). Strains UW1-UW4 designate originally found in the Waterloo region of Ontario, Canada associated with the roots of beans, clover, corn, and reeds, respectively. Strains CAL1-CAL3 designate California strains from fields in San Benito, King City, and Fresno, and were associated with oats, tomato, and cotton, respectively. Each of these seven strains as well as *Pseudomonas putida*

Table 1. Bacterial strains and plasmids used in this work

Strain or plasmid	Description	Source or reference
Strains		
E. coli		
DH5α	*F80d/lacZ M15, recA1, endA1, gyr96, thi-1, hsdR17 (rk-, mk+), supE44, relA1, (lacZYA-a rgF)U169*	Hanahan, 1983
CVR292	DH5α strain contains both plasmids pJQ292 and pACYC184	This study
COR292	DH5α strain contains both plasmids pJQ292 and pACLR	This study
COR195	DH5α strain contains both plasmids pJQ195 and pACLR	This study
COR149	DH5α strain contains both plasmids pJQ149 and pACLR	This study
COR46	DH5α strain contains both plasmids pJQ46 and pACLR	This study
N100	*recA, gal* $E^+galT^+galK^-$	Chirikjian, 1981
S17-1	*recA*, chromosomally integrated RP4 derivative	Simon et al., 1983
E. cloacae		
UW2	Wild-type strain grown on ACC agar isolated from clover soil;	Glick et al., 1995; Shah et al., 1998
UW4	Wild-type strain grown on ACC agar isolated from reeds soil; Amp^r, Cm^r	Glick et al., 1995; Shah et al., 1998
CAL2	Wild-type strain grown on ACC agar isolated from tomato soil; Amp^r,	Glick et al., 1995; Shah et al., 1998
CAL3	Wild-type strain grown on ACC agar isolated from cotton soil;	Glick et al., 1995; Shah et al., 1998
P. putida		
UW1	Wild-type strain grown on ACC agar isolated from bean soil; Amp^r, Cm^r	Glick et al., 1995; Shah et al., 1998
UW3	Wild-type strain grown on ACC agar isolated from corn soil; Amp^r, Cm^r	Glick et al., 1995; Shah et al., 1998
GR12-2	Wild-type strain isolated from grasses in the Canadian High Arctic, grown on ACC agar, has PGPR activity	Lifshitz et al., 1986
ATCC 17399	Psychrophile, no ACC deaminase or PGPR activity	ATCC
P. fluorescens		
CAL1	Wild-type strain grown on ACC agar isolated from oat soil; Amp^r, Cm^r	Glick et al., 1995; Shah et al., 1998
ATCC 17400	Psychrophile, no ACC deaminase or PGPR activity	ATCC

Table 1. Bacterial strains and plasmids used in this work (cont.)

R. meliloti 2011	Wild-type, Nod$^+$Fix$^+$ on *Medicago sativa*, Sm^r	Dénarié, 1977
A. tumefaciens C58	Wild-type nopaline strain (pTiC58m, pATC58)	Otten, et al., 1981
Vectors		
pACYC184	Cloning vector with p15A replicon; Cm^r, Tc^r	Chang et al., 1978
pBR322	Cloning vector with ColE replicon; Amp^r, Tc^r	Bolivar et al., 1977
pUC19	High copy number cloning vector carrying *lacZ'* gene; Amp^r	Yanisch, 1985
pUC18 B/BAP	pUC18 linearized with *Bam*HI and dephosphorylated with bacterial alkaline phosphatase (BAP); Amp^r	Amersham Pharmacia
pGEM-T	PCR product cloning vector; Amp^r	Promega
pKO-1	carries a promoterless *lacK* reporter gene, Amp^r	Mckenney et al., 1981
pQF70	pRO1600-derived broad-host-range vector carrying a promoterless *luxAB* reporter gene downstream from multiple cloning sites, Amp^r	Farinha and Kropinski, 1990
pRK2013	Tripartite conjugation-mobilization plasmid; Km^r	Figurski et al., 1979
pRK415	Broad-host-range cloning vector, *lacZ*α IncP; Tc^r	Keen et al., 1988
pSUP5011	pBR325 derivative, Tn5-*mob* ; Nm^r, Tc^r, Cm^r	Simon, 1984
pTC300	carries a 3.7 kb *npt-sacB-sacR* cassette as a *Bam*HI fragment in pUC19; Amp^r	Constructed by T. Charles
Plasmids		
p4U1-4	6.8 kb *Sau*3A partially digested fragment encoding *acd*S from *E. cloacae* UW4 genomic library cloned into pUC18; Amp^r	Shah et al., 1998
p4U5-7	0.8 kb *Sau*3A partially digested fragment encoding *acd*S from *E. cloacae* UW4 genomic library cloned into pUC18; Amp^r	Shah et al., 1998
p2C2	6.5 kb *Sau*3A partially digested fragment encoding *acd*S from *E. cloacae* CAL2 genomic library cloned into pUC18; Amp^r	Shah et al., 1998
pUCACC	*acd*S from *E. cloacae* UW4 inserted into the *Sac*I and *Xba*I sites in vector pUC18; Amp^r	Shah et al., 1998
pJAB4	Tetracycline resistance gene from pBR322 inserted into the *Bst*EII site in the *acd*S gene on pUCACC; Amp^r, Tc^r	This study
pDRDP	The origin of replication was excised from pJAB4, Amp^r, Tc^r	This study

Table 1. Bacterial strains and plasmids used in this work (cont.)

pDAS1	The *npt*(*sac*R+*sac*B) cassette from pTC300 inserted into the *Sca*I site on pJAB4; Tc^r	This study
pDAS2	A 1.8 kb *Bam*HI fragment carrying a *mob* gene inserted into *Sma*I site in pDAS1	This study
pJQ855	pQF70 derivative carrying the 5' 855 bp upstream region of *acdS* subcloned into the promoterless *luxAB* gene, Amp^r	This study
pJQ741	Deleted the upstream region of *acdS* from -855 bp to -741 bp in pJQ855; Amp^r	This study
pJQ722	Deleted the upstream region of *acdS* from -855 bp to -722 bp in pJQ855; Amp^r	This study
pJQ510	Deleted the upstream region of *acdS* from -855 bp to -510 bp in pJQ855; Amp^r	This study
pJQ404	Deleted the upstream region of *acdS* from -855 bp to -404 bp in pJQ855; Amp^r	This study
pJQ292	Deleted the upstream region of *acdS* from -855 bp to -292 bp in pJQ855; Amp^r	This study
pJQ220	Deleted the upstream region of *acdS* from -855 bp to -220 bp in pJQ855; Amp^r	This study
pJQ195	Deleted the upstream region of *acdS* from -855 bp to -195 bp in pJQ855; Amp^r	This study
pJQ149	Deleted the upstream region of *acdS* from -855 bp to -149 bp in pJQ855; Amp^r	This study
pJQ46	Deleted the upstream region of *acdS* from -855 bp to -46 bp in pJQ855; Amp^r	This study
pJQ588	588 bp *Sau*3A fragment subcloned into the *Bam*HI site in pQF70 in sense orientation; Amp^r	This study
pJQ588-op	588 bp *Sau*3A fragment subcloned into the *Bam*HI site in pQF70 in antisense orientation; Amp^r	This study
pRKLUX	5.0 kb *Sac*I-*Pvu*II fragment containing 855 bp upstream in region of *acdS* with *luxAB* gene cloned into the *Bam*HI site pRK415; Tc^r	This study
pACLR	840 bp *Sma*I-*Eco*RV *acdR* fragment from pJQ855 subcloned into the *Sca*I site in the Cm gene in pACYC184; Tc^r	This study
pWA1	4.0 kb *Sau*3A partially digested fragment from *E. cloacae* UW4 genomic library cloned into pUC18, transformants selected on ACC-agar; Amp^r	This study
pWA2	0.8 kb *Sau*3A partially digested fragment from *E. cloacae* UW4 genomic library cloned into pUC18, transformants selected on ACC-agar; Amp^r	This study

GR12-2 (Lifshitz, 1986) (kindly provided by Dr. Gerry Brown, Agrium, Inc. Saskatoon, Canada) were found to have the ability to promote canola seedling root elongation under gnotobiotic conditions (Glick, et al., 1995). *Pseudomonas putida* ATCC 17399 and *Pseudomonas fluorescens* ATCC 17400, obtained from the American Type Culture Collection, were used as negative controls. Neither is reported to have plant growth-promoting activity. Both *Enterobacter* and *Pseudomonas* strains were grown aerobically in tryptic soybean broth (TSB) (Difco laboratories, Detroit, Mich.) at 28°C. *Rhizobium meliloti* 2011 and *Agrobacterium tumefaciens* C58 (kindly provided by Dr. T. Charles [University of Waterloo, Canada]) were also used as control strains for the megaplasmid isolation. Both strains were cultured at 28°C in TY medium (Beringer, 1974), which contained: 5g tryptone, 3g yeast extract, and 0.7g $CaCl_2 \cdot 2H_2O$, pH 7.2 per liter of water. In order to distinguish *Enterobacter cloacae* from *Escherichia coli* following conjugation, a selective medium, i.e. Simmons citrate agar (DIFCO laboratories, Detroit, Mich.), was used.

Minimal medium with ACC as a sole source of nitrogen was used in the ACC induction experiments (section 2.15.2). *E. coli* strains were grown in M9 minimal medium (Miller, 1972), and *Enterobacter* and *Pseudomonas* strains were cultivated in DF salts medium (Dworkin and Foster, 1958) [$w \cdot v^{-1}$]: 0.4% KH_2PO_4, 0.6% Na_2HPO_4, 0.02% $MgSO_4 \cdot 7H_2O$, 0.2% glucose, 0.2% gluconic acid, 0.2% citric acid and 0.2% $(NH_4)_2SO_4$ with trace elements [per liter]: 1 mg $FeSO_4 \cdot 7H_2O$, 10 µg H_3BO_3, 10 µg $MnSO_4$, 70 µg $ZnSO_4$, 50 µg $CuSO_4$, and 10 µg MoO_3, pH 7.2. Since ACC is heat labile, it was prepared by filter sterilization and added just before inoculation or induction (section 2.15.2.).

E. coli N100 was used as a host cell in the galatokinase expression experiments (section 2.15.1). The recombinant plasmid (*galK*$^+$) in this strain was detected on selective MacConkey galactose medium (Difco Laboratories, Detroit, Mic.), and for the galatokinase assay, a minimal medium M63 (Miller, 1972) was used that contained (per liter): KH_2PO_4 13.6g; $(NH_4)_2SO_4$ 2g; $FeSO_4 \cdot 7H_2O$ 0.5mg; pH 7.2. After autoclaving, 1 mL of 1 M $MgSO_4 \cdot 7H_2O$, 10 mL of 20% glucose or galactose and 20 $\mu g \cdot mL^{-1}$ proline were added.

The sources of plasmid vectors were from our laboratory except for plasmid pQF70 which was obtained from Dr. Anne Glover (University of Aberdeen, Scotland), and plasmids pTC300 and pSUP5011 which were kindly provided by Dr. T. Charles (University of Waterloo, Canada).

2.2. Isolation of plasmid DNA

2.2.1. Alkaline lysis miniprep

Plasmid DNA was routinely prepared by the alkaline lysis procedure (Sambrook et al., 1989). An overnight culture inoculated with a single bacterial colony in a rich medium (such as LB for *Escherichia coli* and TSB for *Enterobacter cloacae*) supplemented with the appropriate antibiotics was distributed into 1.5 mL microcentrifuge tubes, and centrifuged at 16,000 x g for one minute to pellet the cells. The supernatant was removed and the pellet was resuspended in 200 µL of solution I (50 mM glucose, 25 mM Tris-HCl pH8.0, 10 mM EDTA pH8.0) and kept at room temperature for 5 min. Next, 200 µL of freshly prepared solution II (0.2 N NaOH, 1% SDS) was added and the tube was inverted several times and kept at room temperature for 5 min. Solution II serves to rupture the cell membrane and denature the

chromosomal DNA while leaving the tightly curled covalently closed circular plasmid DNA intact. The suspension was then neutralized by the addition of 200 µL of ice-cold solution III (3.0 M potassium acetate, pH 4.8). The tube was inverted several times and kept on ice for 5 min. The low pH of solution III causes the SDS-protein complexes to precipitate and the chromosomal DNA strands to renature and become entangled in one another. The mixture was centrifuged at 16,000 x g for 5 min, and 600 µL of the resulting supernatant was transferred into a new microcentrifuge tube. The plasmid DNA was precipitated by adding 420 µL of isopropanol followed by 10 tube inversions. The plasmid DNA was maintained at room temperature for 10 min and then centrifuged at 16,000 x g for another 10 min. The supernatant was removed and the pellet was washed with 500 µL of ice-cold 70% ethanol followed by centrifugation at 16,000 x g for 3 min at 4°C. The ethanol supernatant was removed and the pellet was dried under vacuum for 5 min. After drying, the plasmid DNA was resuspended in 20 µL of TE buffer (10 mM Tris-HCl, pH 8.0; 0.1 mM EDTA, pH 8.0) and 1 µL of RNase solution (10 $mg \cdot mL^{-1}$) and stored at -20°C.

2.2.2. A modified alkaline-lysis/PEG miniprep

The plasmid DNA isolated by this technique can be used as a DNA template for Taq DyeDeoxy terminator cycle sequencing reaction (Applied Biosystems Inc.). A single bacterial colony was inoculated in Terrific broth (LB medium supplemented with 0.017 M KH_2PO_4 and 0.072 M K_2HPO_4) (Tarrof and Hobbs, 1987) in order to obtain a four- to eight-fold increase in the number of bacteria per milliliter of medium, leading to a higher plasmid

yield. The overnight bacterial culture was centrifuged and the pellet was resuspended in 200 µL of GTE buffer (50 mM glucose, 25 mM Tris-HCl pH 8.0, and 10 mM EDTA pH 8.0). The cells were lysed by the addition of a 300 µL of a freshly prepared 0.2 N NaOH and 1% SDS solution, followed by inversion mixing until the solution became clear. The tubes of lysed cells were incubated on ice for 5 min. Adding 300 µL of 3.0 M potassium acetate (pH 4.8) followed by inversion neutralized the lysates, which were then stored on ice for 5 min. The cellular debris was removed by 10 min of room temperature centrifugation, after which the supernatant was transferred into a clean tube. A 1 µL of RNase solution (10 $mg \cdot mL^{-1}$) was added and the tube was incubated at 37°C for 20 min. After the RNase treatment, the supernatant was extracted twice with 400 µL of chloroform. The layers were mixed by hand for 30 sec and then the phases were separated by centrifugation at 16,000 x g for 1 min. The aqueous phase was collected into a clean tube. The plasmid DNA was precipitated by adding an equal volume of 100% isopropanol and the tube was immediately centrifuged at 16,000 x g for 10 min at room temperature. The DNA pellet was washed with 500 µL of ice-cold 70% ethanol and then dried under vacuum for 5 min. The pellet was dissolved in 32 µL of deionized H_2O, and the plasmid DNA was precipitated by adding 8.0 µL of 4 M NaCl and 40 µL of sterile 13% PEG_{8000}. After thorough mixing, the tube was incubated on ice for 20 minutes and the plasmid DNA was pelleted by centrifugation for 15 min at 4°C. The supernatant was carefully removed and the pellet was rinsed twice with 500 µL of ice-cold 70% ethanol. Then the pellet was dried under vacuum for

5 min and resuspended in 20 µL of deionized H_2O or TE buffer, and stored at -20°C.

2.3. Isolation of large plasmid DNA

2.3.1. Large plasmid isolation

Large plasmids have been found in several bacteria (Burkardt and Burkardt, 1984; Hynes et al., 1985) and methods have been developed which permit the isolation of small as well as large plasmids (Hansen and Olsen, 1978). An overnight bacterial culture was diluted 1:20 into 2 mL of TSB medium and incubated at 28°C for 2 to 3 h with shaking. The cells were harvested by centrifugation at 16,000 x g for 2 min and resuspended in 40 µL of TE buffer. A lysis buffer (4% SDS in TE [pH 12.4]) was freshly prepared and 0.6 mL was added into a centrifuge tube. The cell suspension was then transferred into the lysis buffer and mixed well. The suspension was incubated at 37°C for 20 min to achieve full lysis of the cells and afterward the suspension was neutralized by adding 30 µL of 2.0 M Tris-HCl (pH 7.0). The tube was inverted slowly until a change in viscosity was noted. Chromosomal DNA was precipitated by adding 240 µL of 5 M NaCl and the tube was incubated on ice for 4 h to complete the removal of the chromosomal DNA. Sediment debris was pelleted in a microcentrifuge for 10 min and the supernatant was transferred into another centrifuge tube. The plasmid DNA was precipitated by adding 550 µL of isopropanol to the supernatant and the tube was placed at -20°C for 30 min. After centrifugation for 5 min, the tube was dried under vacuum. The DNA was resuspended in 30 µL of TE buffer. For restriction endonuclease analysis the plasmid isolated by this method was

further purified as followed: the DNA pellet was dissolved in 100 µL of 0.01 M Tris-HCl (pH 8.0) and then 100 µL of phenol and 100 µL of chloroform were added. The mixture was centrifuged at 12,000 x g for 5 min and the upper aqueous phase was transferred into an another microcentrifuge tube. The plasmid DNA was precipitated with 2 volumes of ice-cold 95% ethanol, washed with 70% ethanol and dissolved in 30 µL of TE buffer.

2.3.2. In-well lysis

Large plasmid DNA can be directly detected based on the Eckhardt (1978) method as modified by Hynes et al. (1985; 1986). This technique is rapid, does not require large volumes of cells and allows large plasmid DNAs to be visualized following agarose gel electrophoresis of a crude lysate without any prior isolation. The strict lysis conditions designed by in-well lysis can release large plasmid DNA from the chromosome. It also selectively removes fragments of sheared chromosomal DNA. Since most of the chromosomal DNA remains intact after the lysis, it remains in the well, and therefore less than 5% of the total chromosomal DNA appears in the gel as a band of sheared DNA. In this study, different bacterial strains, such as *Enterobacter, Pseudomonas, Agrobacterium* and *Rhizobium* were grown at 28°C in a nutrient medium (e.g. LB medium) overnight with shaking. They were then diluted and incubated in a minimal medium for an additional 3 h (to OD_{600nm} 0.3-0.4). The bacterial cultures were immediately set on ice. From each strain 1.0 mL of culture was centrifuged at 16,000 x g for 3.5 min and the supernatants were removed. The pellets were resuspended in 500 µL of 0.2% sarkosyl. The tubes were inverted gently and centrifuged for another 3.5 min.

The resulting pellets were carefully resuspended (without pipetting) in 200 µL of loading buffer consisting of 10% sucrose, 100 $\mu g \cdot mL^{-1}$ lysozyme, and 10 $\mu g \cdot mL^{-1}$ RNase in 1x Tris-Borate buffer (TBE) (5x TBE stock contained [per liter]: Tris·base 54 g, boric acid 27.5 g and 10 mM EDTA) and loaded 30 µL into a well of a 0.65% horizontal agarose gel with P1000 micropipette. The gel was prepared in 1X TBE buffer containing 1% SDS. Electrophoresis was carried out at 30V for 30 minutes or until the cells had been lysed and then at 100V for 4-6 h. After electrophoresis, the gel was stained in 1 $\mu g \cdot mL^{-1}$ ethidium bromide solution for 15 min and destained in water for 5 min. The gel was visualized on an ImageMaster® VDS (Pharmacia Biotech) and the image was documented by photography.

2.4. Isolation of total genomic DNA (Meade et al., 1982)

Bacteria were grown in TSB (*Enterobacter cloacae*) or LB (*Escherichia coli*) medium overnight before the cells were distributed into 1.5 mL microcentrifuge tubes and harvested by centrifugation at 16,000 x g for 2 min and washed with 500 µL of TES buffer (10 mM Tris-HCl, 1 mM Na_2EDTA, 50 mM NaCl, pH 8.0). The bacterial cells were ruptured by incubation with 50 µL of a freshly prepared lysozyme solution (2 $mg \cdot mL^{-1}$ in TE) at 30°C for 20 min followed by the addition of 50 µL of sarkosyl/pronase (10% sarkosyl in TE containing 5% pronase) and further incubation at 37°C for 1 h. Lysis became evident as an increase in the viscosity of the suspension. Next, 70 µL of 3 M sodium acetate was added and the suspension mixed gently. The lysate was then extracted twice with 250 µL of a phenol:chloroform (1:1) mixture and twice with 250 µL of chloroform:isoamyl alcohol (24:1). In each

extraction, the tubes were inverted gently for 15-30 min and then centrifuged at 12,000 x g for 10 min. The supernatant from each centrifugation was transferred into a clean microcentrifuge tube. The DNA in the supernatant was precipitated with an equal volume of isopropanol. After centrifugation, the supernatant was discarded and the DNA pellet was washed twice with 70% ice-cold ethanol, dried under vacuum and resuspended in 40 μL of TE buffer.

2.5. DNA manipulation

2.5.1. Phenol-chloroform DNA purification

Crude DNA obtained from alkaline lysis as well as DNA that had been subjected to restriction endonuclease treatment was purified using phenol-chloroform extraction procedures. Each DNA sample that was dissolved in TE buffer was diluted and treated with an equal volume of phenol-chloroform and vigorously vortexed for 30 sec. The suspension was centrifuged at 16,000 x g for 2 min and the top layer was transferred into a clean microcentrifuge tube using a micropipette. The DNA solution was then added with 0.1 volume of 3 M sodium acetate (pH 5.2) followed by 2.0-2.5 volumes of ethanol to precipitate the DNA. The precipitating DNA was kept at -20°C for at least 30 min and then centrifuged at 16,000 x g for 10 min at room temperature. The resulting pellet was washed with ice-cold 70% ethanol and centrifuged at 16,000 x g for 3 min at 4°C. The ethanol supernatant was discarded and the pellet was dried under vacuum for 5 min and then resuspended in a volume of TE buffer.

2.5.2. Restriction endonuclease digestion

For a single enzyme digestion, the DNA sample was incubated with the restriction enzyme and its buffer according to instructions from the manufacturer. For a double enzyme digestion, the restriction buffer was chosen according to the table in the Roche Molecular Biochemicals catalogue. The digestion was carried out in a total volume of 30 µL that consisted of 100 ~ 1000 ng of the DNA sample, 3 µL of the appropriate 10X buffer, 2 units of the enzyme. After mixing the digestion mixture was incubated for at least 1 h or overnight at 37°C with the exception that *Sma*I digestion was carried out at 25°C. Once digestion was complete, the enzymes were inactivated by phenol purification or by heating for 10 min at either 65°C or 85°C depending on the manufacturer's instructions.

2.5.3. DNA extraction from agarose gels

DNA was extracted from agarose gels using either the GENECLEAN® II kit (Bio 101, Inc., La Jolla, Calif., USA) or the DNA Extraction kit (MBI Fermentas Inc., Ontario, Canada). Restriction endonuclease digested DNA was loaded onto an agarose gel and run long enough to allow sufficient separation between bands. DNA bands were visualized using an ultraviolet light, after which the band of interest was cut out of the gel using a razor blade. The excised band was placed into a 1.5 mL microcentrifuge tube and weighed. The agarose was dissolved by adding three volumes of saturated NaI solution followed by incubation at 50°C for 10 min, or until the agarose was completely dissolved. Five µL of resuspended glassmilk or silica powder suspension was added to the DNA-containing NaI solution. The tube was

inverted gently several times and then placed on ice for at least 5 min to allow binding of the DNA to the matrix. The glassmilk or silica powder/DNA complex was pelleted by centrifugation for 5 sec, and the supernatant was discarded. The pellet was then washed three times with 500 µL of ice-cold wash buffer (provided by the manufactures). During each wash, the pellet was resuspended gently in the wash buffer, and the suspension was centrifuged for 5 sec. After the supernatant was removed from the last wash, the tube was centrifuged briefly and any remaining liquid was removed with a micropipette. To elute the DNA into TE buffer, the pellet was resuspended in 5 µL of TE buffer and incubated at 50°C for 5 min. After incubation, the tube was centrifuged for 1 min, and the supernatant was transferred into a fresh tube. To remove small matrix particles that may have been transferred with the supernatant, the tube was spun again for 1 min, and the supernatant was then transferred into a new tube.

2.5.4. Klenow fill-in reaction

E. coli Klenow polymerase, along with a mixture of dNTPs, was used to fill in the DNA overhangs produced by 5′ overhang-producing restriction endonucleases (Maniatis et al., 1982; Sambrook et al., 1989). Digested DNA fragments were first purified using the phenol-chloroform purification procedure (section 2.5.1), and dissolved in 9 µL of TE buffer. The DNA solution (50 ng/µL) was incubated at 37°C, and 0.5 µL Klenow DNA polymerase (2.5 units/µL) and 0.5 µL 1x Klenow buffer (20 mM Tris-HCl, pH 8.0, 100 mM $MgCl_2$) were added. The mixture was incubated at 37°C for 3 min before 1 µL of the dNTP mix (0.25 µM each dNTP) was added followed

by 10 min of incubation at 37°C. The reaction was stopped by adding EDTA to 10 mM final concentration and heating at 75°C for 10 min, and then the mixture was stored at 4°C or used directly for DNA ligation.

2.5.5. Dephosphorylation of plasmid DNA

Alkaline phosphatase catalyzes the removal of 5'-phosphate residues from DNA vector fragments to prevent the vector self-ligation (Sambrook et al., 1989). Before the plasmid vector was treated, it was digested with the appropriate restriction enzyme and then diluted to 50 µL in the same buffer. After inactivation of the restriction enzyme, an alkaline phosphatase (Amersham Pharmacia) solution was diluted 20-fold and 0.1 unit was added. The reaction was incubated at 37°C for 30 min and once the reaction was completed, the alkaline phosphatase was inactivated by heating it at 85°C for 15 min. The dephosphorylated vector can be used directly for ligation.

2.5.6. DNA ligation

Ligation of digested DNA fragments was performed using T4 DNA ligase according to the manufacturer's instructions. If DNA directly from the digestion solution was used for ligation, the restriction enzymes had to be inactivated. If DNA was isolated from an agarose gel, it was first purified with phenol-chloroform. Successful ligation was obtained when a molar ratio of insert DNA to vector DNA was kept at three to one. During a blunt-ended DNA fragment ligation, 2 µL of 50% PEG and 4 units of T4 DNA ligase (2 units for cohesive-end) to 20 µL of ligation mixture was added. The ligation mixture contained 1x ligation buffer (66 mM Tris-HCl, 5 mM $MgCl_2$, 5

mM dithiothreitol, 1 mM ATP, pH 7.5) and was incubated at room temperature at least 1 h for blunt-end ligation or overnight at 16°C for cohesive-end ligation. For a PCR product ligation, the PCR product was purified from a gel and a vector pGEM®-T (Promega, Madison, Wis., USA) was used. Since *Taq* polymerase usually adds a single adenosine to the 3′ ends of PCR products (Mezei and Storts, 1994), the pGEM-T vector is designed to add a 3′ terminal thymidine-overhang at the insertion site. This improves the efficiency of ligation of a PCR product into the plasmid by preventing recircularization of the vector and providing a compatible overhang for PCR products. After ligation, the T4 DNA ligase was inactivated by heating at 65°C for 10 min. Inactivating the enzyme may increase the number of transformants by two orders of magnitude (Michelsen, 1995). For electrotransformation, it was necessary to extract the ligated DNA with chloroform and precipitate it from the reaction mixture with ethanol. The precipitated DNA was dissolved in deionised water.

2.6. Construction of genomic DNA libraries

Isolated total genomic DNAs from *Enterobacter cloacae* UW4 and CAL2 were partially digested with restriction endonuclease *Sau*3AI. The digestion conditions were controlled such that the DNA was digested into fragments between 3 and 10 kb. The vector, plasmid pUC19, was digested to completion with restriction endonuclease *Bam*HI and subsequently treated with alkaline phosphatase to prevent its recircularization. An alternative vector, pUC18 *Bam*HI/BAP (Amersham Pharmacia Biotech, Quebec, Canada) was purchased and used directly for the genomic library construction. The partially digested

DNA fragments were ligated with *Bam*HI-cut dephosphorylated plasmid pUC19 or pUC18 *Bam*HI/BAP. After ligation, the ligation reaction mixtures containing different genomic DNA fragments were transformed into *E. coli* DH5α cells (section 2.11.1). The transformants were plated on LB medium containing 100 $\mu g \cdot mL^{-1}$ ampicillin. In order to select for the presence of inserts, 40 µL of X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) from a 20 $mg \cdot mL^{-1}$ stock in dimethylformamide, and 4 µL of IPTG (isopropylthio-β-D-thiogalactoside) from a 200 $mg \cdot mL^{-1}$ stock, were spread over the surface of LB-ampicillin plates 30 min prior to use. The plates were incubated overnight at 37°C. The white colonies that represented the clones containing genomic DNA inserts were selected and re-plated on LB-ampicillin plates for additional screening.

2.7. Screening of the genomic libraries

2.7.1. Screening by protein activity

Colonies from LB-ampicillin plates were replicated on M9-ACC plates, which contained M9 minimal medium with 3 mM ACC as the sole nitrogen source, and incubated at 37°C for 2 days. Only the clones that contained ACC deaminase could use ACC as a nitrogen source and grow on M9-ACC plates. The clones obtained from the ACC-plates were purified as a single colony, and the plasmids from each clone were isolated and digested with restriction enzymes to confirm the presence of an insert.

2.7.2. *In situ* colony hybridization

Colonies from both *E. cloacae* UW4 and CAL2 libraries were transferred from the plates onto nylon membranes (Gelman Sciences, Ann Arbor, MI., USA). The membranes were marked with the proper orientation and were used to overlay the colonies on the plates. When the membrane became wet, it was gently lifted free of the plate. The bacteria on the membrane were lyzed by soaking the membrane in a 10% SDS solution for 2 min. The released DNA was denatured with 0.5 N NaOH and 1.5 M NaCl for 2 min, and then neutralized with 1 M Tris-HCl (pH 7.5) and 1.5 M NaCl for 5 min. Cell debris was removed by rinsing the membranes thoroughly with 2X SSC solution (0.3M NaCl, 0.03M Na-citrate). The DNA was cross-linked to the membrane by exposing the wet membrane to 150 mJ of UV light (GS Gene linker™, UV Chamber, Bio-Rad Laboratories; kindly provided by Dr. J.J. Heikkila), and then hybridized with either ^{32}P- or DIG-labeled DNA probes (section 2.12.). The colonies that contained DNA that hybridized to the probe were selected and grown on another LB-ampicillin plate for secondary screening. Finally, plasmids were isolated from the positive colonies and digested with appropriate restriction endonucleases. The inserts from these plasmids were further characterized by Southern hybridization (section 2.12.).

2.8. DNA sequence analysis

Preparation of DNA for sequencing is followed by the instructions from the MOBIX Central Facility, McMaster University, Hamilton, Ont., Canada. Plasmid DNA was isolated by either the alkaline lysis-PEG miniprep method (section 2.2.2.) or the alkaline lysis miniprep method (section 2.2.1.).

In the latter case the isolated plasmid DNA was further purified using phenol-chloroform (section 2.5.1.). With these preparations it is important to ensure that no salt or organic compounds are in the final product. Removal of salt and organic compounds is facilitated by a number of washes with 70% ethanol and the final DNA pellet is resuspended in water as opposed to TE buffer. The concentration of the DNA used for sequencing was kept higher than 200 $ng \cdot \mu L^{-1}$, except for chromosomal DNA walking where the concentration of genomic DNA was usually not higher than 300 $ng \cdot \mu L^{-1}$ and sequenced with specifically designed primers (higher Tm). The primer design for both DNA sequencing and PCR is described in section 2.9.

DNA sequencing was performed at the MOBIX Central Facility, McMaster University, Hamilton, Ont., Canada. Sequencing reactions utilized the modified Taq-FS enzyme from Perkim-Elmer with fluorescently labeled dideoxy-terminator chemistry (Prober et al., 1987). The sequencer, an Applied Biosystems 373A Stretch, was used for running the gel and detecting the fluorescent bands. Sequence data was analyzed using the Macintosh program DNA Strider v. 1.2 (Marck, 1988) and similar sequences were identified from the GenBank databases, e.g. NBCI (Benson, et al., 1998), EMBL (Stoesser, et al., 1997) and DDBJ (Tateno, et al., 1998), by use of the BLAST network service and BLOCK SEARCH program (Henikoffs', 1991).

2.9. **DNA amplification by PCR** (Rashtchian, 1995)

The polymerase chain reaction (PCR) was performed in a Perkin Elmer Cetus DNA thermal cycler using synthetic oligonucleotides as primers that were synthesized by MOBIX, McMaster University, Hamilton, Ont. To

amplify the DNA fragments, bacterial cells or extracted DNA were used as templates. Each PCR reaction mixture (in a 0.5 mL microcentrifuge tube) contained: 1X PCR buffer, 0.2 mM dNTPs, 0.2 pmol primers, 100 pg~1 ng plasmid DNA or one bacterial colony, and 3 units of either *Taq* DNA polymerase or Expand™ High Fidelity PCR system (Roche Molecular Biochemicals, Quebec, Canada). The Expand™ High Fidelity PCR system is composed of a mixture containing thermostable *Taq* DNA and PWO DNA polymerase. PWO DNA polymerase has 3'-5' exonuclease proofreading activity allowing a 3-fold increase in the fidelity of DNA synthesis compared to *Taq* DNA polymerase. After the reaction mixture was added, each PCR reaction tube was vortexed and centrifuged briefly, and 30 µL mineral oil was added to the top of the mixture. Amplification was started from denaturation at 94°C for 5 min, and then performed with thirty cycles consisting of 1 min at 94°C, 1 min at 52°C and 2 min at 72°C. Once this was completed, the reaction proceeded to 72°C for 10 min followed by cooling to 4°C. For fragments less than 1.0 kb, *Taq* DNA polymerase was used; for fragment greater than 1.0 kb, the Expand High Fidelity PCR system was used with extension occurring at 68°C for 3 min. For bacterial cell amplification, one bacterial colony from an agar plate was added along with 1 µL of 0.1% sterile Tween 20. The oligonucleotide primers used in the experiments were as follows:

(1) AB7955 5- CTTCACAGGCGGCTTTCCAGC-3'

(2) AB7986 5'-GCCTCGGCATGCTGCCATGG-3'

(3) AB9647 5'-CGACTCTAGAGGATCCTGAGAC-3';

(4) AB9648 5'-CGATTCAGGTTCTAGACGCTGTTCC-3';

(5) AB11442 5'-CCATTCCCGCATCCAGTGCTTC-3';

(6) 5'-CCTTGAAGGGCATCAGCATGG-3'

(7) 5'-CGCTGTTCCTTGATTGTTGTG-3'

(8) 5'-CGGGCTGCATGGAATGCCACG-3'

Factors that were considered in designing oligonucleotide primers include (Dieffenbach et al., 1995): (1) primers were 19 to 22 nucleotides long; (2) the optimal G+C content was 50% with no more than 12 G+C; (3) in all cases one or two G or C were used at the 3' end to provide an anchor for the site of primer extension; (4) an attempt was made to minimize the possible extent of intra- and inter- primer homology (Pallansch et al., 1990).

2.10. DNA deletion

The DNA 5' upstream region of the ACC deaminase gene was cloned into the multiple cloning site of promoter-probe vector pQF70 to construct deletions from 5' end using the Promega Erase-a-Base® System kit. The system is based on the procedure developed by Henikoff (1984), in which exonuclease III (ExoIII) is used to specifically digest insert DNA from a 5' protruding or blunt end restriction site but not a 4-base 3' overhanging end. The plasmids of interest were isolated using the alkaline lysis miniprep method (section 2.2.1.), and purified with phenol-chloroform (section 2.5.1.). The pellet was dissolved in water, and 2 M sodium acetate (pH 4.0) and 2 M NaCl were added to final concentrations of 50 mM and 75 mM, respectively. An equal volume of acid-phenol (pH 4.1) (prepared by equilibration of phenol [pH 5.2] with 50 mM sodium acetate [pH 4.0] several times) was added and mixed thoroughly to reduce the amount of nicked plasmid to less than 20% present in the starting preparation. After centrifugation at 12,000 x g for

5 min, the aqueous phase was recovered. Two or three extractions were needed to remove most of the nicked and linear DNA, after which the closed circular DNA was extracted with 0.05 volume of 1 M Tris-HCl (pH 8.6) and 1 volume of chloroform:isoamyl alcohol (24:1). The aqueous phase was recovered and the DNA was precipitated again in 0.1 volume of 2 M NaCl and 2 volumes of ethanol, washed with ice-cold 70% ethanol and dried under vacuum. Finally, the DNA (30 μg) was dissolved in 50 μL of TE buffer. The configurations of the plasmid DNA were checked on an agarose gel. To initiate deletion, about 10 μg of the closed circular DNA was digested with two different restriction enzymes: *SacI* that generated a 3′ protruding end resistant to Exo III, and *SmaI* that left a blunt end adjacent to the insert from which deletion was to proceed. Since Exo III is strongly inhibited by NaCl (Promega, 1989), the plasmid DNA was extracted with phenol-chloroform and precipitated with ethanol following restriction digestion. The DNA pellet was then resuspended in 10 μL of TE buffer, and 1 μL of this DNA was run on an agarose gel to estimate its concentration. Five μg of DNA was added to 6 μL of the 10X Exo III buffer and adjusted with water to a volume of 60 μL. Based on the expected Exo III digestion rate of about 80 $bp \cdot min^{-1}$ at room temperature, the deletion series was set up for 10 time points at 45 sec intervals each time point. Ten microcentrifuge tubes were placed on ice and each tube corresponded to one time point. To begin, 7.5 μL of S1 nuclease mixture (which consisted of 86 μL sterile water, 13.5 μL S1 buffer and 30 units of S1 nuclease) was added into the ten tubes. A 2.5 μL DNA sample (from the 60 μL) was transferred into an S1 tube as a zero time control. Deletion digestion started by adding 2 μL (500 units) of Exo III into the DNA sample

(57.5 µL) and mixed as rapidly as possible. Every 45 seconds, a 2.5 µL DNA sample was removed and placed into an S1 nuclease tube on ice with pipetting up and down briefly to mix. After all the samples were taken, the tubes were transferred to room temperature for 30 min. During this time, S1 nuclease cut the single strand that was produced after Exo III digestion. To stop the reaction, 1 µL of S1 nuclease stop buffer (0.3 M Tris base and 0.05 M EDTA) was added to each tube and the tubes were heated at 70°C for 10 min. The DNA was then precipitated by adding 0.3 volume of 7.5 M ammonium acetate and 2 volumes of 100% ethanol. After centrifugation, the supernatant was removed and the DNA pellet was washed with ice-cold 70% ethanol, dried under vacuum and resuspended in 9 µL of TE buffer. All tubes for each time point were transferred to 37°C with 1 µL of Klenow mix (which consisted of 15 µL Klenow buffer and 5 units of Klenow DNA polymerase), and the tubes were incubated at 37°C for 3 min, followed by the addition of 1 µL of the dNTP mix (0.25 µM each). The tubes were incubated for an additional 5 min at 37°C and heated at 65°C for 10 min to inactivate the enzyme. For ligation, 40 µL of Ligase mix (consisting of 395 µL deionized water, 50 µL Ligase 10X buffer, 50 µL 50% PEG, 5 µL 100 mM DTT and 4 units of T4 DNA Ligase) was added to each tube. The ligation tubes were incubated at room temperature for at least 1 h, then 10 µL of the ligation products from each time point were transformed into *E. coli* DH5α competent cells (section 2.11.1.).

2.11. Introduction of foreign DNA into bacterial cells

2.11.1. $CaCl_2$ mediated transformation

The plasmids constructed in all experiments were first transformed into and maintained in *E. coli* DH5α competent cells. The competent cells were prepared based on the Maniatis et al. (1982) method. A flask containing 30 mL of LB medium was inoculated with 300 µL of overnight culture and incubated at 37°C until the OD600nm of the culture rose to 0.4 (about 3.5 h). After shaking, the cells were chilled on ice for 2 h and then collected by centrifugation (3000 x g; 10 min; 4°C). The pellet was resuspended gently in 1~2 mL of ice-cold Trituration buffer (Promega) (which consisted of 100 mM $CaCl_2$, 70 mM $MgCl_2$, and 40 mM sodium acetate [pH 5.5], freshly prepared), and then diluted to 25 mL with the same buffer followed by standing on ice for 45 min. After chilling, the treated cells were centrifuged at 1,800 x g for 10 min at 4°C and then gently resuspended in 2 mL of ice-cold Trituration buffer on ice. To prepare the cells for cold storage, 80% glycerol was added with mild swirling to a final concentration of 15% ($v \cdot v^{-1}$). The competent cells were then distributed into 200 µL aliquots, transferred to 1.5 mL microcentrifuge tubes, quick-frozen in liquid nitrogen, and stored at -80°C. Before transformation, the required aliquots of competent cells were thawed on ice. For each transformation, 5-10 µL of ligation product or isolated plasmid DNA (about 500 ng of DNA) was added to the competent cells, mixed gently, and incubated for 30-60 min on ice. The mixture was heated in a 42°C water bath for 45-90 sec (for *E. cloacae*, heat shock was performed at 42°C for 5 min) (Berry and Kropinski, 1986) and then placed on ice for 2 min. To recover the transformed competent cells, 800 µL of SOC medium (per liter: 10 g tryptone, 5 g yeast extract, 5 g NaCl, 10 mM $MgSO_4$ and 10 mM $MgCl_2$, pH 7.0 and 20 mM sterilized glucose was added after autoclaving) was added and

warmed to 37°C for 5 min before shaking for 1 h at 37°C (for *E. cloacae*, recovery was performed for 2 h with shaking or overnight without shaking at 30°C) (Berry and Kropinski, 1986). The recovered cells were plated on selective medium containing the appropriate antibiotic and incubated overnight at 37°C (30°C for *E. cloacae*, strains).

2.11.2. Electrotransformation

Electrocompetent cells were prepared by washing with ice-cold sterile water (Dower et al., 1988). To prepare the cells, 250 mL of nutrient medium was inoculated with 2.5 mL of an overnight culture and grown until an OD600nm of 0.4 was reached. At which point, the cells were quickly harvested by pouring the culture into chilled centrifuge tubes and placing them on ice for 10 min. The culture was then centrifuged in a Sorval RC5C centrifuge using a prechilled GSA rotor (4°C) at 6,000 x g for 15 min. The supernatant was poured off and the bacterial pellet was resuspended in a few milliliters of the ice-cold MilliQ water. After resuspension, the centrifuge tube was filled with ice-cold MilliQ water, mixed and centrifuged again. As above, the supernatant was discarded, and the pellet was resuspended in a small amount of water. The tubes were then half-filled with water and spun again. Once again, the supernatant was discarded, and the pellet was resuspended and centrifuged. Finally, the cells were resuspended in 5 mL of ice-cold 10% glycerol and centrifuged in an SS34 rotor at 6,000 x g for 15 min. The cells were resuspended in 800 µL of ice-cold 10% glycerol and then distributed in 40 µL aliquots to chilled 500 µL microcentrifuge tubes and quickly frozen in liquid nitrogen before being stored at -80°C. Before electroporating, the

aliquots of electrocompetent cells were removed from the -80°C freezer and allowed to thaw on ice. The cuvettes and the cuvette holder were also placed on ice. After the cells were thawed, the Electroporator (Bio-Rad Gene Pulser™, kindly provided by Dr. B.A. Moffatt) was turned on and the conditions were set at 2.5KV, 25 μF capacitance and 200 ohms. The DNA sample, dissolved in water rather than TE buffer, was added to the cells, mixed gently and left briefly on ice. The mixture was then transferred into a chilled cuvette. Any water or condensation on the cuvette or cuvette holder was wiped off and the cuvette was tapped on the bench to dislodge any bubbles. The cuvette was then placed into the sliding holder and both were placed into the electroporator. The cells were then pulsed at the set voltage by pressing the two red buttons simultaneously and holding them down until the buzzer sounded. Immediately after the pulse, the cuvette was removed and 1 mL of SOC medium was added. The suspension was mixed by inverting the cuvette and then transferred into a sterile growth tube. The cells were incubated at 37°C (or 30°C for *Enterobacter*) with shaking for 1 h and then plated on selective plates. After overnight incubation, the colonies grown on the selective plates were further purified and identified.

2.11.3. Tripartite conjugation (Simon et al., 1983)

All three organisms, a donor cell, a cell containing a helper plasmid and a recipient cell, were grown separately overnight in nutrient medium with appropriate antibiotics. One microcentrifuge tube was used to pellet all of the three organisms one by one in the same volume (or three times volume from recipient), and after each centrifugation the cell pellet was washed with

saline to remove antibiotics. The pellets containing all three organisms were gently resuspended in 75 µL of saline and the resuspension was dropped on a sterile filter membrane which was placed on a nutrient medium plate. The plate was incubated at 30°C for 24 h to allow mating to occur. The cell mixture with the filter was then removed from the plate and transferred into a sterile microcentrifuge tube that contained 1 mL saline solution. After vortexing, the cells were removed from the filter into the saline and 100 µL aliquots of the suspension were plated onto selective medium containing appropriate antibiotic(s). Both donor and recipient cells were also plated on nutrient agar and selective agar as a control for spontaneous resistant mutant and/or experimental errors.

2.12. Southern hybridization

2.12.1. **Southern blotting** (Sambrook, 1989)

DNA in the gel was photographed with a fluorescent ruler on the Imagemaster® VDS (Amersham Pharmacia Biotech., Quebec, Canada). Two pieces of Whatman 3MM paper and a piece of nylon membrane (Bio-Rad Laboratories, Mississauga, Ont) were cut to exactly the same size as the gel and the latter was soaked in water. Another two pieces of Whatman 3MM paper were cut longer and wider than the gel as a bridge and soaked in transfer solution (0.4N NaOH) that also served to denature the DNA during the transfer (personal communication with Dr. S. Shah). The wetted bridge paper was placed on a support and connected to a reservoir of transfer solution. The air bubbles were removed by rolling a glass test tube over the surface. The gel was placed upside down on the bridge paper and the

presoaked membrane was placed on top. Two pieces of Whatman 3MM paper exactly the same size as the gel were soaked in transfer solution and then placed on top of the membrane, and the air bubbles were removed gently. A few pieces of paper towel cut to the same size as the gel were immediately placed on the top covering the Whatman 3MM paper. To prevent short-circuiting of fluid between the towels and the bridge paper, the gel was surrounded by a water-tight border of parafilm. A stack of paper towels (5-8 cm high) were placed on top, and then a glass plate was put on top of the towels and weighted down with a 500 g weight. After an overnight transfer, the gel with the membrane were turned over and the position of the wells were marked. The membrane was then removed, soaked in 2X SSC (0.3 M NaCl plus 0.03 M Na-citrate) solution for 2 min and placed on a piece of filter paper. The DNA was cross-linked to the membrane by exposing it to a UV light with 150 mJ (GS Gene linker™, UV Chamber, Bio-Rad Laboratories), and then hybridized with ^{32}P- or DIG-labeled DNA probes.

2.12.2. DNA labeling

2.12.2.1. ^{32}P-radioactive random primer labeling

Probes were radiolabeled using the random primer labeling kit (Roche Molecular Biochemicals, Quebec, Canada). The DNA probe (at least 25 ng) was linearized and denatured for 5 min in a boiling water bath and immediately cooled on ice. In a separate microcentrifuge tube the reaction mixture was added as follows: 2 µL of hexanucleotide mixture (10x concentrated hexanucleotide mixture); 1 µL of mixed dNTPs (0.5 mM each); 1 µL of BSA (1 $mg \cdot mL^{-1}$); 10 µL of denatured probe; 5 µL [α–^{32}P]-dCTP (3000

Ci·mM^{-1}) (ICN, Costa Mesa, CA., USA) and 1 µL of Klenow polymerase (3 units·µL^{-1}). The probe mixture was incubated at 37°C for approximately 3 h and the labeling reaction was stopped by adding 1 µL of 0.5 M EDTA. The labeled probe was separated from unlabeled and labeled nucleotides by diluting the reaction mixture into 100 µL volume with TE buffer and passing it through a 1 mL Sephadex G50 column. The probe was eluted from the column with TE buffer and eluted fractions were monitored with a Geiger counter. The fractions with higher counts were collected and used as probes for hybridization.

2.12.2.2. DIG-nonradioactive random primer labeling

Non-radioactive labeling employed the DIG random primer labeling kit (Roche Molecular Biochemicals, Quebec, Canada). Linear probe DNA (500 ng to 3 µg) in 15 µL of H_2O was boiled in a water bath for 10 min and quickly transferred to a wet ice bath. Next, 2 µL of hexanucleotide mix (10 x concentrated hexanucleotide mix), 2 µL of dNTP labeling mixture (10 x concentrated the mixture containing 1 mM dATP, 1 mM dCTP, 1 mM dGTP, 0.65 mM dTTP and 0.35 mM DIG-11-dUTP) and 1 µL of Klenow polymerase (2 units·µL^{-1}) were added and incubated at 37°C for at least 1 h. Finally, 2 µL of 200 mM EDTA (pH 8.0) was added to terminate the reaction.

2.12.3. Southern hybridization

2.12.3.1. Hybridization with ^{32}P-labeled probe (GelmanSciences)

The membrane containing denatured DNA was soaked in prehybridization fluid (BEPS buffer: 1% BSA; 1 mM EDTA; 0.5 M

Na-phosphate, pH 7.2 and 7% SDS, dissolved at 65°C) and incubated at 65°C for 30 min with gentle agitation. Before the labeled probe was added into BEPS buffer, the probe was denatured in a boiling water bath for 5 min and quickly chilled on ice. The prehybridization fluid was replaced with hybridization solution (BEPS buffer containing the denatured labeled probe) and hybridization was allowed to continue for 16-20 h at 65°C with constant shaking. After hybridization, the hybridization solution was transferred to a plastic tube and kept at -20°C for reuse and the membrane was washed twice with a solution of 2X SSC and 0.1% SDS at 65°C for 15 min. Further washing was performed twice with a solution of 1X SSC and 0.1% SDS at 65°C for 20 min and twice with a solution of 0.1X SSC and 0.1% SDS at 65°C for 20 min. A hand held monitor was used to give an indication of the decrease in radioactivity of the background. Finally, the membrane was placed on a sheet of Whatman 3MM paper, wrapped with Saran Wrap and exposed to Kodak X-OMAT-AR film for autoradiography. The orientation of film was marked with a pen at a corner. Following exposure, the film was developed in a dark room by first soaking the film in GBX developer for 5 min, followed by a 15 sec rinse in water and 15 min in Kodak fixer (kindly provided by Dr. B.A. Moffatt). After developing, the film was washed in running water for 5 min and air-dried.

2.12.3.2. Hybridization with DIG-labeled probe (Roche Molecular Biochemicals, Quebec, Canada)

The membrane was prehybridized in a hybridization tray with 50 mL of prehybridization buffer (5X SSC, 0.1% [$w \cdot v^{-1}$] N-lauroylsarcosine, 0.02%

[$w \cdot v^{-1}$] SDS, 1% blocking reagent) for at least 1 h at 65 °C. The labeled probe was boiled for 10 min, quickly cooled on ice and spun down. The hybridization buffer was made by diluting 20 µL of labeled probe in 10-15 mL of prehybridization buffer. The prehybridization buffer was removed and hybridization buffer was added to the membrane. Hybridization was performed at 65°C overnight with constant agitation. After the hybridization, the buffer containing probe was collected and stored at -20°C for reuse. The membrane was washed twice with 2X SSC and 0.1% SDS solution at room temperature for 15 min and twice with 0.1X SSC and 0.1% SDS solution at 68°C for 15 min. The membrane was rinsed briefly in maleic acid buffer (0.1 M maleic acid, 0.15 M NaCl, pH 7.5) to remove SDS. Next, the membrane was incubated in 100 mL blocking solution at room temperature with gentle agitation for 1 h. The antibody solution was prepared by diluting anti-DIG-AP conjugate to 75 $mU \cdot mL^{-1}$ (1:10,000) in 20 mL blocking solution. After removing the blocking solution, the membrane was incubated with the diluted antibody at room temperature for 30 min. The antibody solution was then poured off and the membrane was washed twice with 100 mL maleic acid buffer for 15 min. Before the colour reaction, the membrane was equilibrated with 20 mL detection buffer (0.1 M Tris-HCl, 0.1 M NaCl and 50 mM $MgCl_2$, pH 9.5) for 2-5 min and followed by incubation in freshly prepared colour solution (45 µL NBT [nitroblue tetrazolium; 75 mg NBT in 1 mL 70% dimethylformamide] and 35 µL BCIP [5-bromo-4-chloro-3-indolyl phosphate; 50 $mg \cdot mL^{-1}$] in 10 mL detection buffer) in the dark until the bands were coloured. The reaction was stopped by washing the membrane with water for 5 min. Results were documented by photographing the wet filter.

2.13. Northern hybridization

2.13.1. RNA extraction

The most important consideration in any procedure that involves purification of RNA is to avoid contamination with RNase. All glassware was therefore oven-baked (240°C for 4 h or overnight at 180°C) before use or treated with 0.1% diethyl pyrocarbonate (DEPC) overnight at room temperature and then autoclaved. All solutions were prepared with DEPC-treated water and autoclaved. Total RNA was isolated from *E. cloacae* UW4, CAL2 and *E. coli* DH5α that contained plasmid p4U2 using the following three different methods.

2.13.1.1. Phenol extraction (Boulnois, 1987)

Cells grown in either nutrient medium or ACC induction medium were collected by centrifugation at 4°C. Each cell pellet was resuspended in 1 mL of water and 1 mL of lysis buffer (1% SDS, 0.2 M Tris-HCl, pH 7.5, 0.2 M NaCl, and 40 mM EDTA). The mixture was heated in a boiling water bath for 1-2 min and extracted once with preheated (64°C) phenol and once with cold phenol (4°C). The RNA in the top layer, was precipitated with 2.5 volumes (around 5 mL) of ice-cold absolute ethanol at -20°C for 16 h. Following centrifugation at 18,000 x g for 20 min, the RNA pellet was washed twice with 70% ice-cold ethanol, air dried, and then dissolved in water. The concentration and quality of RNA extracts were assessed by determining the absorbance at 260 nm and 280 nm (OD_{260nm} of 1.0 40 μg RNA·mL $^{-1}$ and the

ratio of OD_{260nm}/OD_{280nm} should range between 1.7 and 2.0 for pure nucleic acids). The extracted RNA sample was aliquoted and stored at -80 °C.

2.13.1.2. RNeasy mini microspin

Total bacterial RNA was also isolated using RNeasy Mini Kits (Qiagen Inc., Chatsworth, Calif. USA). The RNeasy Mini procedures are fast and simple and allow the preparation of up to 100 µg total RNA within 30 min. Initially, bacterial cells were harvested at the end of log-phase growth and the supernatant was discarded. The cell pellet was resuspended in 100 µL lysozyme-containing TE buffer (400 µg lysozyme per mL) by vortexing followed by incubation for 5 min to digest the bacterial cell wall prior to lysis. After the addition of 350 µL of GITC (guanidinium isothiocyanate)-containing lysis buffer (Buffer RLT: 50 mM Tris-HCl, pH8.0, 140 mM NaCl. 1.5 mM $MgCl_2$, 0.5% Nonidet P-40; 10 µL of β-mercaptoethanol was added into 1 mL of buffer RLT before use), the sample was vortexed vigorously and centrifuged for 2 min. The lysate was mixed with 250 µL ethanol by pipetting. The mixture (around 700 µL) including any precipitate was then applied onto an RNeasy mini column in a 2 mL collection tube and centrifuged for 15 sec at 8000 x g. In this step the RNAs in the mixture were adsorbed to the RNeasy silica-gel membrane. To remove contaminants the column was washed first with 700 µL Buffer RW1, and then 500 µL Buffer RPE (Both buffers were supplied by Qiagen Inc.,) with centrifuging 15 sec at 8,000 x g. To dry the RNeasy membrane 500 µL Buffer RPE (supplied by Qiagen Inc.,) was added onto the RNeasy column and the column was centrifuged for 2 min at maximum speed. Following the spin, the RNeasy column was transferred

into a new 1.5 mL collection tube and the RNAs were eluted by adding 50 µL of RNase-free water and centrifuging for 1 min at 8,000 x g. The concentration and quality of RNA extracts were assessed as described in section 2.13.1.1. RNA samples were aliquoted and stored at -80°C.

2.13.1.3. CsCl ultracentrifugation (Chirgwin et al., 1979)

Bacterial cells were lysed as described in Section 2.13.1.2. and the lysate was carefully decanted into ultracentrifuge tubes (14 x 89 mm for SW41 rotor in a BECKMAN L8-70 Ultracentrifuge) containing 3.3 mL of 5.7 M CsCl buffer (95.97 g CsCl and 0.83 mL 3 M sodium acetate, pH 6.0, in 100 mL of a final volume adjusted with DEPC-treated water and filter sterilized). The tubes were top-filled with 4 M GIT buffer (which consisted of 47.26 g Guanidine isothiocyanate, 0.835 mL 3 M sodium acetate, pH 6.0, and 0.835 mL β-mercaptoethanol in 100 mL DEPC-treated water) and balanced to within 0.01 g. Ultracentrifugation was performed at 42,000 x g (with a SW41 rotor) for 23 h at 20°C. After centrifugation, the tubes were carefully removed from the rotor without agitation and the GIT buffer layer (containing protein) and the CsCl layer (containing DNA) were carefully poured off. The tubes were inverted to drain and then about 20 mm from the bottom of each tube was removed by cutting. The remaining CsCl was rinsed with 1 mL 70% ice-cold ethanol and the tubes were inverted to drain for 5-10 min. The RNA pellet was dissolved in 360 µL TES buffer (10 mM Tris-HCl, pH 7.4; 5 mM EDTA and 1% SDS) and kept on ice for 10 min. The RNA was transferred into a microcentrifuge tube and precipitated by adding 40 µL 3 M sodium acetate (pH 5.2) and 1 mL RNase-free ice-cold ethanol. The precipitated RNA was

pelleted by centrifugation at 16,000 x g at 4°C and then dissolved in 360 µL DEPC-treated water. Once again the RNA was precipitated with 40 µL 3 M sodium acetate (pH 5.2) and 1 mL RNase-free ice-cold ethanol. The tubes were kept on dry ice for 30 min and then centrifuged at 16,000 x g at 4°C. If a sample contained high amounts of extraneous protein, it could be purified with phenol and chloroform. The pellet was finally dissolved in 50 µL DEPC-treated water. The quantity of RNA in the sample was determined as described in section 2.13.1.1. RNA samples were stored in aliquots at 4°C as an aqueous solution or as a pellet under 100% ethanol at -80°C.

2.13.2. RNA gel electrophoresis (Sambrook et al., 1989)

Prior to starting, the electrophoresis tank was cleaned with 0.5% SDS and rinsed with water. Once dry the tank was treated with 3% H_2O_2 for 10 min and then rinsed thoroughly with DEPC-treated water. The RNA formaldehyde agarose gel consisted of 1.2-1.4% ($w \cdot v^{-1}$) agarose, 1X MOPS buffer (0.2 M 3-morpholino propanesulphonic acid, 50 mM sodium acetate and 10 mM EDTA, pH 7.0), and 6% ($v \cdot v^{-1}$) formaldehyde, which was added after the agarose was melted and cooled to 50 °C. About 5-20 µg of the RNA sample was resuspended in 10 µL of sample buffer (50% ($v \cdot v^{-1}$) formamide, 0.1 volume 10X MOPS and 6% ($v \cdot v^{-1}$) formaldehyde). The samples were incubated at 65°C for 15 min, chilled on ice, and then 2 µL of loading buffer (50% glycerol and 0.1% ($w \cdot v^{-1}$) bromophenol blue) was added. Samples were then mixed with ethidium bromide (10 $mg \cdot mL^{-1}$) and loaded onto the gel. Electrophoresis was carried out at 5 $volts \cdot cm^{-1}$ in 1X MOPS buffer (pH 7.0) in a fume-hood. To mix the running buffer, the electrophoresis apparatus was

gently shaken every hour. When the bromophenol blue indicator dye migrated three-fourths down the gel, the gel was removed and covered with Saran Wrap to prevent contamination. In order to detect the ribosomal RNA bands, the gel was stained in ethidium bromide solution (25 µL of 10 $mg \cdot mL^{-1}$ stock in 500 mL water) for 45 min and photographed with a ruler on a UV transilluminator (ImageMaster® VDS, Amersham Pharmacia Biotech., Quebuc, Canada).

2.13.3. Northern blotting and hybridization

After electrophoresis, the RNA gel was washed three times in DEPC-treated water for 5 min each, followed by two twenty minutes washes in 10X SSC buffer on a rotating platform to remove the formaldehyde. The RNA was then transferred to a nylon membrane in a manner similar to Southern blotting as described in section 2.12.1. with the exception that the transfer buffer for Northern blotting was 10X SSC. After overnight blotting, the membrane was marked for slots and orientation and rinsed with 2X SSC buffer to remove residual agarose. RNA was cross-linked to the membrane by treatment with 150 mJ of UV light (GS Gene linker™, UV Chamber, Bio-Rad Laboratories). The blot was ready for subsequent hybridization.

The DNA probe for Northern hybridization was linearized by restriction enzyme digestion and purified from a gel using the Geneclean kit (Bio 101, Inc., La Jolla, Calif). The probe was then radiolabeled with ^{32}P-dCTP using random primed labeling as described in section 2.12.2.1.

With gloves on, the membrane was prehybridized in a tray with 20-50 mL of prehybridization solution (40% formamide, 6X SSC buffer, 2.5X

Denhardts's reagent, 0.1% SDS and 100 $\mu g \cdot mL^{-1}$ denatured, fragmented salmon sperm DNA). The tray was incubated at 42°C for 2 to 4 h with gentle shaking. The prehybridization buffer was replaced with 20 mL of hybridization buffer, which consisted of the denatured radiolabeled probes in prehybridization buffer. The tray was then incubated overnight at 42°C with gentle agitation.

After hybridization, the membrane was washed with freshly prepared buffers in the following order: (1) Two washes with 100 mL of 2X SSC and 2% SDS at room temperature for 15 min; (2) Two washes with 200 mL of 0.5X SSC and 1% SDS at 42°C for 15 min; and (3) two washes with 100 mL 0.2X SSC buffer and 0.1% SDS at 42°C for 10 min. The filter was then wrapped with Saran Wrap and exposed to Kodak X-OMAT-AR X-ray film at -70°C with an intensifying screen for overnight or longer. Following exposure, the cassette was warmed to room temperature and the film was developed in a dark room (section 2.12.3.1.).

2.14. Protein assay

2.14.1 Crude protein extraction

Cells were grown in 300 mL nutrient or minimal medium to late-logarithmic phase (with or without inducer) on an orbital shaker at 200 rpm and harvested by centrifugation in a Sorval RC5C centrifuge using a GSA rotor at 8,000 x g for 10 min. The supernatant was discarded and the tubes were kept on ice. The cell pellet was washed and resuspended in a small volume of buffer containing 2.2 mM dithiothreitol. The crude cell-free extracts were prepared by rupturing bacterial cells (i) with three freeze-thaw

cycles (Shah, et al., 1998): each consisting of immersion in liquid nitrogen for 1 min and in a 25°C water bath for 5 min; and (ii) by sonicating (Jacobson, et al., 1994), the chilled cell suspension was sonicated using three alternating 60-s periods at 140-160 W with a sonicator (Braun-Sonic 2000). The disrupted samples from either method were centrifuged at 100,000 x g for 30 min at 4°C. The supernatant containing the total soluble protein fraction was distributed in small volume aliquots and stored at -20°C.

2.14.2. Protein concentration assay

The protein concentrations in all enzyme assays were measured according to the Protein Assay instruction from Bio-Rad Laboratories which is based on the method of Bradford (Bradford, 1976). The concentrated dye reagent (Coomassie Brilliant Blue G-250) was diluted with distilled deionized water in a 1:4 ratio and filtered to remove particles. For a standard concentration curve, a series of known BSA (bovine serum albumin) concentrations were prepared with a linear range from 0.2 to 0.9 $mg \cdot mL^{-1}$ in a 100 µL standard solution. Each point of the series was assayed in duplicate or triplicate. Each 100 µL of standard solution was transferred into a test tube and 5.0 mL of diluted dye reagent was added. The tube was vortexed and incubated at room temperature for at least 5 min but no more than 1 h. The absorbance of each sample was measured at 595 nm in a Beckman DU-7 spectrophotometer (kindly provided by Dr. J.J. Heikkila). A standard curve was obtained by drawing the best straight line through absorbance data plotted against concentration (Appendix I).

2.14.3. SDS-PAGE gel electrophoresis

Crude protein extracts were analyzed by SDS (sodium dodecyl sulfate)-PAGE (polyacrylamide gel electrophoresis) electrophoresis with a Mini-PROTEIN II electrophoresis cell (Bio-Rad laboratories, Mississauga, Ont.) according to Laemmli (1970). Glass plates for the gel apparatus were cleaned with soap and ethanol and the gel sandwich was assembled with a short glass plate facing outward and a longer plate facing inward with two spacers and an alignment card in between. The gel sandwich was aligned in the alignment slot and clamp screws were tightened. After removing the alignment card, a separating gel solution (2.0 mL H_2O, 1.25 mL of 1.5 M Tris-HCl pH 8.8, 1.7 mL 30% acrylamide/bis, 50 µL 10% SDS, 50 µL 10% ammonium persulfate, 5 µL TEMED [N,N,N',N'-Tetramethylethylene-diamine]) was carefully added between the glass plates using a pipet along with a spacer until the gel solution reached 5 cm from the bottom. A thin layer of isopropanol was added to the top of the gel to keep the gel surface flat. When the separating gel polymerized, the layer of isopropanol was poured off, and the gel was rinsed with water and dried with a filter paper. The stacking gel was then prepared (1.7 mL H_2O, 313 µL of 1.0 M Tris-HCl pH 6.8, 416 µL 30% acrylamide/bis, 25 µL 10% SDS, 13 µL 10% ammonium persulfate, 2.5 µL TEMED) and added on top of the separating gel until the solution reached the top of the front plate. Immediately, a comb was inserted at a slight angle between the two glass plates being careful not to set bubbles trapped on the end of teeth. After the stacking gel polymerized, the comb was carefully removed and the gel was placed into the electrophoresis chamber. Electrophoresis buffer diluted in 1X concentration (5X buffer in one

liter consisting of 15 g Tris base, 72 g glycine and 5 g SDS) was added to the inner and outer reservoir. Protein samples and protein size markers were mixed with 5X sample buffer to give a final concentration of 1X (5X sample buffer contained 60 mM Tris-HCl pH 6.8, 25% glycerol, 2% SDS, 14.4 mM β-mercaptoethanol and 0.1% bromophenol blue) and heated at 95°C for 5 min. The samples were loaded into the wells and the gel was run at 100 volts until the bromophenol blue dye front reached the bottom of the gel. Once the power source was disconnected, the gel was removed from the plates, a small corner was cut off from the marker lane and the gel was stained with Coomassie blue (1.0 g Coomassie brilliant blue R-250 in 450 mL methanol, 450 mL H_2O and 100 mL glacial acetic acid) at 60°C for 10 min with gentle shaking. The gel was destained by incubation with destaining solution (100 mL methanol and 100 mL glacial acetic acid in 800 mL H_2O) overnight at room temperature. The gel was kept either in 7% acetic acid in a sealed bag or dried on filter paper under vacuum after photographing on the ImageMaster® VDS (Amersham Pharmacia Biotech., Quebec, Canada).

2.15. Measurements of enzyme levels

2.15.1 Galactokinase assay

Galactokinase gene (*galK*) is a promoterless reporter gene on plasmid pKO-1 constructed by Rosenberg and co-workers (Mckenney et al., 1981; Rosenberg et al., 1983) that is used for isolating and characterizing promoters. A bacterial cell with a mutation in the gene for galactokinase (*galK*$^-$) (*E. coli* N100) does not grow on minimal medium containing galactose as the sole source of carbon and grows as a white colony on MacConkey galactose agar.

However, when those mutants (*galK*$^-$) are complemented with a *galK*$^+$ plasmid, the cells can grow on minimal galactose medium and produce red colonies on MacConkey galactose agar (*galK*$^+$ cells are capable of fermenting galactose producing a localized pH drop which, followed by absorption of the neutral red, imparts a red color to the colony). Thus, if a pKO-1 vector contains a promoter-active insert and complements a *galK*$^-$ host cell, it allows the host cell to become *galK*$^+$. The transformants with red colour on MacConkey galactose agar can be selected for a promoter insertion. Not only can promoter insertion be selected, but also the levels of galactokinase activity in transformed *E. coli* cells can be determined in a galactokinase assay (McKenney et al., 1981).

Levels of galactokinase were determined by measurement of the phosphorylation of ^{14}C-galactose. Cells were grown to log phase in M56 medium with galactose as a carbon source. Proteins were then extracted from bacterial cells as described in section 2.14.1. and the protein concentration was determined. For galactokinase activity assay, 20 µL of the extract mixture containing ~40 µg crude proteins from each sample was warmed to 30°C and combined with 80 µL of prewarmed assay mixtures (stock solution I:II:III was mixed up by the ratio 1:2:5): solution I: 8 mM $MgCl_2$, 200 mM Tris-HCl, pH 7.2, 10 mM ATP; solution II: 5 mM D-galactose and 22.5 µCi·mL^{-1} D-^{14}C-galactose; solution III: 5 mM dithiothreitol and 16 mM NaF (freshly made). A sample for a zero time point was immediately removed (30 µL) and placed on a DE81 filter which rested on a block of dry ice to stop the reaction. After 15 min, duplicate 30 µL volumes were transferred onto the filters resting on dry ice. The filters were washed by vacuum filtration three times with 20 mL of

distilled water and dried. The dried filters were then added into scintillation vials that contained 10 mL scintillation fluid. The vials were counted against ^{14}C-standards in a Beckman LS1701 liquid scintillation counter. The enzyme activity is expressed as nanomoles of product (galactose 1-phosphate) formed per minute per mg of cellular protein.

2.15.2. ACC deaminase assay

As ACC deaminase can be induced by ACC, the bacterial culture was grown in a minimal medium with ACC as a sole source of nitrogen. There were two different culture-induction methods employed. In the first method, the bacterial culture was grown in nutrient medium overnight, and then 1% of the culture was transferred into a minimal medium which contained 3 mM ACC. Bacteria in ACC medium were grown for another 2 days with shaking. In the second method to induce ACC deaminase, once the bacterial culture grown in a nutrient or an ammonium salt-containing medium reached late-log phase, the cells were harvested by centrifugation. After washing with sterile saline water (0.85% NaCl), the cell pellet was pooled in a one-tenth volume of a minimal medium with 5 mM ACC as the sole source of nitrogen followed by incubation for another 6 h. After induction by either method, the cells were harvested, washed and resuspended in a small volume of 0.1 M Tris-HCl (pH 8.5). The crude protein extracts were prepared either as described in section 2.14.1 or with toluene (personal communication with Dr. G. Burd) in which case, 50µL of toluene was added to 1 mL of bacterial suspension and vortexed vigorously for 30 sec to permeabilize the cells. The protein concentration for each crude extract was determined, and then ACC

deaminase assay was quantified by measuring the amount of α-ketobutyrate that was produced by the deamination of ACC as described by Honma and Shimomura (1978). About 100 µg of a protein extract in a 200 µL volume was incubated with 50 mM ACC at 30°C for 20 min. Two negative controls were also run at the same time, (i) buffer without the protein extract and (ii) the same amount of protein extract without additional ACC. The reaction was stopped by adding 1.8 mL of 0.56N HCl. After addition of 0.3 mL of 0.1% 2,4-dinitrophenylhydrazine in 2N HCl (a colorimetric indicator), the mixture was incubated at 30°C for 15 min, and then 2 mL of NaOH was added and the absorbance was measured at 540 nm (Honma and Shimomura, 1978). One unit of the ACC deaminase activity indicated the formation of 1 nmole of α-ketobutyrate per mg protein per hour under these conditions. An α-ketobutyrate standard curve was obtained by measuring absorbances of a series of different α-ketobutyrate concentrations at 540 nm (Appendix II).

A Km value of ACC deaminase from *E. cloacae* UW4 was determined by incubating the protein extracts with ACC at a series of different ACC concentrations (from 1 mM to 5 mM and 10 mM, 20 mM, 30 mM, and 40 mM). Procedures for the ACC deaminase assay were the same as mentioned in this section. The Km value was determined by a Lineweaver-Burk plotting and the samples were shown in Appendix V.

2.15.3. Luciferase assay:

2.15.3.1. X-ray film assay

pQF70 is a promoter-probe vector containing the *luxAB* (luciferase) genes from *Vibrio harveyi* (Farinha and Kropinskin, 1990). The promoterless

luxAB genes acting as reporter genes on this plasmid do not give light unless a promoter is inserted ahead of them. The promoter-insertion positive clones of pQF70 can be easily detected by swabbing 8 µL of n-denyl aldehyde inside a petri dish lid. This volatile long-chain aldehyde is utilized by bacterial luciferase to produce a blue-green light (490 nm). The light output from those positive colonies can be observed on the petri dishes in a darkened room or can be recorded by brief exposure to X-ray film (Shaw and Kado, 1986).

2.15.3.2. CytoFluor assay

The light output (490 nm) of luminescence can be quantitated with a Fluorometric detector. Overnight cultures were diluted 100-fold in M9 minimal ammonium salt-containing medium supplemented with ampicillin (50 $\mu g \cdot mL^{-1}$) and cultivated in triplicate with shaking at 37°C. When cultures were grown to early exponential growth phase (OD 600 nm = 0.2, since the LuxAB activity requires the cell energy, the active cells were needed), ACC was added as an inducer at a final concentration of 1 mM and the cells were grown for another 1.5 h. The optical density of each bacterial culture at 600 nm was recorded and then 1.0 mL of the culture from each tube was centrifuged and resuspended in 100 µL of 0.85% NaCl. From each tube, two 50 µL aliquots were added to wells of microwell plate (NUNC™). Light output was measured, after the addition of 3 µL of n-decyl aldehyde, on a CytoFluor Series 4000-Fluorescence Multi-well Plate Reader (PerSeptive Biosystems). The detection conditions were set at 30°C, plate continuous mixing 99 sec, excite at lamp-off and measure emission with 460/40 nm filter. After reading, the data was analyzed by presenting the mean of the data

obtained in triplicates and the standard errors of the means. The activity of luminescence was expressed by relative fluor units per $1x10^8$ CFU (colony-forming units) which were measured by relating the data at OD_{600nm} of the growth culture to the numbers of their CFU from the plates (see standard curves from the appropriate strains in Appendix III and IV).

2.16. Gnotobiotic root elongation assay

The protocol for assessing the effect of various bacterial strains on the elongation of canola roots is based on the method described by Lifshitz et al. (1987) with some modifications (Caron et al., 1995). The bacterial cells were grown in either M9-salt broth + NH_4Cl (1.5 $g \cdot L^{-1}$) or M9-salt broth + ACC (5 mM) for 24 h with shaking. Cells were pelleted by centrifugation at 5000 rpm for 10 min, washed, and resuspended in 100 mM $MgSO_4$. The absorbance of the bacterial suspension was adjusted to an optical density of 0.15 at 600 nm prior to incubation with the seeds. Seed-pack growth pouches (Northrup King CO., Minneapolis, MN, USA) were wetted with 10 ml of deionized water and sterilized by autoclaving. Canola seeds (*Brassica campestris cv. Reward*, kindly provided by Dr. G. Brown, Agrium, Inc.) were surface disinfected by immersing in 70% ethanol for 1 min and 1% sodium hypochlorite for 10 min, followed by thorough rinsing with sterilized water immediately prior to use. Once cleaned, equal numbers of the disinfected seeds were incubated for 1 h at room temperature with 15 mL of either 100 mM $MgSO_4$ (as a control) or a bacterial suspension in 100 mM $MgSO_4$. Six to twelve seeds of the same treatment were placed in each sterile growth pouch and 10 pouches were used in each treatment for statistical analysis.

The pouches were placed upright in a rack with two empty pouches at each end of a row. The box containing the growth pouches was covered with Saran Wrap to prevent drying and wrapped with aluminum foil to promote low light conditions (0.97 $\mu mol \cdot m^{-2} \cdot sec^{-1}$) for the first three days. All experiments were performed in a growth chamber (Conviron CMP 3244) which was maintained at a temperature of 20 ± 1°C with a photoperiod of 12 h light (18 $\mu mol \cdot m^{-2} \cdot sec^{-1}$) and 12 h darkness. After 3 days (root emergence) the aluminum foil was removed and the seedlings were kept in the growth chamber for another two days. The root lengths were measured on the fifth day. For each experiment, the data were analyzed using a one-way ANOVA and a unpaired t-test by the computer program Systat^{tm} (version 2.1).

3. RESULTS

3.1. Isolation and characterization of ACC deaminase genes

3.1.1. Selection of PGPR strains for gene isolation

In 1978 Honma first reported that some soil microorganisms that contain ACC deaminase were able to use ACC as a sole source of nitrogen and since then, ACC deaminase has been found in many different soil bacteria (Penrose and Glick, 1997). Rhizosphere soil samples from Waterloo, Ontario, Canada, and several locations in California, U.S.A. were screened in our laboratory using minimal medium containing ACC as a sole source of nitrogen. From these samples, seven strains were isolated based on their growth on the minimal medium and designated UW1, UW2, UW3, UW4, CAL1, CAL2 and CAL3 (section 2.1) (Glick et al., 1995b; Shah et al., 1997; 1998). After a more complete characterization, the seven strains were found to comprise two different genera, UW1 and UW3 were identified as strains of *Pseudomonas putida*, CAL1 as *Pseudomonas fluorescens*, and UW2, UW4, CAL2 and CAL3 were found to be strains of *Enterobacter cloacae* (Shah et al., 1997; 1998) (Table 1).

Although all seven strains were able to grow on minimal medium with ACC, we did not know whether or not they all contained ACC deaminase. In order to isolate the ACC deaminase genes from one or two of the seven strains, the selection of strains was based on the ability of the bacteria to grow in ACC medium, and the results of ACC deaminase assays and SDS-PAGE analysis. ACC deaminase genes isolated from *Pseudomonas*

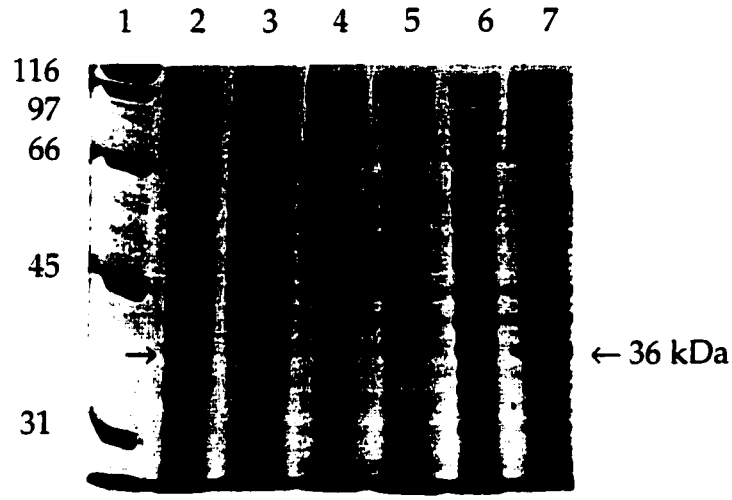
spp. (Klee et al., 1991; Sheehy et al., 1991) and partially purified ACC deaminase enzyme from *Pseudomonas putida* GR12-2 (Jacobson et al., 1994) served as controls.

Initially, the seven strains were cultivated in 300 mL DF-minimal medium supplemented with 3 mM of ACC for 48 h at 30°C. After incubation, the absorbance at 600 nm was recorded for each strain as a measure of the growth rate of each bacterium. Of the seven strains, *Enterobacter cloacae* CAL3 was found to grow the slowest compared with other strains, whereas *E. cloacae* UW4 and CAL2 were the most prolific of the seven strains and grew better than *P. putida* GR12-2 (Table 2). ACC deaminase assays were performed on all of the strains with the exception of *P. putida* UW3, as it did not pellet during the assay and hence, the data from this strain was not included. The results are shown in Table 2, and indicate that *P. putida* UW1 and *E. cloacae* UW2 had very little ACC deaminase activity, whereas the activity for *E. cloacae* CAL2 was much higher, followed by *E. cloacae* UW4 and *P. fluorescens* CAL1. Although the data from *E. cloacae* CAL3 showed the highest enzyme specific activity, it may be anomalous due to the cell-free protein extract of this sample turned to milky (not clarity) after ACC incubation. Thus, according to these preliminary results, *E. cloacae* UW4 and CAL2 were found to have the highest ACC deaminase specific activity and the greatest ability to grow in ACC medium compared to the other strains. Further confirmation of the presence of ACC deaminase in both strains UW4 and CAL2 was provided by SDS-PAGE, in which crude protein samples from both strains, along with *P. putida* GR12-2, cultivated with either ammonia or ACC as the source of nitrogen, were extracted

Table 2. Characterization of seven PGPR strains for the ability to utilize ACC as a sole source of nitrogen and for their level of ACC deaminase activity.

Strains	OD_{600nm} DF-ACC medium, 48 h	ACC deaminase activity nmoles/mg protein/h
Pseudomonas putida UW1	1.19	58.8
Enterobacter cloacae UW2	1.11	58.8
Pseudomonas putida UW3	2.01	–
Enterobacter cloacae UW4	2.17	278
Pseudomonas fluorescens CAL1	1.14	260
Enterobacter cloacae CAL2	2.01	566
Enterobacter cloacae CAL3	0.58	690
Pseudomonas putida GR12-2	1.35	407

Fig. 4. SDS polyacrylamide gel electrophoretic analysis for ACC deaminase from extracts of *E. cloacae* UW4 and CAL2 cultured with different media, using strain *P. putida* GR12-2 as a positive control. Lane 1, protein molecular mass standard: β-galactosidase (116 kDa), phosphorylase B (97.4 kDa), bovine serum albumin (66 kDa), ovalbumin (45 kDa), and carbonic anhydrase (31 kDa); lane 2-3, strain GR12-2 incubated with either ammonia or ACC (3 mM); lane 4-5, strain UW4 incubated with either ammonia or ACC (3 mM); lane 6-7, strain CAL2 incubated with either ammonia or ACC (3 mM); the arrow indicates around 36,000 daltons for the ACC deaminase bands from each strain incubated with ACC in lanes 3, 5 and 7.


1 2 3 4 5 6 7
116
97
66
45
31
← 36 kDa

following sonication of the cell suspension. After SDS-PAGE, extracts of cells grown in the presence of ACC produced bands with an increased intensity at a molecular weight of around 36,000 daltons (Fig. 4, lane 3, 5 and 7) in comparison to cells grown in the presence of ammonia (lane 2, 4, and 6). Close inspection of this gel revealed that each band from the ACC-grown cells actually contains two bands with the lower one representing ACC deaminase. Thus, *E. cloacae* UW4 and CAL2 were the two strains selected for ACC deaminase gene isolation.

3.1.2. Isolation of ACC deaminase genes

Two genomic libraries were constructed using either the pUC19 or pUC18/*Bam*HI/BAP plasmid vectors (Table 1). When pUC19 was used as a vector it was first digested with *Bam*HI and then treated with alkaline phosphatase to ensure that recombinant plasmids accounted for over 80% of the ligation products. Total genomic DNA was isolated from *E. cloacae* UW4 and CAL2 and partially digested with *Sau*3A. Nearly all partially digested fragments were between 1 kb and 10 kb in size. After ligation into a vector the recombinant ligation products were used to transform competent *E. coli* DH5α cells. Transformed cells that grew on selective agar (LB containing 100 μg/mL ampicillin) were further screened by using either M9 minimal medium with ACC (3 mM) as a sole source of nitrogen, or *in situ* DNA hybridization. In the latter case, the oligonucleotide probe was provided by Dr. S. Shah who designed the primers according to the published DNA sequences of ACC deaminase genes amplified from *E. cloacae* UW4 by PCR

(Shah et al., 1998). The results from the screening on ACC plates are shown in section 3.5.3.

According to digested fragment size (x), a library size (N, number of colonies) that has a 0.99 probability (P) of containing any particular DNA sequence from a genome (y) can be calculated as N=In (1-P)/In (1-x/y). In this study, the average size of *Sau*3A partially digested fragments was 5 kb, thus, in order to cover the entire genome, the minimal size of the library constructed from each strain should be around 4,000 clones. After the third screening with *in situ* DNA hybridization, seven positive clones were isolated from 7300 clones of the *E. cloacae* UW4 library and two positive clones, out of 5,800 clones, were obtained from the *E. cloacae* CAL2 library. The plasmids were isolated from the nine positive clones and designated as p4U1, p4U2, p4U3, p4U4, p4U5, p4U6, p4U7 from strain UW4, p2C1 and p2C2 from strain CAL2. The nine plasmids were digested with *Hind*III and *Sma*I to release the inserts, and separated on an agarose gel. The insert fragments were analyzed by Southern hybridization with PCR-generated ACC deaminase gene probes (provided by Dr. S. Shah). Results of the Southern hybridization demonstrated that all of the plasmids contained the insert and they all hybridized to the probe (Fig. 5). However, among all the plasmids, three of them, p4U1, p4U2 and p4U4 contained the same size insert (around 6.8 kb) and p4U5-p4U7 contained the same insert but the size was different from other plasmids (around 0.7 kb). Plasmid p4U3 contained a different insert, around 4.4 kb but had a 3.8 kb fragment that was present in p4U1, p4U2 and p4U4 as well. When hybridized with the ACC deaminase gene, the 3.8 kb fragment from p4U3 bound with the same probe

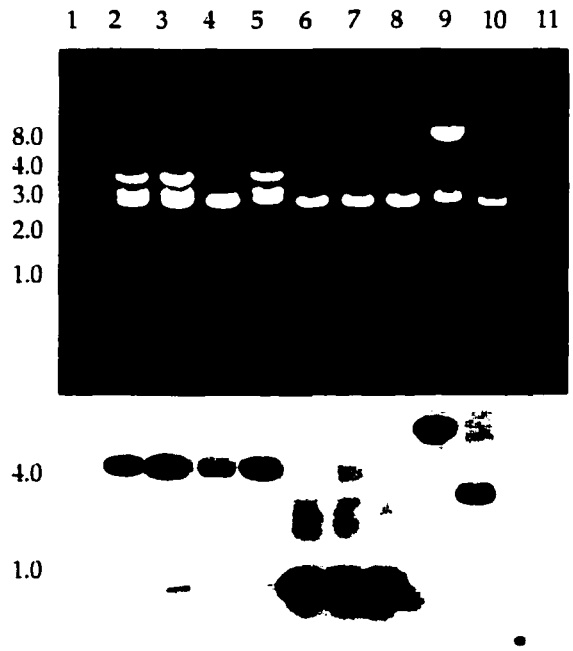
as the 3.8 kb fragments from p4U1, p4U2 and p4U4 (Fig. 5). Plasmid p2C1 had an 8 kb insert while the insert in p2C2 was close to 3.1 kb and both inserts hybridized to the probe.

3.1.3. Analysis of the DNA sequences

Sequencing of the open-reading frames (ORF) of the ACC deaminase genes from p4U2, p4U3 and p2C2 representing both *E. cloacae* UW4 and CAL2 was performed in both directions (Shah et al., 1998). Both sequences contained 1014 nucleotides are predicted to encode proteins of 338 amino acids in size. The amino acid sequences derived from both DNA sequences using the computer program DNA Strider 1.2 were aligned with the published ACC deaminase sequences using the Align program (www.genome.eerie.fr; from Pedro's BioMolecular Research Tools, or DDBJ malign program, www.ddbj.ac.jp) (Fig. 6). So far, the known sequences of ACC deaminase genes have been reported from *Pseudomonas* sp. strains 6G5, 3F2 (Klee et al., 1991), *P.* sp. strain ACP (Sheehy et al., 1991) and *P. fluorescens* F17 (Campbell and Thompson, 1996). When the sequences of two isolated genes were aligned with the known sequences, it was found that the sequences from all of the aligned strains except *P.* sp. strain ACP were highly homologous to each other with an 85-95% identity at the nucleotide level and a 96-99% identity at the amino acid level. The sequence from *P.* sp. strain ACP, on the other hand, was only 74-75% identical at the nucleotide level and 81-82% identical at the amino acid level to the other sequences. The ACC deaminase sequences from both strains UW4 and CAL2 have been published (Shah et al., 1998) and the accession numbers of both genes

Fig. 5. **A**: Southern hybridization analysis of two separate ACC deaminase genes present in nine clones. Upper panel: Agarose gel showing the inserts from the nine plasmids digested with *Hind*III and *Sma*I. Lane 1 and lane 11: 1 kb DNA marker. Lane 2 - lane 10: plasmids p4U1, p4U2, p4U3, p4U4, p4U5, p4U6, p4U7, p2C1 and p2C2. Lower panel: Southern hybridization of the nine positive plasmids shown as upper panel using a PCR-generated ACC deaminase gene as a probe. The numbers beside each panel indicate the DNA size in kb. **B**: PCR analysis of the insert of p4U2 with different combinations of primers. lane 1, DNA marker; lane 2-5, PCR products from different primers: P-forward and P1 (lane 2); P-forward and P2 (lane 3); P-reverse and P1 (lane 4); and P-reverse and P2 (lane 5).

A



B

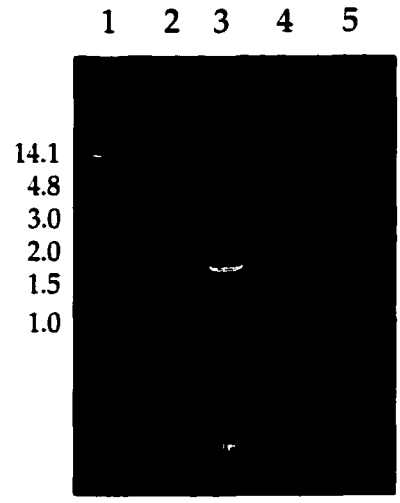


Fig. 6. Alignment of the deduced amino acid sequences of the ACC deaminase genes from *E. cloacae* UW4 and CAL2 with those of *Pseudomonas* spp. strains 6G5, F17, 3F2 and ACP. The bold letters represent amino acids that are not identical to those found in the sequence of strain UW4. Asterisks indicate the conserved region beside the putative active site Lys-51.

UW4: *Enterobacter cloacae* UW4, accession: AF047710 (Shah et al., 1998).

CAL2: *Enterobacter cloacae* CAL2, accession: AF047840 (Shah et al., 1998).

6G5: *Pseudomonas* sp. strain 6G5; accession: P30297 (Klee et al., 1991).

F17: *Pseudomonas fluorescens* F17; accession: Q51813 (Campbell & Thompson, 1996).

3F2: *Pseudomonas* sp. strain 3F2; (Klee et al., 1991).

ACP: *Pseudomonas* sp. ACP; accession: Q00740 (Sheehy et al., 1991).

```
UW4   MNLNRFERYPLTFGP SPITPLKRLSEHLGG KVELYAKREDCNSGL AFGGNKTRKLEYLIP
CAL   MNLNRFERYPLTFGP SPITPLKRLSQHLGG KVELYAKREDCNSGL AFGGNKTRKLEYLIP
6G5   MNLNRFERYPLTFGP SPITPLKRLSQHLGG KVELYAKREDCNSGL AFGGNKTRKLEYLIP
F17   MNLNRFKRYPLTFGP SPITPLKRLSEHLGG KVELYAKREDCNSGL AFGGNKTRKLEYLIP
3F2   MNLNRFKRYPLTFGP SPITPLKRLSEHLGG KVELYAKREDCNSGL AFGGNKTRKLEYLIP
ACP   MNLQRFPRYPLTFGP SPIQPLARLSKHLGG KVHLYAKREDCNSGL AFGGNKTRKLEYLIP
                                        ************ ***************

UW4   EAIEQGCDTLVSIGG IQSNQTRQVAAVAAH LGMKCVLVQENWVNY SDAVYDRVGNIEMSR
CAL   EAIEQGCDTLVSIGG IQSNQTRQVAAVAAH LGMKCVLVQENWVNY SDAVYDRVGNIEMSR
6G5   EAIEQGCDTLVSIGG IQSNQTRQVAAVAAH LGMKCVLVQENWVNY SDAVYDRVGNIEMSR
F17   EALEQGCDTLVSIGG IQSNQTRQVAAVAAH LGMKCVLVQENWVNY SDAVYDRVGNIEMSR
3F2   EALEQGCDTLVSIGG IQSNQTRQVAAVAAH LGMKSVLVEENWVNY SDAVYDRVGNIEMSR
ACP   EALAQGCDTLVSIGG IQSNQTRQVAAVAAH LGMKCVLVQENWVNY SDAVYDRVGNIQMSR
      **
UW4   IMGADVRLDAAGFDI GIRPSWEKAMSDVVE RGGKPFPIPAGCSEH PYGGLGFVGFAKELR
CAL   IMGADVRLDAAGFDI GIRPSWEKAMSDVVE QGGKPFPIPAGCSEH PYGGLGFVGFAKKLR
6G5   IMGADVRLDAAGFDI GIRPSWEKAMSDVVE QGGKPFPIPAGCSEH PYGGLGFVGFAEEVR
F17   IMGADVRLDAAGFDI GIRPSWEKAMNDVVE RGGKPFPIPAGCSEH PYGGLGFVGFAEEVR
3F2   IMGAEVRLDAAGFDI GIRPSWEKAMSDVVA RGGKPFPIPAGCSEH PYGGLGFVGFAEEVR
ACP   ILGADVRLDVPDFDI GFRRSWEDALESVRA AGGKPYAIPAGCSDH PLGGLGFVGFAEEVR

UW4   QQEKDLGFKFDYIVV CSVTGSTQAGMVVGF AADGRSKNVIGVDAS AKPEQTKAQILRIAR
CAL   QQEKELGFKFNYIVV CSVTGSTQAGMVVGF AADGRSKNVIGVDAS AKPEQTKAQILRIAR
6G5   QQEKELGFKFDYIVV CSVTGSTQAGMVVGF AADGRSKNVIGIDAS AKPEQTKAQILRIAR
F17   EQEKQLGFKFDYIVV CSVTGSTQAGMVVGF AADGRSKNVIGIDAS AKPEKTKAQILRIAR
3F2   EQEKQLGFTFDYIVV CSVTGSTQAGMVVGF AADGRSKNVIGIDAS AKPEQTKAQILRIAR
ACP   AQEAELGFKFDYVVV CSVTGSTQAGMVVGF AADGRADAVIGVDAS AKPAQTREQITRIAR

UW4   HTAELVELGREITEE DVVLDTRFAYPEYGL PNEGTLEAIRLCGSL EGVLTDPVYEGKSMH
CAL   HTAELVELGREITEE DVVLDTRFAYPEYGL PNEGTLEAIRLCGSL EGVLTDPVYEGKSMH
6G5   HTAELVELGREITEE DVVLDTRFAYPEYGL PNEGTLEAIRLCGSL EGVLTDPVYEGKSMH
F17   HTAELVELGREITEE DVVLDTPFAYPEYGL PNEGTLEAIRLCGSL EGVLTDPVYEGKSMH
3F2   HTAELVELGREITED DVVLDTRFAYPEYGL PNEGTLEAIRLCGSL EGVLTDPVYEGKSMH
ACP   QTAEKVGLERDIMRA DVVLDERFAGPEYGL PNEGTLEAIRLCART EGMLTDPVYEGKSMH

UW4   GMIEMVRRGEFPDGS KVLYAHLGGAPALNA YSFLFRNG
CAL   GMIEMVRRGEFPEGS KVLYAHLGGAPALNA YSFLFRDG
6G5   GMIEMVRRGEFPEGS KVLYAHLGGAPALNA YSFLFRNG
F17   GMIEMVRRGEFPEGS KVLYAHLGGAPALNA YSFLFRNG
3F2   GMIEMVRRGEFPEGS KVLYAHLGGAPALNA YSFLFRNG
ACP   GMIEMVRNGEFPEGS RVLYAHLGGVPALNG YSFIFRDG
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deposited at the GenBank database are AF047710 and AF047840, respectively. Since the sequence encodes ACC deaminase, the gene is hereafter referred to as *acdS*. From the alignment results, the sequence differences suggest that ACC deaminase from *P.* sp. strain ACP may fall into a different category than the other five enzymes (Shah et al., 1998). However, when all six ACC deaminase sequences were aligned together, the active site of the ACC deaminases, PLP-binding lysine-51 residue was identical across all of the aligned sequences as was a conserved region of 28 amino acids flanking the lysine-51 residue (Fig. 6). The sequence of the conserved region is as follows, with Lys-51 bolded: LYAKREDNSGLAFGGN**K**TRKLEYLIEA.

A recent study reported that the ACC deaminase gene from the yeast, *Hansenula saturnus*, has been isolated and sequenced (Minami et al., 1998). The sequence was only 60-63% identical at the amino acid level to those of the other reported ACC deaminase genes. However, the clone, when expressed in *E. coli* has comparable ACC deaminase activity. Another two putative ACC deaminase genes were reported from genomic sequencing, one was from the archaebacterium *Pyrococcus horikoshii* (Kawarabayasi et al., 1998) and another from *E. coli* (Itoh et al., 1996). Both putative ACC deaminase genes had very low sequence identity (around 30-37%) at the amino acid level with the reported bacterial ACC deaminase sequences (Table 3) and both sequences have not yet been cloned or assayed for ACC deaminase activity so that it is not known whether or not these are bona fide ACC deaminase genes. The G+C contents of ACC deaminase genes from different soil microorganisms were determined and compared

Table 3. Comparison of identity in ACC deaminase genes at amino acid level.

UW4: *Enterobacter cloacae* UW4, accession AF047710; (Shah et al., 1998).

CAL2: *Enterobacter cloacae* CAL2, accession AF047840; (Shah et al., 1998).

6G5: *Pseudomonas* sp strain 6G5, accession P30297; (Klee et al., 1991).

3F2: *Pseudomonas* sp strain 3F2; (Klee et al., 1991).

F17: *Pseudomonas fluorescens* F17, accession Q51813; (Campbell & Thompson, 1996).

ACP: *Pseudomonas* sp strain ACP, accession Q00740; (Sheehy et al., 1991).

Hsa: *Hansenula saturnus* (yeast), accession (Minami et al., 1998).

Eco: *Escherichia coli* K-12/MG1655, accession P76316; (Itoh et al., 1996).

Pho: *Pyrococcus horikoshii* OT3, accession AP000001; (Kawarabayasi et al., 1998).

	UW4	CAL2	6G5	F17	3F2	ACP	*Hsa*	*Eco*	*Pho*
UW4	100%	97.9%	97.9%	96.4%	95.6%	82.0%	62.0%	33.0%	29.9%
CAL2		100%	98.2%	95.3%	94.4%	82.2%	62.0%	33.0%	29.3%
6G5			100%	97.0%	96.2%	82.8%	63.2%	33.0%	30.2%
F17				100%	97.3%	82.2%	62.6%	32.2%	30.5%
3F2					100%	81.7%	64.4%	31.8%	30.2%
ACP						100%	59.7%	34.2%	30.6%
Hsa							100%	30.1%	29.5%
Eco								100%	36.5%
Pho									100%

Table 4. Comparison of the G+C contents (mol%) of ACC deaminase genes.

UW4: *Enterobacter cloacae* UW4, accession AF047710; (Shah et al., 1998).

CAL2: *Enterobacter cloacae* CAL2, accession AF047840; (Shah et al., 1998).

6G5: *Pseudomonas* sp strain 6G5, accession P30297; (Klee et al., 1991).

F17: *Pseudomonas fluorescens* F17, accession Q51813; (Campbell & Thompson, 1996).

ACP: *Pseudomonas* sp strain ACP, accession Q00740; (Sheehy et al., 1991).

Hsa: *Hansenula saturnus* (yeast), accession (Minami et al., 1998).

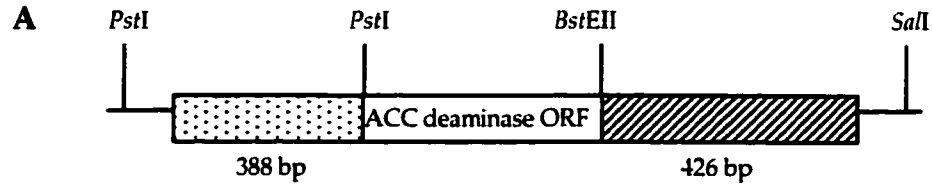
Strains	G+C content of ACC deaminase gene	G+C content of bacterial genome strain (genus)
Enterobacter cloacae UW4	57.3%	52-54% (52-60%)
Enterobacter cloacae CAL2	58.3%	52-54% (52-60%)
Pseudomonas sp. 6G5	57.1%	(58-71%)
Pseudomonas fluorescens F17	59.8%	59-61% (58-71%)
Pseudomonas sp. ACP	63.9%	(58-71%)
Hansenula saturnus (yeast)	44.8%	-

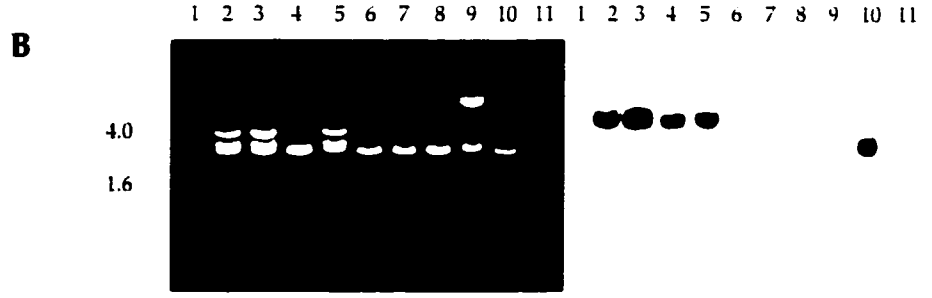
with the G+C content of the entire genome. The G+C data indicated that the highly homologous ACC deaminase genes, such as the genes from *E. cloacae* CAL2 and *P.* sp. 6G5, had similar G+C contents. While the genes with lower homology had different G+C contents, such as the case with *P.* sp. ACP. However, the G+C contents of all ACC deaminase genes were still similar to the G+C content of their respective genomes (Table 4).

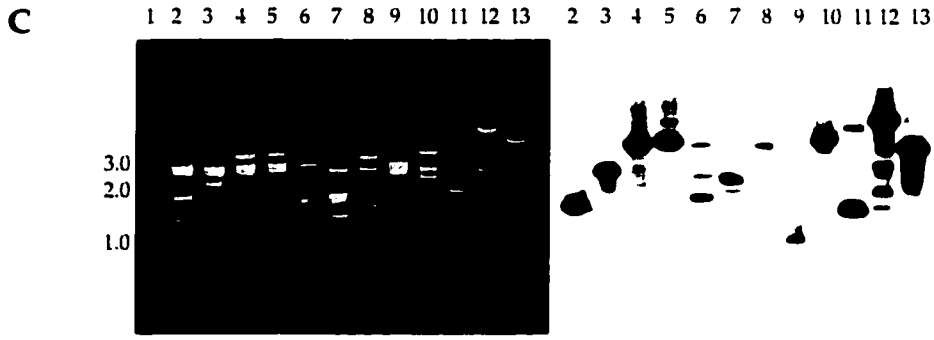
Prior to sequencing the flanking regions of both genes, all nine plasmids were hybridized with two different 5′ and 3′ end probes, to determine the extent of the upstream and downstream regions of the *acdS* gene in each clone. From ACC deaminase sequence data of p4U2, a 388 bp *Pst*I digestion fragment, adjacent to the 5′ end, and a 426 bp *Bst*EII-*Sal*I digestion fragment close to the 3′ end were selected as probes (Fig. 7A). The 5′ end probe hybridized to p4U1-p4U4 and p2C2, displaying distinct hybridization bands. There were no hybridization signals between the 5′ probe and p4U5-p4U7 and p2C1 (Fig. 7B). Since the inserts of p4U5-p4U7 were only around 0.7 kb, it was likely that they contained the incomplete 5′ end of the ACC deaminase gene. Among plasmids p4U1-p4U4, p4U2 was selected for further analysis. When p4U2 was digested with different restriction enzymes and hybridized with the 5′ end probe, results showed that the probe could hybridize with a 1.1 kb *Eco*RV fragment, a 2.1 kb *Sal*I fragment, a 3.8 kb *Sma*I fragment, a 3.9 kb *Hind*III fragment, a 1.2 kb *Hae*II fragment, and a 4.0 kb *Bgl*II fragment (Fig. 7C). The *Pst*I digestion gave a hybridization band around 0.6 kb that suggested a fragment of at least 200 bp in the region upstream of the ATG start codon in p4U2. Regarding hybridization with the 3′ end probe, p4U2, p2C1 and p2C2 were digested

Fig. 7. Analysis of possible presence of both flanking regions of ACC deaminase gene by two different DNA hybridization probes.

A: The two probes were obtained by digestion of the UW4 ACC deaminase gene with different restriction enzymes to obtain either the 5′ or 3′ end. **B**: Agarose gel and its corresponding Southern hybridization with the 388 bp *Pst*I-*Pst*I fragment as probe for the nine plasmids that were obtained from both UW4 and CAL2 libraries. Lane 1 and 11, 1 kb DNA marker; lane 2-10, p4U1, p4U2, p4U3, p4U4, p4U5, p4U6, p4U7, p2C1 and p2C2. **C**: Agarose gel and its corresponding Southern hybridization with the 388 bp *Pst*I-*Pst*I fragment as probe for plasmid p4U2 digested with different restriction enzymes: *Eco*RV+*Sma*I+*Hind*III (lane 2); *Sal*I+*Sma*I (lane 3); *Sma*I+*Hind*III (lane 4); *Hind*III+*Sma*I (lane 5); *Bam*HI+*Hind*III+*Sma*I (lane 6); *Sph*I+*Sma*I (lane 7); *Nco*I+*Hind*III+*Sma*I (lane 8); *Pst*I+*Sma*I (lane 9); *Bgl*II+*Hind*III+*Sma*I (lane 10); *Hae*II (lane 11); *Kpn*I+*Hind*III (lane 12); *Dra*I (lane 13); lane 1 and lane 14, 1 kb DNA marker. **D**: Agarose gel and its corresponding Southern hybridization with the 426 *Bst*EII-*Sal*I fragment as probe for plasmid p4U2 (lane 2), p2C1(lane 3) and p2C2 (lane 4). Lane 1 and lane 6, 1 kb DNA marker. All three plasmids were digested with *Sma*I and *Hind*III.


A
PstI
PstI
BstEII
SalI
ACC deaminase ORF
388 bp
426 bp


B
1 2 3 4 5 6 7 8 9 10 11 1 2 3 4 5 6 7 8 9 10 11
4.0
1.6

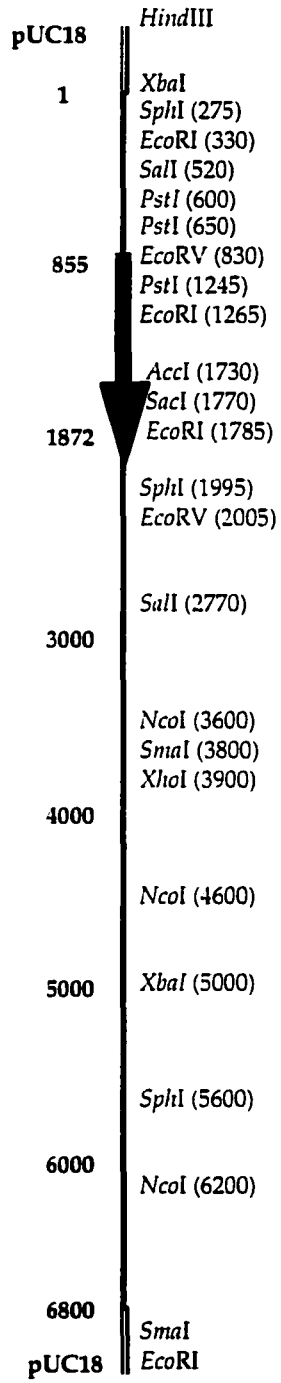

C
1 2 3 4 5 6 7 8 9 10 11 12 13 2 3 4 5 6 7 8 9 10 11 12 13
3.0
2.0
1.0


D
1 2 3 4 5 6 2 3 4 5 6
5.0
2.0
1.6

with *Sma*I and *Hind*III and both p4U2 and p2C2 showed positive hybridization bands whereas p2C1 still failed to hybridize with the probe (Fig. 7D). The reasons for the lack of hybridization of both probes to p2C1 was unclear. However, these results suggested that each of p4U2 and p2C2 may contain both upstream and downstream regions of the *acdS* coding region.

The data from the hybridization experiments together with the results from partial and double digestions of p4U2 with different restriction enzymes (results not shown), provided the information necessary to construct a restriction map of p4U2 (Fig. 8). Before the 5′ upstream region was completely sequenced, the location of the insert and its orientation were confirmed by PCR with four different primers. Two primers, P1 and P2, designed from the reported ACC deaminase genes and amplified an 800 bp fragment from *acdS* (Shah et al., 1998) and the other set of primers annealed with the vector, i.e. forward and reverse (kindly provided by Dr. B. Moffatt). The four primers were used in different combinations, such as P1 and P-forward, P1 and P-reverse, P2 and P-forward and P2 and P-reverse. Since the insert of p4U2 was 6.8 kb, and if the ACC deaminase gene was located towards one end of the insert, at least one PCR product should be obtained. Information gathered from the size of PCR product, and the set of primers used for the amplification, provided information regarding the location and orientation of the *acdS* gene on the insert. As expected, a PCR product of around 1750 bp was amplified from primers P2 and P-forward (Fig. 5B). Since primer P2 is inwards about 900 bp from the ATG start codon, the results suggested that the *acdS* coding region is located near the primer

Fig. 8. Restriction map of the insert of p4U2. The arrowhead indicates the direction and location of the ACC deaminase ORF in the 6.8 kb insert. Restriction endonuclease sites are as labeled and the numbers indicate the position of the site (in nucleotides) from the *Xba*I end of the cloned DNA.


pUC18
HindIII
1
XbaI
SphI (275)
EcoRI (330)
SalI (520)
PstI (600)
PstI (650)
855
EcoRV (830)
PstI (1245)
EcoRI (1265)
AccI (1730)
SacI (1770)
1872
EcoRI (1785)
SphI (1995)
EcoRV (2005)
SalI (2770)
3000
NcoI (3600)
SmaI (3800)
XhoI (3900)
4000
NcoI (4600)
5000
XbaI (5000)
SphI (5600)
6000
NcoI (6200)
6800
SmaI
pUC18
EcoRI

forward side on the vector and the upstream region is around 850 bp (1750 bp minus 900 bp). Furthermore, the results indicated that the *acdS* gene is located in an antisense orientation with respect to the *lac* promoter. This analysis was later confirmed by DNA sequencing.

Using the DNA sequences of the *acdS* coding regions obtained from p4U2 and p2C2, both flanking regions (upstream and downstream regions) were further sequenced by primer walking. The primers used for sequencing of the flanking regions were designed by Shah et al. (1998) and another primer, AB11442, was also designed to complete the sequencing for two DNA strands of the upstream region in p4U2. The sequence obtained from p4U2 is 3000 bp long and contains an 855 bp upstream region and a 1128 downstream region from the *acdS* ORF (Fig. 9); a total of 1715 bp was obtained from p2C2, which contains a completed *acdS* ORF and a 629 bp upstream region as well as a 69 bp downstream region (Fig. 10). A putative Shine-Dalgarno consensus ribosome-binding sequence (AAGGA) (Stormo et al., 1982) was identified 13 nucleotides upstream (-13) of the translation initiation codon (ATG) in both p4U2 and p2C2. In the 3'-noncoding region of the *acdS* ORF from strain UW4, an inverted repeat containing 12 base pairs in length was found (with a single underline in Fig. 9). Since this repeat sequence is potentially capable of forming a hairpin-like structure with eight GC pairs, it may represent a transcription termination site.

To search for multiple copies of the *acdS* gene in strain CAL2, the genomic DNA was separately digested with the restriction enzymes *Hind*III and *Sma*I loaded on an agarose gel and hybridized with the UW4 ACC deaminase gene (Fig. 11). The hybridization pattern yielded a single major

Fig. 9. Nucleotide sequence of the ACC deaminase from p4U2, a positive clone screened from the *E. cloacae* UW4 genomic library. The open reading frame corresponding to the ACC deaminase sequence (1017 bp) is shown in uppercase letters. A putative Shine-Dalgarno consensus ribosome-binding sequence was recognized and is labeled in lower case bold face type in the upstream region. In the downstream region an inverted repeat sequence signified by a single underline may represent a transcription termination site. Two inverted repeats located in the upstream region are indicated by the single underline; and two direct repeats, one linked to each other is represented with double underline, and another is indicated with the dotted underline.

tgtggacacaaatccttgaagggcatcagcatgggggggagcaacaaagaggggggggtca 60
aattgggcatcagtcggagcaccgtgcggaccaaacgtggagagggtgttccgcaaactg 120
caatgcaccacccgtgcggccgcaacgttgaaggcattgacgctgggggtgatttgagtt 180
tcacaggggaatttgcggggttgacaggaattggcgcagccccatgaacgagcggatttg 240
cctgacgcccggcaggtacaacaattggtcggcatgcaggcggttgaaaatatggctgtc 300
cttggtccgcaacaacatgaagtagttgaattcgcctgtcaccacgtggcattccatgca 360
gcccgagaatttttgcgcggcggttttgaaggcggcaaacgattccggggtggagcggtc 420
cagcaccacaccgatcaataccaccattcccgcatccagtgcttcggtgtcgagcagggc 480
cacgatggccttgatcaagccggcctgcttgagtcggtcgacgcgacgcagacaggcagg 540
cggactcaatttcacctttccgccaacgccacgttggagatggacgcgtctcgctgcag 600
cgccttcagaatggcacggtcggttcgatcaagctgcaacggctctgcagctttttcgtt 660
tgctttaacatggtttttttattgcgttcatattgtttttgcacaataacgttagtgttt 720
tgtttttatgtacggaataaagcatctattaattgctgtaatttgcaacacaaatttttca 780
ggtttttttcttaagattgattcatcgaagcctccgtaaaagagcttggatatcacaacaa 840
tc**aagga**acagcgcc**ATG**AACCTGAATCGTTTTGAACGTTATCCGTTGACCTTCGGTCCA 900
TCCCCCATCACTCCCTTGAAACGCCTCAGCGAGCACCTGGGCGGCAAGGTGGAACTGTAT 960
GCCAAGCGTGAAGACTGCAATAGCGGCCTGGCCTTCGGCGGGAACAAAACGCGCAAGCTC 1020
GAATATCTGATTCCCGAGGCCATCGAGCAAGGCTGCGACACCTTGGTGTCCATCGGCGGT 1080
ATCCAGTCGAACCAGACCCGCCAGGTCGCTGCGGTCGCCGCCCACTTGGGTATGAAGTGT 1140
GTGCTTGTGCAGGAAAACTGGGTGAACTACTCCGACGCTGTATATGACCGCGTCGGCAAC 1200
ATCGAGATGTCGCGGATCATGGGAGCGGATGTGCGGCTTGATGCTGCAGGTTTCGACATT 1260
GGAATTCGGCCGAGCTGGGAAAAgGCCATGAGCGATGTCGTGGAGCGCGGCGGCAAACCG 1320
TTTCCAATTCCGGCGGGCTGTTCCGAGCATCCCTATGGAGGGCTCGGGTTTGTCGGCTTC 1380
GCTAAGGAATTGCGGCAGCAGGAAAAGGATTTGGGCTTCAAGTTTGACTACATCGTGGTG 1440
TGCTCGGTGACCGGCAGTACCCAGGCCGGCATGGTCGTCGGTTTCGCGGCTGACGGTCGC 1500
TCGAAAAACGTGATCGGGGTCGATGCTTCGGCGAAACCGGAGCAAACCAAGGCGCAGATC 1560
CTGCGTATCGCTCGACATACCGCTGAACTGGTGGAGCTGGGGCGCGAAATCACTGAAGAG 1620
GATGTGGTGCTCGATACGCGTTTCGCCTATCCGGAATATGGCTTGCCCAACGAAGGGACG 1680
CTGGAAGCGATTCGCCTGTGCGGCAGTCTTGAGGGGGTGTTGACCGATCCGGTCTACGAG 1740
GGCAAATCCATGCACGGCATGATTGAAATGGTACGCCGCGGGGAATTCCCTGACGGCTCC 1800
AAAGTTCTTTATGCCCACCTGGGCGGCGCACCTGCGTTGAACGCCTACAGCTTCTTGTTT 1860
CGCAACGGC**TGA**gcttggcgccgccaggaaaacgcaggactgggtaggagcgggcaagcc 1920
cgcttctacagtaattgcgtcttccagtcgttccctttgccaactcaagtccaactcacg 1980
cgggagtaccgccatgcatgcgatatcgcagttcgccgtctgggtcaaagagcctctggc 2040
gaatgccggggttgtcatcgttaccagtgccgcgttacccaaatacgtgattgacaagct 2100
gcatgtggcgatcgacgactgggatcaggttgcctatctggtcgtcaagcaacccaggga 2160
gttgatgctcgactggttgcgtttcgggttcaattcagtggagtcaccacaggtctctag 2220
ttgtcacgcccagtgaacttgctgcgtttcggtatccaaaggctgcttttttactcatgtt 2280
gactcagcccataccgaacttgcctggctggggtctgtctgcgccatccattgcgtgtcg 2340
tgaatccgggaaatacaggccagtccaccgctgcgatggataaacaggtggagatgattt 2400
tatcgatgacccggaccttggcgaaaagcgtattgcaagaacgctgcgtcatttgacgaa 2460
gacagcatcaattcacctgttgaatcaaccactcaaataagaaagacaacgcctgtgagc 2520
cgaacttccgactatcgctttaaatccaggaagtgggtgcgcgagccttccagttgtacg 2580
cgcgtcgttgttgtcgcgatagggctcagtagtgaccacgttgatctgatacatcacggt 2640
ttcagttgcttcgacaacatcgccgctgatgcatgttcgacgccagccggacgccgggtt 2700
tctagtccgtcttgcaaccccggaattgtgactacttccctttgttatcccccgggaact 2760
tggataaccttttttttacatgaatttatcgaagccgccaaaacaagcatctcccaaata 2820
ccaaaggcgccccgaaccaactttccctcctttacccgtggaaccaattttaaccccctg 2880
gccccggtaatttcttcccccccctaaatttaaccccccggttttgcttcccttcccac 2940
ctttaaccattcccctaaaccttttttctccaggacccctcaaataaatccctttcacc 3000

Fig. 10. Nucleotide sequence of the ACC deaminase from p2C2, a positive clone screened from the *E. cloacae* CAL2 genomic library. The open reading frame corresponding to the ACC deaminase sequence (1017 bp) is shown in uppercase letters. A putative Shine-Dalgarno consensus ribosome-binding sequence was recognized and is indicated in lower case bold face type in the upstream region.

ggggcaagattcggaggggaggaggcacgcaaaaataattttaacatccg 50
ggaacttttttattgcatgcgctgtttgtaaaatggccaggattttggat 100
ggattccattaatttcgggcaggttgctggcaaacaaacagggtttcgtt 150
cacgattggcggattgcttaggagtttgtttttggcaattgagccggtta 200
cacgttggtgtttgcgacctttgccgaatgcctaaacgcaatacagatg 250
gatgatgtgcggagcgctctgggtcatctcgatccgtcccagtgacttcg 300
gtgaggacgatgccagagcgtagaggcttttaacgggtagccaagtatct 350
gtgtcgtttcggtagagatccagtggatgcggcactgggtctgtggtgcc 400
tggcttcttgtgaatatttgttgctttcatatcgatttctacgcaatgaa 450
agtatgttttgtacggcgtttgaaaagttatgtttctttatgtgatggaa 500
acttgcaacacatatttttggattcttcttaagattgaactcatcgaag 550
cccctcctgaaagagcctggaatacggccctttcccttttcggtctggcgc 600
ttggatatccaatatc**aagga**gcagagtc**ATG**AATCTGAATCGTTTTGAA 650
CGTTATCCATTGACCTTCGGTCCTTCTCCCATCACGCCCTTGAAGCGCCT 700
CAGTCAACATCTGGGGGGCAAGGTCGAGCTGTATGCCAAACGTGAAGACT 750
GCAACAGTGGCCTGGCCTTTGGTGGGAACAAGACGCGCAAGCTCGAATAC 800
CTCATTCCCGAAGCGATCGAGCAAGGTTGCGATACGCTGGTCTCCATCGG 850
CGGCATTCAGTCGAACCAGACCCGCCAGGTCGCTGCCGTCGCTGCCCACT 900
TGGGCATGAAGTGCGTGTTGGTGCAGGAAAACTGGGTGAACTATTCCGAT 950
GCGGTGTATGACCGAGTCGGCAACATCGAAATGTCGCGGATCATGGGCGC 1000
TGACGTGCGGCTTGACGCCGCTGGCTTCGATATTGGCATCCGGCCAAGTT 1050
GGGAAAAGGCCATGAGCGATGTCGTGGAACAGGGGGGTAAACCGTTTCCG 1100
ATTCCGGCGGGCTGCTCCGAGCATCCCTATGGCGGCCTCGGTTTCGTCGG 1150
CTTTGCCAAAAAATTGCGGCAGCAGGAAAAGGAACTGGGCTTCAAGTTTA 1200
ACTACATCGTGGTCTGCTCGGTGACCGGCAGTACGCAGGCGGGCATGGTT 1250
GTCGGTTTCGCGGCTGACGGTCGTTCTAAGAATGTGATTGGTGTCGATGC 1300
TTCGGCCAAGCCGGAACAGACCAAGGCACAGATCCTGCGCATCGCCAGAC 1350
ACACGGCTGAGCTGGTGGAGTTGGGGCGCGAGATTACGGAAGAGGACGTG 1400
GTGCTCGATACGCGTTTCGCCTACCCGGAATATGGCTTGCCCAACGAAGG 1450
TACATTGGAAGCAATCCGACTGTGCGGCAGCCTTGAAGGGGTGCTGACAG 1500
ATCCGGTGTACGAAGGTAAATCGATGCACGGCATGATTGAAATGGTCCGT 1550
CGTGGCGAATTCCCTGAAGGCTCCAAGGTGCTTTACGCACACCTGGGTGG 1600
GGCGCCGGCGCTGAACGCCTACAGCTTCCTATTCCGTGATGGC**TGA**atca 1650
ctagtgcggccgcctgcaggtcgaccatatgggagagctcccaacgcgtt 1700
ggatgcatagcttga

band approximately 3.2 kb in size for the *Sma*I digestion, and a single 7.5 kb fragment for the *Hind*III digestion. These results suggest that there is a single copy of the *acdS* gene in strain CAL2. The same conclusion for strain UW4 was obtained by Shah et al. (1998). However, the presence of an additional weak hybridization band in strain UW4 has been found when the stringency of hybridization was lower. On the other hand, it was surprising to find that no hybridization signals were found from other strains, such as CAL1, CAL3 and GR12-2, when the UW4 *acdS* gene was used as a probe regardless of whether high or low stringency was employed. The fact that these other PGPR strains have been shown to have ACC deaminase activity suggests that there may be different versions of ACC deaminase among the various strains (Shah et al., 1998).

3.1.4. Expression of an ACC deaminase gene in *Escherichia coli*

The plasmid p4U2, containing the ACC deaminase gene from *E. cloacae* UW4 together with its flanking regions on a 6.8 kb fragment, was transformed into *Escherichia coli* DH5α. When the host cell *E. coli* DH5α alone and the transformant containing p4U2 were grown on M9 minimal medium containing 3 mM ACC as the sole source of nitrogen, it was observed that native *E. coli* DH5α did not grow, while the p4U2 transformant gained the ability to use ACC as a sole source of nitrogen and was able to grow on ACC-minimal medium (overnight culture can reach 1.0 of OD600nm).

The specific activity of ACC deaminase from *E. coli* cells harboring p4U2 was determined at different cultural conditions and compared with

Fig. 11. Southern hybridization analysis of *Hind*III- or *Sma*I-digested genomic DNA of strain CAL2 using the UW4 *acdS* gene as a DNA hybridization probe. **A**: Agarose gel electrophoresis of genomic DNA. **B**: Autoradiogram of the nylon membrane-blotted DNA from the gel shown in panel A after hybridization with the ^{32}P-labeled *acdS* DNA probe. Lane 2-4, genomic DNA samples were digested with *Hind*III and loaded in the gel wells at three different quantities, 0.3, 0.8 and 1.5 μL; lane 5-7, genomic DNA samples were digested with *Sma*I and loaded as lane 2-4. Lane 7, 1 kb DNA ladder.

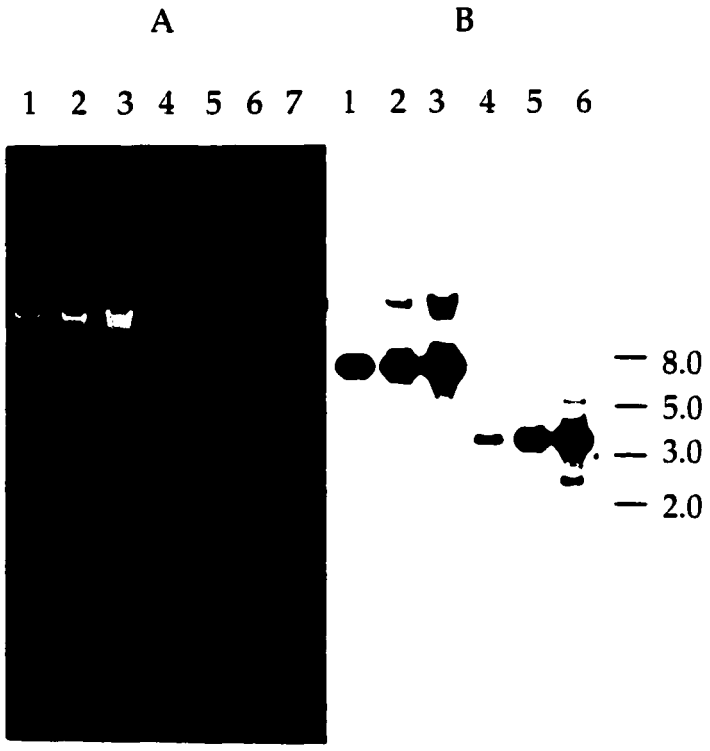

A
B
1 2 3 4 5 6 7 1 2 3 4 5 6
8.0
5.0
3.0
2.0

Table 5. ACC deaminase activity* of cell-free extracts of *E. coli* cells carrying either pUC18 or p4U2 (the ACC deaminase gene from *E. cloacae* UW4 inserted into pUC18) and *E. cloacae* UW4 grown in different media as indicated. For ACC induction, the cells from each treatment was inoculated at 1% and incubated in M9-minimal medium supplemented with 3 mM ACC for 2 days.

*: ACC deaminase activity is expressed in nanomoles per milligram of protein per hour.

nd*: indicates the data was undetectable since the cells did not grow in ACC medium.

nd: represents not determined.

IPTG: isopropyl-β-D-thiogalactoside. When it was used, the concentration of IPTG was 1 mM.

Medium	ACC deaminase activity* Strains/plasmid		
	E.coli /pUC18	*E. coli* /p4U2	*E. cloacae* UW4
Nutrient medium	14	104	27
Nutrient medium +IPTG	13	80	nd
Nutrient medium +ACC	nd	169	nd
Minimal medium with NH_4^+	24	74	52
Minimal medium with NH_4^++IPTG	17	69	64
Minimal medium with NH_4^++ACC	nd*	67	79
Minimal medium with ACC	nd*	328	346
Minimal medium with ACC+IPTG	nd*	305	nd

wild type *E. cloacae* UW4, with *E. coli*/pUC18 as a negative control. All three strains were grown in either nutrient (LB or TSB) or minimal medium, and either with or without ammonia, with or without ACC, and either with IPTG or not. The results (Table 5) showed that *E. coli*/p4U2 had a higher level of ACC deaminase activity in the medium with ACC as the sole source of nitrogen (328 nmoles/mg protein/h) and a lower activity level in the nutrient medium (104 nmoles/mg protein/h) as well as ammonia medium. The control *E. coli*/pUC18 had only a background level (14-24 nmoles/mg protein/h) and the activity was undetectable in ACC medium since they are unable to use ACC. These results indicate that the cloned *acdS* gene has been expressed in *E. coli* cells. The addition of IPTG, an inducer of the *lac* promoter, did not induce any reproducible increase in ACC deaminase activity in strain *E. coli*/p4U2, suggesting the *E. coli lac* promoter is not responsible for the observed expression of ACC deaminase in *E. coli*. The results agreed with previous analysis by PCR and DNA sequencing that showed that the *acdS* gene on p4U2 was inserted in an antisense orientation to the *lac* promoter in pUC18. Moreover, it was observed that when *E. coli*/p4U2 was grown in minimal medium with ACC acting as the sole source of nitrogen, it had the same high level of ACC deaminase activity as that of wild-type *E. cloacae* UW4, suggesting that the ACC deaminase gene from *E. cloacae* can be expressed normally in *E. coli* and that the insert on p4U2 contains the *acdS* gene promoter which can be recognized by *E. coli* RNA polymerase. The Km for ACC from the wild-type UW4 strain was determined by measuring ACC deaminase activity at different ACC

concentrations, and plotting the data as a Lineweaver-Burk plot, to be 10.7 ± 1.8 mM (Appendix V).

3.2. Manipulation of the ACC deaminase promoter

3.2.1. Analysis of the upstream sequences

The regions upstream of the *acdS* gene from both strains UW4 and CAL2 were sequenced (Fig. 9 and Fig. 10) and aligned with the upstream regions of ACC deaminase genes from other organisms (Fig. 12) (Shah et al., 1998). One distinct feature of the 5′ untranslated upstream region from strain UW4 is that it lacks 35 nucleotides (from -28 to -63) present in strains CAL2, ACP, and F17. Among these latter upstream regions there is a high degree of homology up to -130 bp from the ATG translation start codon (-93 in the case of strain UW4). Beyond this point, the homology is considerably lower, except for one small stretch of eight nucleotides located at position -175 (-140 in strain UW4). Therefore, it is possible that the promoter lies within -130 bp from the ATG start codon (-93 in strain UW4). However, sequences resembling the -35 (TTGACA) and -10 (Pribnow box: TATAAT) regions recognized by *E. coli* RNA polymerase (Deretic et al., 1989) were not found. The sequence GATATC, a putative Pribnow box, was found -23 bp from the ATG start codon.

Interestingly, several unusual repetitive sequences, including direct and inverted repeats, were found (using DNA Strider 1.2; Marck, 1988) within the -230 bp upstream region as indicated in Fig 9. Of the two inverted repeats, indicated by the underline in Figure 9, one is a 13 bp repeat with a two base pair mismatch (CGATCAAGCTGCA) and, the other is a

Fig. 12. Alignment of upstream regions of ACC deaminase genes. The numbering scheme shown decreases from the ATG start codon. Dashes represent nucleotides identical to those found in the *E. cloacae* UW4 sequence (or to *E. cloacae* CAL2 in the region of the 35-bp gap in *E. cloacae* UW4) and dots represent gaps in the sequences. The putative Shine-Dalgarno sequence (AAGGA) is double underlined. A sequence similar to -10 sequence (GATATC) is single underlined. For *Pseudomonas* sp. strain F17 and 6G5, all of the available sequence data is shown. The symbol ♦ indicates -130 position from the ATG translation start codon.

```
UW4    TCCGCCAACGCCACGTTGGAGATGGACGCGTCTCGCTGCAGC  -254
CAL2   AGA-A-G-T----GAGC-T---G-CTTTTAA-GG-TA--CAA  -285
ACP    G----G-G--AG--A-GT-CA-CT------C-GGCT-T--A-  -284

UW4    GCCTTCAGAATGGCACGGTCGGTTCGATCAAGCTGCAACGGC  -212
CAL2   -TA-CTGTGTC-TTT----A-A...----C--TG-ATG----  -246
ACP    CG-C--GAG--C--C--A---AGGC-C--G-T-GATTG-A--  -242

UW4    TCTGCAGCTTTTTCGTTTGCTTTAACATGGTTTTTTATTGCG  -170
CAL2   A---GGT--G-GGT-CC--GC--CTTG--AA-A---G----T  -204
ACP    ...-G-C--GCCAGCACT--AACGG-AA--G-AAGGGCAA-C  -203

UW4    TTCATATTGTTTTTTGCACAATAACGTTAGTGTTTTGTTTTT  -128
CAL2   -------C-A---C-A-G----G-AAG--.--------ACGG  -163
ACP    GCAG-CCGAC--GGGT-GTC-CG-AT--T.----GCA-CCA-  -162
                                        ♦
UW4    ATGTACGGAATAAAGCATCTATTAATTGCTGTAAT.TTGCAA   -87
CAL2   CGT-TGAA--GTT-TGT-TCT---TG--A--G--AC------  -121
ACP    -....--CATC-TT-C--ATCC--TG--AA-TC--A.----C-  -124

UW4    CACAAATTTTCAGGTTTTTTCTTAAGATTGAT.TCATCG.AA   -47
CAL2   ----T-----TT--A--C-------------AC------.--   -80
ACP    T--CT-------AAA--A-G.-CT--T-AA--T-TT---C--   -83


UW4    GCCTC.CGTAAAAGAGCTTG.....................   -28
CAL2   ---C-TCCTG-------C--GAATACGGCCCTTTCCCTTTCG   -38
ACP    --ACATTTCC--T-CAT---CCTAC-ATTT--A-A--GCGAA   -41
F17    ---C-T-.-G----CA-C-----CG-A---T---G-----G-   -39
6G5

UW4    ............GATATCACAACAA..TCAAGGAACAGCGCCATG 3
CAL2   GTCTGG.CGCTTG------.---T-...-------G---A-T---- 3
ACP    CGGAT-CT-T-C-CG-C--TTTTACCGA-------G-T--..---- 3
F17    ------.---------G--.---T--..A------G---A-T---- 3
6G5                ------C--T..-..-------G---A-T---- 3
```

9 bp repeat, with a two base pair mismatch (AATTGCTGT). Two direct repeats that are in close proximity to each other are separately represented with either dotted underline or double underline, as shown in Figure 9. One direct repeat is 11 bp with only a one base pair mismatch and contains eight thymines within the 11 base pairs. It is intriguing that the upstream region from -55 to -205 bp is a thymine-rich region that contains 48.7% thymine. Another direct repeat of 27-31 base pairs was found and contains 12 to 15 thymine residues. Although the function of these repeats is unclear, they may represent regions that interact with transcriptional factors. Furthermore, the GC content within the 205 bp upstream region was only 32.2%.

3.2.2. Detecting promoter activity in pKO-1

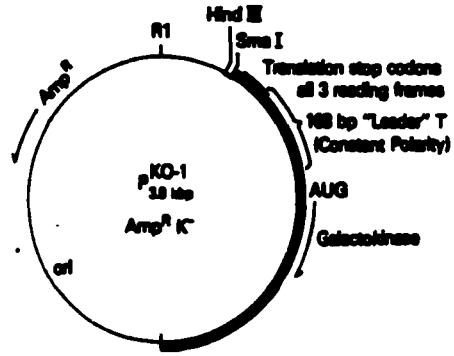
The vector pKO-1 is a transcriptional-fusion promoter-probe vector that carries a promoterless *E. coli galK* gene as a reporter gene (McKenney et al., 1981). The *galK* gene encodes the enzyme galactokinase, one component of the galactose operon, that catalyzes conversion of galactose into galactose-1-phosphate in the presence of ATP. The vector contains 168 bp of the region preceding the *galK* coding sequence. Ahead of this region are several unique restriction sites, such as *Eco*RI, *Hind*III and *Sma*I, that can be used to insert foreign DNA into the vector. Translation stop codons in all three reading frames, immediately ahead of the 168 bp region, are also present on this plasmid vector (Fig. 13A). The *galK* gene by itself is not expressed in plasmid pKO-1. Only when a DNA fragment that carries a promoter sequence inserted in the proper orientation is *galK* transcribed, and when the

plasmid is transformed into a host cell with the genotype $E^+T^+K^-$ (i.e., galactose epimerase positive, galactose transferase positive and galactose kinase negative), the expression of *galK* on a plasmid would complement the galactokinase deficient in the host cell, resulting in cells that can either grow on minimal medium containing galactose as a carbon source or grow on MacConkey galactose agar as red colonies.

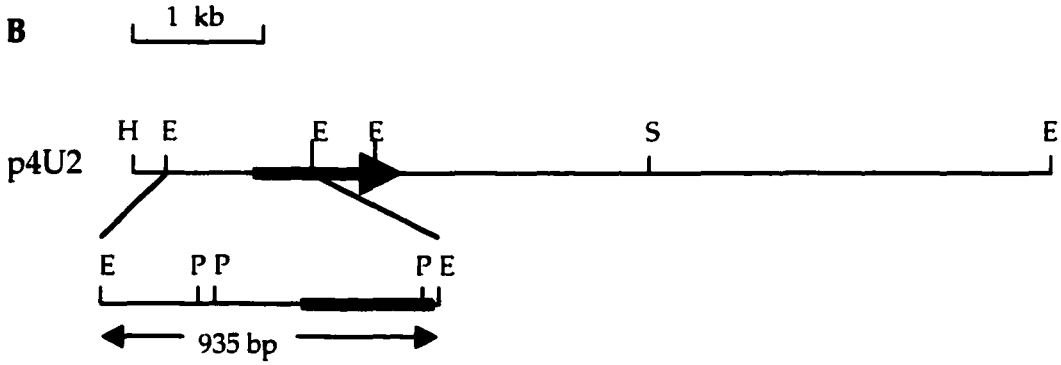
To use pKO-1 to detect the transcriptional activity of the upstream region of *acdS*, p4U2, carrying the upstream region of *acdS*, was digested with *Eco*RI. Four *Eco*RI-*Eco*RI fragments were obtained with sizes of, 4.9 kb, 3.0 kb, 0.9 kb and 0.6 kb. Among the digestion fragments, the 0.9 kb and 0.6 kb bands hybridized with an *acdS* probe. Since the 0.6 kb fragment was known to be located entirely within the *acdS* ORF, it should not contain the upstream region of *acdS*. According to the *Eco*RI partial digestion pattern, all *Eco*RI-*Eco*RI fragments were mapped in the p4U2 insert as shown in Fig. 13B, indicating that the 0.9 kb fragment contained 0.5 kb upstream from the ATG translation start codon. The mapping of p4U2 was confirmed later by DNA sequencing and the exact size of the upstream region within a 935 bp *Eco*RI fragment was 528 bp. The 935 bp fragment was ligated into pKO-1 and transformed into *E. coli* N100 ($E^+T^+K^-$), and transformants were selected on MacConkey plates as red colonies (Fig. 13C). The orientation of each insert was determined by digesting the plasmid with *Eco*RI and *Pst*I, respectively. Two different orientational inserts in relation to the ACC deaminase gene were obtained, named pJP92 (in an antisense orientation) and pJP96 (in a sense orientation) (Fig. 13D).

Fig. 13. Construction of pJP92 and pJP96 using vector pKO-1 and the preliminary analysis of the promoter upstream of the *acdS* gene. **A**: Plasmid pKO-1. **B**: The 935 bp *Eco*RI-*Eco*RI fragment from p4U2 was subcloned into pKO-1 to construct pJP92 and pJP96, respectively. The arrowhead indicates the direction and location of the ACC deaminase ORF. Plasmid pJP96 included the 935 bp fragment with the portion of *acdS* gene in the same the orientation as *galK* while in pJP92 it was in the opposite orientation. H: *Hind*III, E: *Eco*RI, S: *Sal*I, and P: *Pst*I. **C**: *E. coli* cells containing either pKO-1 (C1), pJP92 (C2) or pJP96 (C3) grown on MacConkey galactose agar plates. Cells with either pJP92 (C2) or pJP96 (C3) grew well and produced a red colour. **D**: Both plasmids pJP92 and pJP96 were digested with *Pst*I, and then separated by agarose gel electrophoresis to confirm that they were inserted in different orientations in pKO-1. Lane 1 was a 1 kb DNA ladder, lane 2: pJP92 digested with *Pst*I and lane 3 and 4: pJP96 digested with *Pst*I.

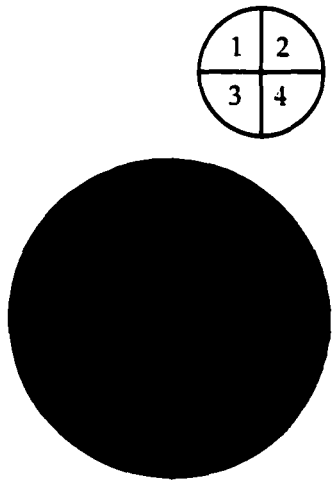
A


Hind III
Sma I
Translation stop codons
all 3 reading frames
168 bp "Leader" T
(Constant Polarity)
AUG
Galactokinase
ori

B


1 kb
p4U2
H E
E E
S
E
E P P
P E
935 bp

C


1 2
3 4

D


1 2 3 4
3.0
2.0
1.0
0.75

Table 6 Growth and β-galactokinase activity of different pKO-1 construct in different media.

*: β-galactokinase activity was determined in M36 minimal medium supplemented with galactose as a carbon source and expressed as nanomoles of galactose phosphorylated per mg protein.

**: Specific β-galactokinase activity, expressed as nanomoles of galactose phosphorylated per mg protein per min.

++: bacteria grew well

±: bacteria grew poorly

−: no bacterial growth

Medium	*E. coli* N100/pKO-1	*E. coli* N100/pJP92	*E. coli* N100/pJP96
MacConkey-galactose	± (white)	++ (red)	++ (red)
MacConkey-galactose + ACC	± (white)	++ (red)	++ (red)
M36 + galactose	_	++	++
β-galactokinase activity* (0 min)	68	221	81
β-galactokinase activity* (15 min)	137	561	179
β-galactokinase activity**	4.6	22.7	6.5

Both clones, *E. coli*/pJP92 and *E. coli*/pJP96, containing the 528 bp upstream region in different orientations, together with *E. coli*/pKO-1, were tested for their ability to grow on M36 minimal medium with galactose as the sole carbon source, and for galactokinase activity. Interestingly, both clones were able to grow on M36 minimal medium and both of their colonies were red on MacConkey galactose agar plates (Table 6). These results suggest that there are at least two promoters located in different orientations within the 528 bp upstream region of *acdS* gene. However, the results from galactokinase activity (Table 6) showed that the expression of *galK* from pJP96, the insert in the sense orientation, was very low (but never the less reproducible) when compared with the background level obtained from pKO-1. Surprisingly, the expression of *galK* gene was three-fold higher in pJP92, which contained the same insert as pJP96 except that in pJP92 it was the antisense orientation. Both putative promoters can be recognized by *E. coli* RNA polymerase, and one initiates transcription from the opposite strand of the ACC deaminase gene and is stronger.

3.2.3. Manipulation of the upstream region in pQF70

An alternative promoter-probe vector, pQF70, was also used in this study to analyze the activity of the ACC deaminase promoter. In this case, the promoterless luciferase genes (*luxAB*), from *Vibrio harveyi*, ligated into a partially deleted pBR322 to construct a new vector, pQF70, in which *luxAB* act as reporter genes for inserted bacterial promoters. A large multiple cloning site (MCS) derived from pUC18 and translational stop codons in all three reading frames were also present on the vector ahead of the *luxAB*

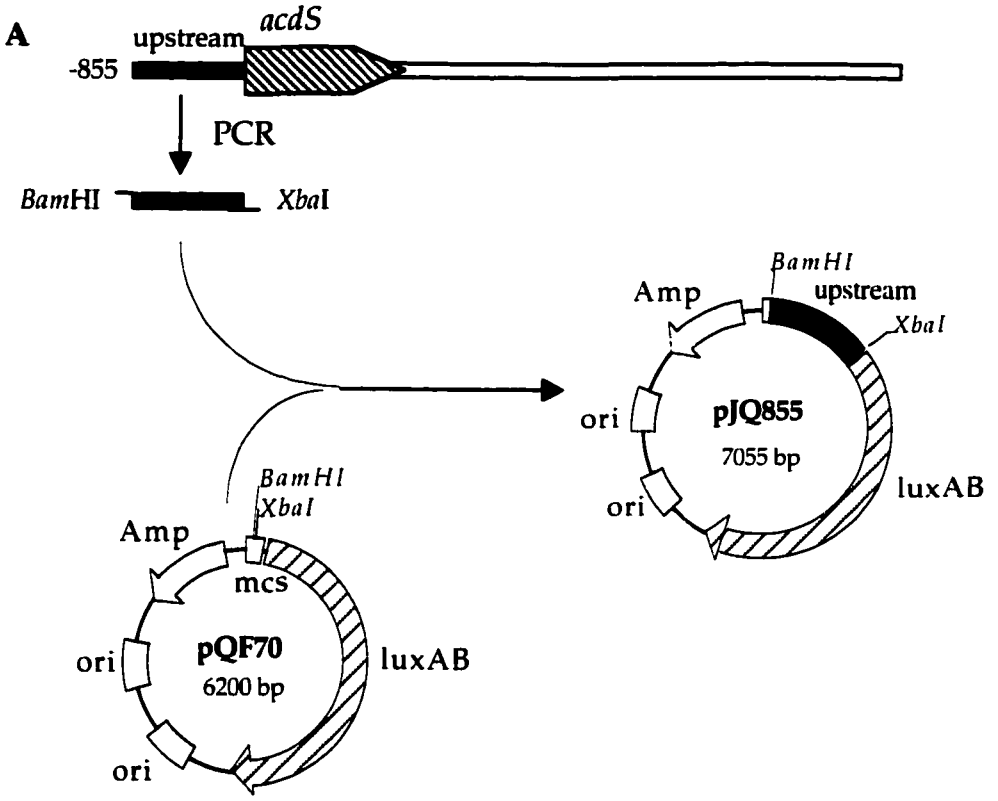
genes (Farinha and Kropinskin, 1990). The pQF70 promoter-probe construct used for detecting promoter activity has several advantages over other reporter vectors, including the absence of natural background bioluminescence in both soil and plants (Meighen, 1991), the presence of a broad-host-range origin of replication, the detection of luminescence is rapid in real-time analysis without the need for protein extraction, the system is 1,000-fold more sensitive than the commonly used *lacZ* marker (Prosser, 1994), and it can be easily quantified. It was reported that the *luxAB* genes, when used as reporter genes, provide a direct linear relationship between light output and cell concentration. However, the relationship can only be obtained from exponentially growing cells because the output of light needs energy (in the form of $FMNH_2$) from the cell (Rattray et al., 1990). Luminescence-marked organisms can be detected by several techniques: by eye in a darkened room, by X-ray film, by luminometry, by a scintillation counter and by charge-couple device (CCD) microscopy (Stewart and Willliams, 1992, Prosser, 1994).

In this study, the region of DNA that includes 855 bp upstream of the *E. cloacae* UW4 *acdS* gene in clone p4U2 was subcloned into pQF70 to construct a transcriptional fusion with the *luxAB* genes. The fusion was accomplished by using PCR primers to introduce restriction sites into the upstream region to facilitate the cloning of a completed upstream region. Two oligonucleotide primers, AB9647 carrying a *Bam*HI site from the -855 end, and another primer, AB9648, carrying a *Xba*I site from the +12 bp at ATG start codon, were used in PCR with p4U2 as the template. A 870 bp PCR product containing the 855 bp upstream region was amplified, purified,

digested with *Bam*HI and *Xba*I, and ligated into the same sites on pQF70. The plasmid that was generated by these manipulations designated as pJQ855 (where the number 855 represents the number of base pairs within the upstream region) (Fig. 14A), and transformed into *E. coli* DH5α.

Clones containing pJQ855 were selected on LB agar containing ampicillin and the plasmids from several colonies were isolated and digested with *Kpn*I and *Xho*I. Two digestion bands were obtained, 6.2 kb from pQF70 and a 870 bp band for the insert. Since the insert and ligation were performed with double restriction enzymes digestion, only the sense orientation could be obtained. Both *E. coli* DH5α/pJQ855 and *E. coli* DH5α/pQF70 were streaked on the same M9 minimal agar plate with ammonia, in duplicate, where one plate was supplemented with ACC (3 mM) and the other was not. The light output of the grown cells was detected by swabbing n-denyl aldehyde, a substrate for LuxAB, onto the inside of the petri dish lid followed by observation in a dark room. As expected, only *E. coli* DH5α/pJQ855 grown on M9 minimal medium with ACC produced light. On the same plate with ACC, *E. coli* DH5α/pQF70 did not produce any light. Furthermore, no light output was detected from cells containing either pJQ855 or pQF70 grown on M9 minimal agar without ACC. When the plates were placed on X-ray film for 1 min, the image of the light output from *E. coli* DH5α/pJQ855 on M9-ACC agar produced a dark black exposure on X-ray film (Fig. 14B). These results demonstrate that the 855 bp upstream region contains a promoter sequence that can initiate transcription of the ACC deaminase gene and that ACC is a necessary inducer for the promoter. Since the minimal medium used for detection of

Fig. 14. Cloning of the upstream region of the *acdS* gene from UW4 using vector pQF70 and analysis of the clones. **A**: A representation of the scheme used to clone the 855 bp upstream region of the ACC deaminase gene (*acdS*) from p4U2 into pQF70 by PCR to form pJQ855. Amp: ampicillin, ori: origin of replication, mcs: mutilple-cloning sites, *luxAB*: *luxAB* genes. **B**: Both *E. coli*/pJQ855 and *E. coli*/pQF70 were grown on the same M9-ammonia-containing agar plates. The strain on the top of each plate in each panel was *E. coli*/pJQ855 while in the lower part of plate it was *E. coli*/pQF70. The plates on the left in each panel contained ACC and plates on the right did not. The upper panel shows the photograph of the plates with incident light, and lower panel is an X-ray film resulting from exposure to the bioluminescence from *E. coli*/pJQ855 and *E. coli* /pQF70.


A
upstream
acdS
-855
PCR
BamHI
XbaI
BamHI
XbaI
Amp
mcs
pQF70
6200 bp
ori
ori
luxAB
BamHI
upstream
XbaI
Amp
pJQ855
7055 bp
ori
ori
luxAB

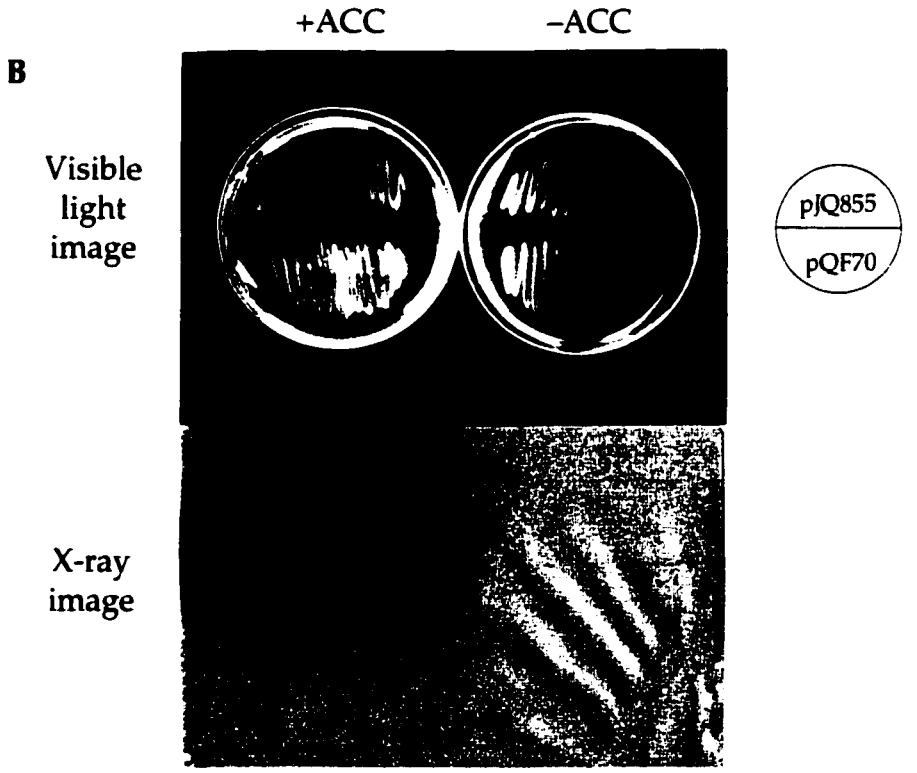
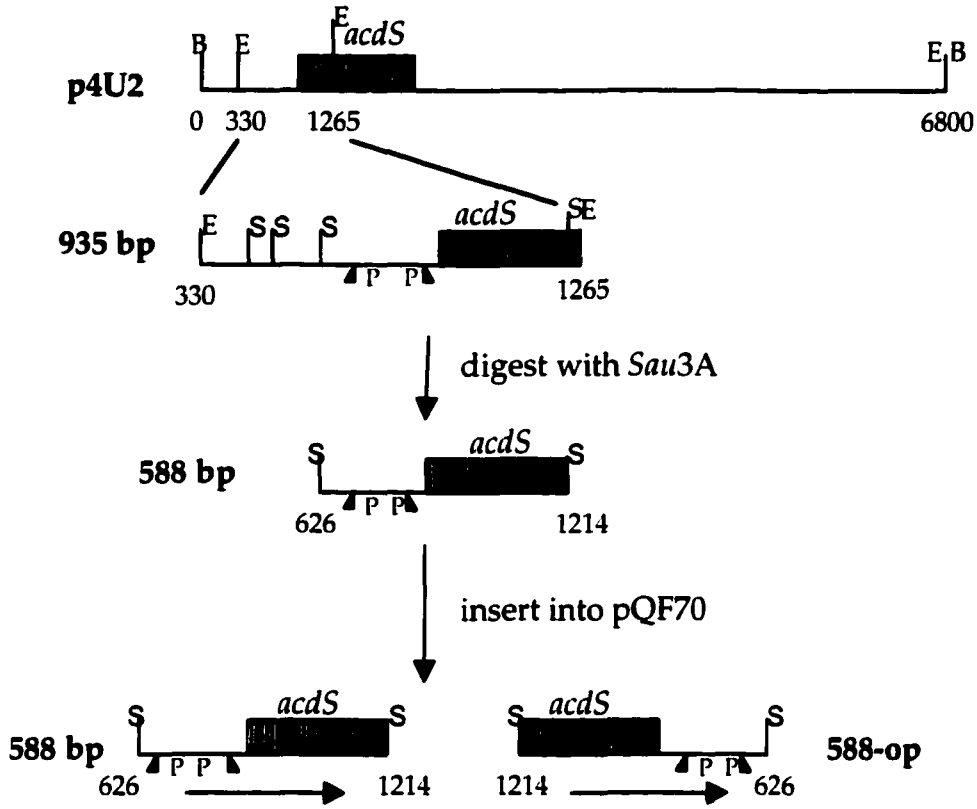
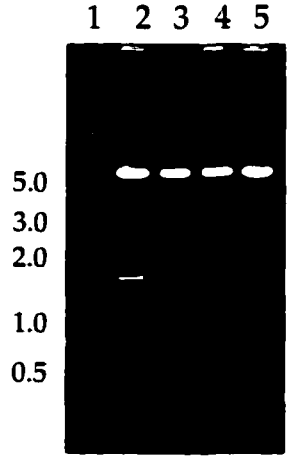

+ACC
-ACC
B
Visible light image
X-ray image
pJQ855
pQF70

Fig. 15. **A:** The scheme used to subclone a portion of the ACC deaminase upstream region into pQF70. The 935 bp *Eco*RI-*Eco*RI fragment was digested with *Sau*3A and the 588 bp *Sau*3A fragment was subcloned into pQF70 in both orientations to form pJQ588 and pJQ588-op. E: *Eco*RI, B: *Bam*HI, and S: *Sau*3A, P: promoter. **B**: The plasmids pJQ588 and pJQ588-op were digested with *Pst*I to confirm the orientation. Lane 1, 1 kb DNA ladder, lane 2, pJQ588, lane 3-4, pJQ588-op, lane 5, pQF70.

A


p4U2
B
E
acdS
E B
0 330 1265 6800
935 bp
E S S S acdS S E
330
P P
1265
digest with Sau3A
588 bp
S acdS S
626
P P
1214
insert into pQF70
588 bp
S acdS S
626
P P
1214
S acdS S
1214
P P
626
588-op

B


1 2 3 4 5
5.0
3.0
2.0
1.0
0.5

luminescence contained ammonia which was required for the bacteria to grow, and luminescence was obtained from the minimal medium that contained ACC, it indicated that induction of ACC deaminase can also occur in the presence of ammonia as a source of nitrogen.

3.2.4. Deletion of the upstream region in pJQ855

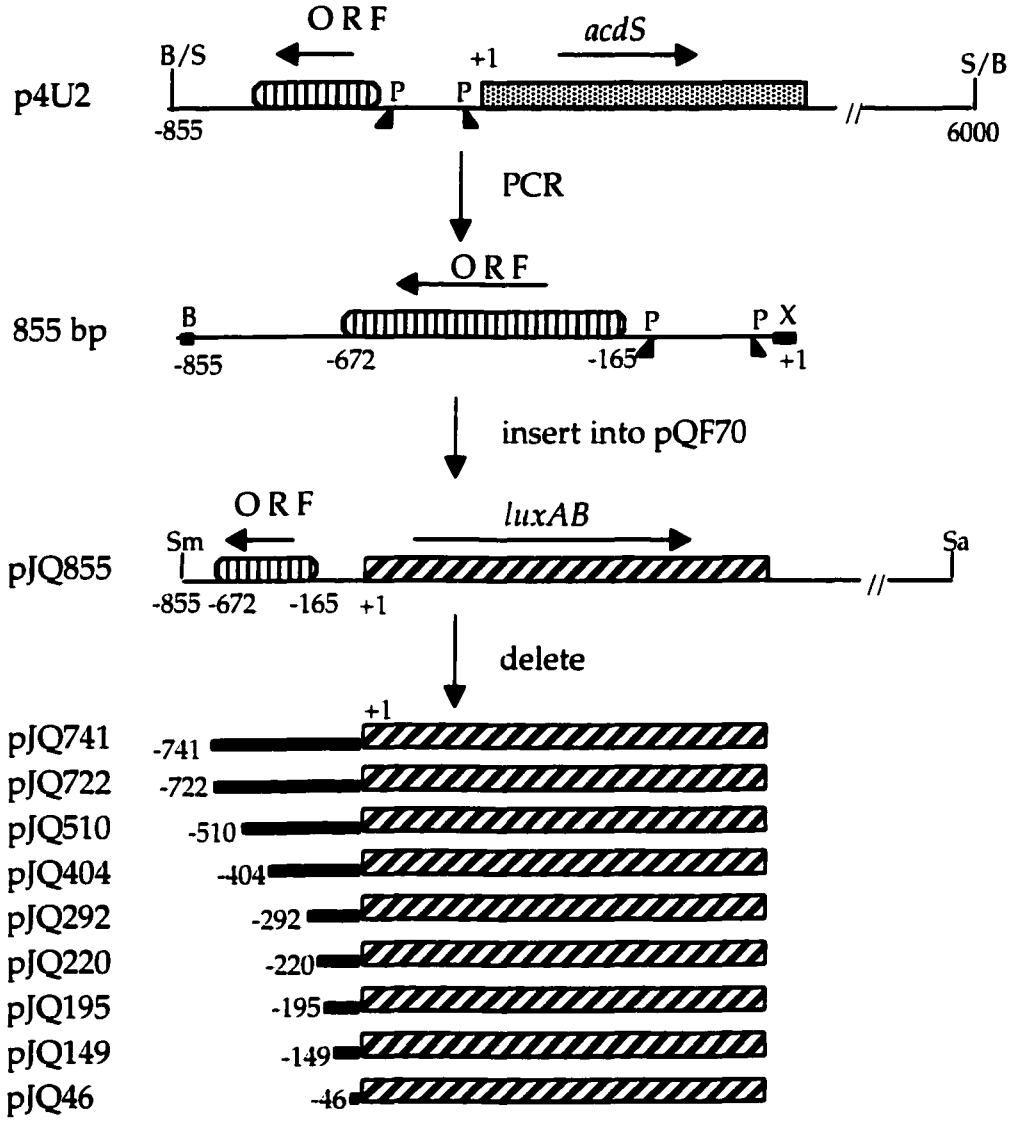
In order to precisely identify the location of the promoter within the 855 bp upstream region, the 935 *Eco*RI-*Eco*RI fragment from p4U2 containing the *acdS* upstream region was further digested with *Sau*3A. The five fragments obtained from the digestion were 115 bp, 60 bp, 134 bp, 588 bp and 47 bp in size (Fig. 15A), as expected from the sequence. The 588 bp fragment contained the ATG start codon and 229 bp directly upstream from the *acdS* gene. When the 588 bp fragment was subcloned into pQF70, two opposite orientations were obtained, named pJQ588 and pJQ588-op (the latter represents the insert in an antisense orientation; Note: pJQ588 is different from pJQ855 and the number behind pJQ represents the number of base pairs within the upstream region) (Fig 15B). Both transformants were analyzed for light output on M9-ACC plates, and only the cells containing pJQ588-op produced detectable light. As with pJP92 and pJP96, activity of the reporter gene (*galK*) was higher where the insert was in the antisense orientation, compared to the low activity measured when the insert was in the sense orientation. These results confirm that there is one promoter on the opposite strand and it is localized within the 229 bp upstream region of the ACC deaminase gene. However, the location of the ACC deaminase promoter was unclear.

Since light output was obtained from pJQ855 in which the whole 855 bp upstream region was cloned into pQF70 in the sense orientation, more specific localization of the promoter region could be achieved by deleting the 855 bp portion from the -855 position toward the ATG start codon of the ACC deaminase gene while monitoring the light production. Using the "Erase-a-base" method (see material and methods), a series of deletions were created, and each deletion construct was transformed into *E. coli* DH5α. The plasmids from each deletion construct were isolated and digested for size determination on an agarose gel. Accurate determination of the position of the deletions was made using DNA sequencing with primer 6, primer 7 and primer 8 (section 2.9). In total, nine deletion constructs, with precise sizes calculated from DNA sequencing were obtained; they were: pJQ741, pJQ722, pJQ510, pJQ404, pJQ292, pJQ220, pJQ195, pJQ149 and pJQ46 (the number after JQ represents the number of base pairs of the upstream region remaining) (Fig. 16). The activities of LuxAB, as output of light, from the nine clones were quantified on a CytoFluor Reader.

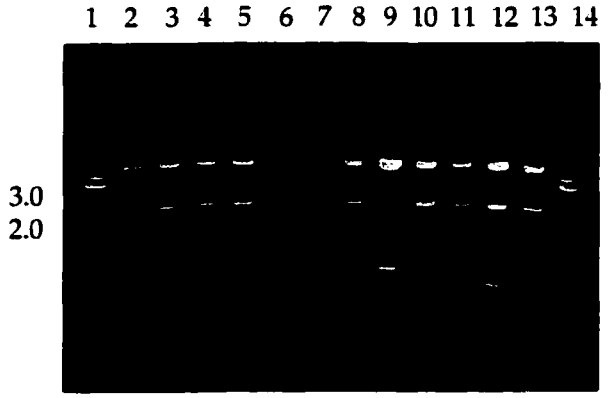
Before quantification of the luminescence from ACC promoter-driven LuxAB expression, the optimal conditions for determination of luminescence on the CytoFluor Reader had been determined and set up for later use in the experiments (see Materials and Methods). Several factors that affected the induction of the ACC promoter and optimal detection were also tested. For example, ACC induction time, duration of induction, and bacterial concentration were all optimized. For each test, the bacterial cells were freshly inoculated and grown in M9 minimal medium containing

Fig. 16. Deletion analysis of the upstream region of the ACC deaminase gene in pJQ855. **A**: Schematic representation of the deletion of the upstream region of the UW4 ACC deaminase gene (*acdS*) in pJQ855. The 855 bp upstream region from p4U2 was subcloned into pQF70, which contains promoterless *luxAB* genes. The upstream region contains two putative promoter regions (P) that both could initiate transcription, each gene in a different direction. Sequence analysis revealed the presence of a small ORF that starts from -165 and ends at -672. Nine constructs that include the different deleted fragments in the upstream region were obtained and named according to the positions of the deletions. B: *Bam*HI, S: *Sma*I, X: *Xba*I, Sm: *Sma*I and Sa: *Sal*I, P: promoter region. **B**: An agarose gel showing the deleted plasmids digested with *Hind*III and *Sca*I to estimate the size of the deletion.

A


ORF
acdS
B/S
+1
S/B
p4U2
P
P
-855
6000
PCR
ORF
855 bp
B
P
P X
-855
-672
-165
+1
insert into pQF70
ORF
luxAB
Sm
Sa
pJQ855
-855 -672 -165 +1
delete
+1
pJQ741
-741
pJQ722
-722
pJQ510
-510
pJQ404
-404
pJQ292
-292
pJQ220
-220
pJQ195
-195
pJQ149
-149
pJQ46
-46

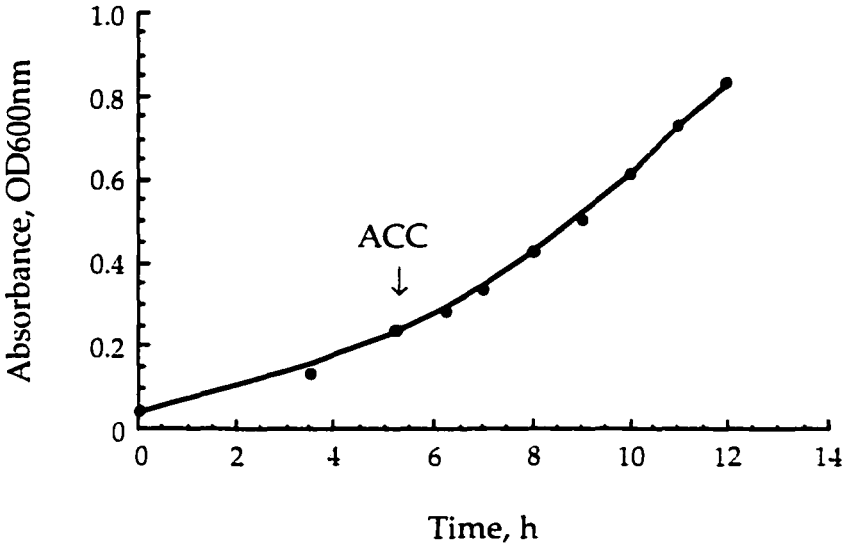
B


1 2 3 4 5 6 7 8 9 10 11 12 13 14
3.0
2.0

ammonia with shaking. Tests were carried out using different periods of incubation prior to the addition of ACC. ACC was added to separate cultures at 4 h, 6 h and 8 h after initial inoculation and it was found that the highest level of luminescence due to ACC promoter-driven *luxAB* expression was obtained from induction at 4 hours after inoculation, while the lowest luminescence was observed at 8 h (Fig. 17B) and even lower when induction occurred at stationary phase (data not shown). Thus, the best time for ACC induction is at 4 hours after bacterial inoculation, and at that time the bacterial growth is close to an OD_{600nm} of 0.2 (Fig. 17A). Furthermore, after induction, the cell culture was continuously grown with shaking, and samples were taken at different time intervals for the detection of luminescence. The results showed that with *E. coli* DH5α/pJQ855 the highest luminescence (750 fluor unit) was obtained at 1.5 h after induction. No luminescence was detected from *E. coli* DH5α/pQF70 and *E. coli* DH5α/pJQ855 that was incubated without ACC induction (Fig. 18A). This indicated that the 855 bp upstream region contained a promoter that was induced by ACC. The width of the luminescence peak may depend on the strength of the promoter and metabolic activity of cells. A direct relationship between luminescence and culture concentration was obtained by comparing the luminescence of different cell concentrations. Results showed that the direct relationship was maintained for cell concentrations up to 10^8 cfu (colony-forming unit) per milliliter (Fig. 18B), and no luminescence at all was detected at this concentration from *E. coli* DH5α/pQF70. The luminescence activity was expressed as fluor units

Fig. 17. Induction time of ACC for luminescence detection of *E. coli*/pJQ855. **A**: An *E. coli* DH5α growth curve in M9-ammonia minimal medium. Bacteria growth at each point was obtained by an average value of duplicate measurements. The arrow marks the time at which ACC was added (at a final concentration of 1 mM) and the time the culture reached an OD_{600nm} of 0.2. **B**: Luminescence of *E. coli* DH5α/pJQ855 induced by ACC (1 mM) at different times during the cell incubation. All experiments were carried out in triplicate. The error bars indicate standard error.

A


Absorbance, OD600nm
1.0
0.8
0.6
0.4
0.2
0
ACC
0
2
4
6
8
10
12
14
Time, h

B

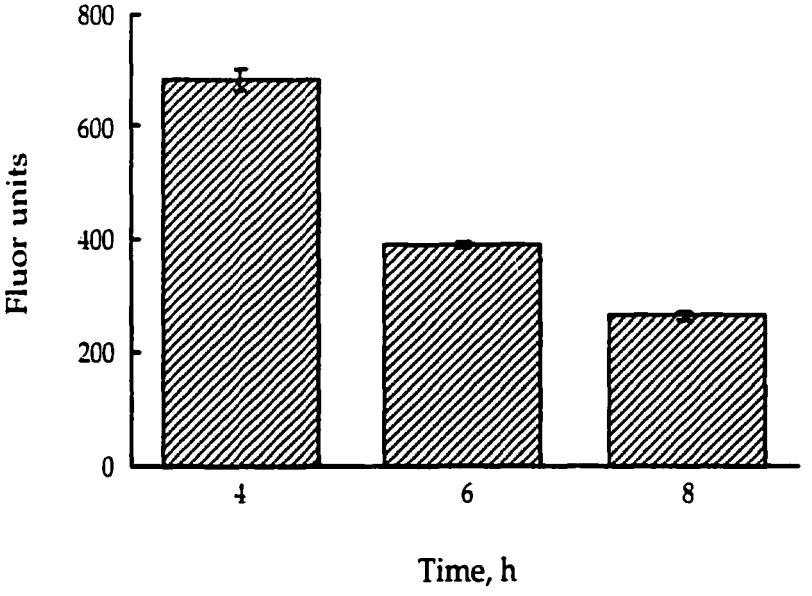

Fluor units
800
600
400
200
0
4
6
8
Time, h

Fig. 18. Luminescence of *E. coli*/pJQ855 and *E. coli*/pQF70 determined with induction time course and at different bacterial concentrations. **A**: Luminescence of *E. coli* DH5α/pJQ855 (♦) and *E. coli* DH5α/pQF70 (●) following ACC induction (at 5 h). **B**: Luminescence of *E. coli* DH5α/pJQ855 (♦) and *E. coli* DH5α/pQF70 (●) under different cell concentrations. The bacterial cells were cultured in M9-ammonia minimal medium with 1 mM ACC as an inducer. The experiments were carried out in triplicate. The error bars represent the standard error at each point.

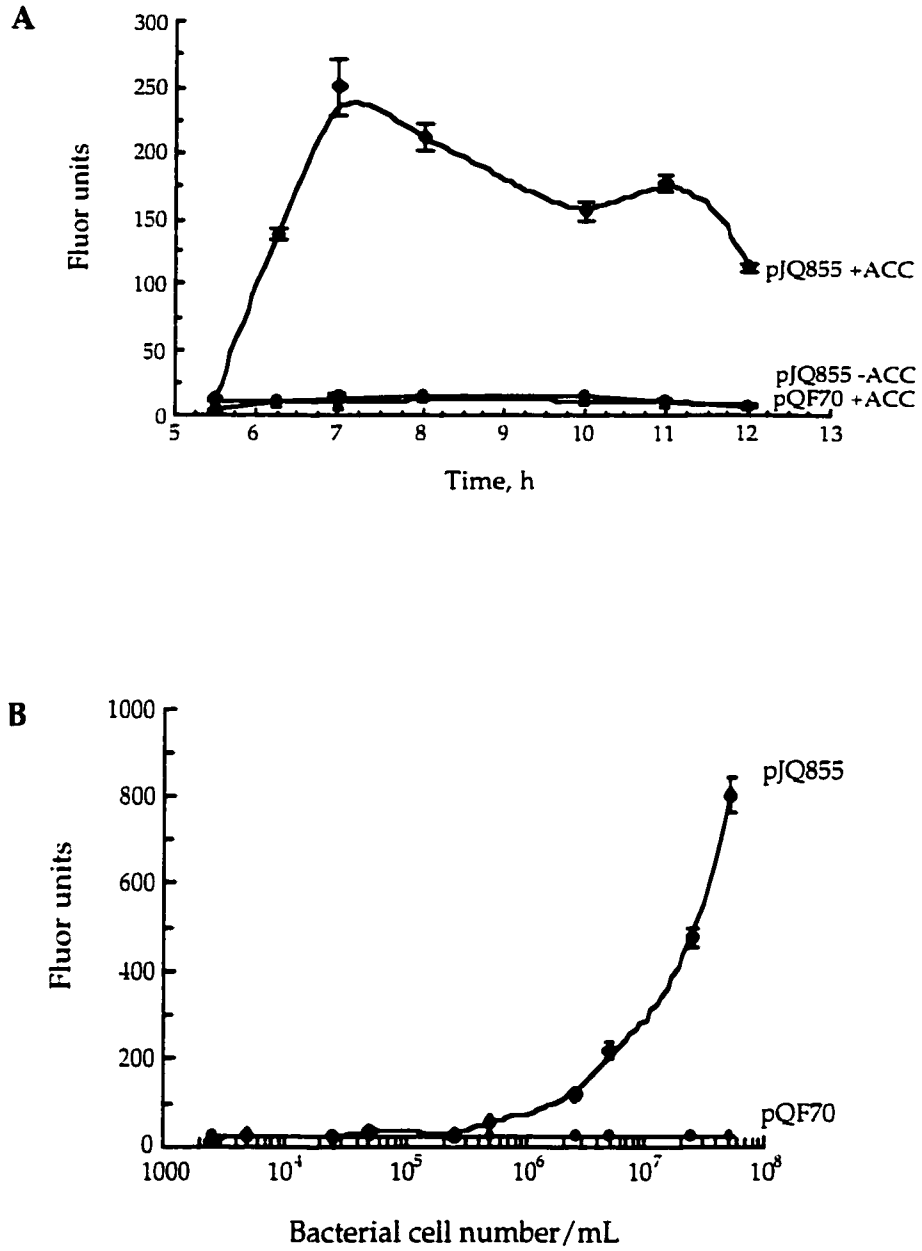
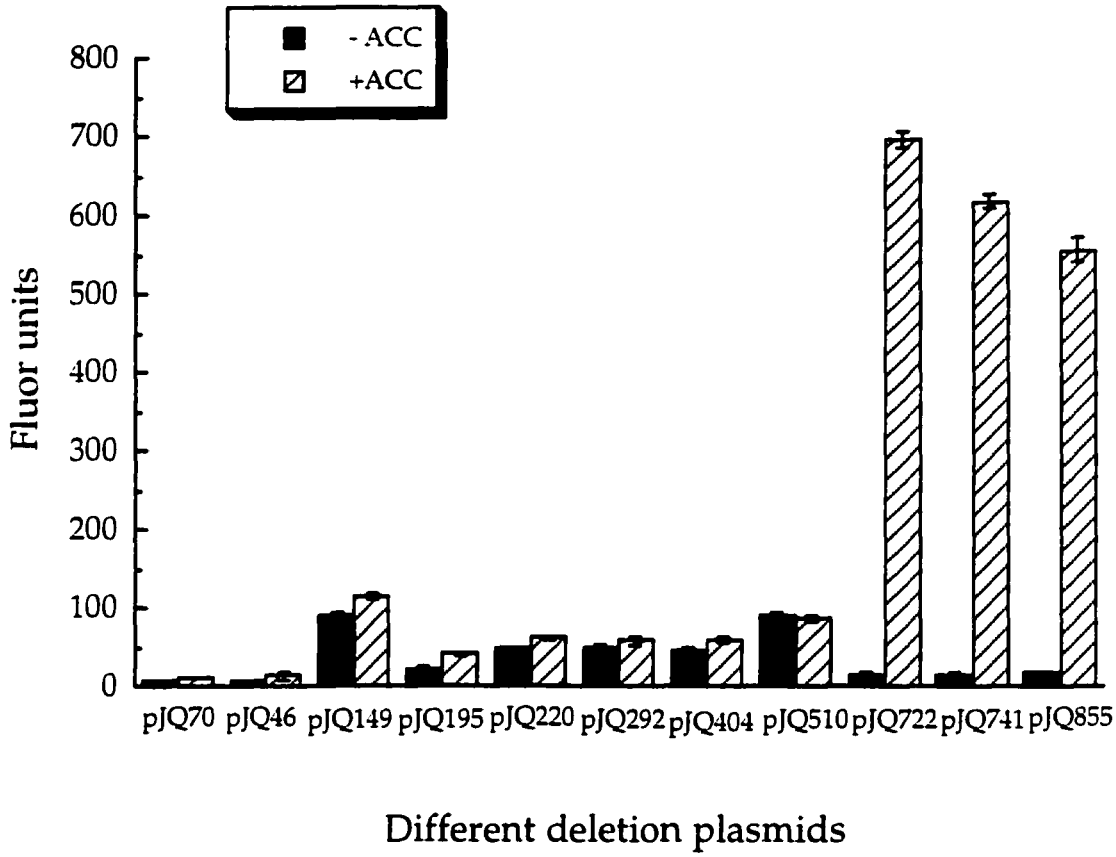

A
Fluor units
300
250
200
150
100
50
0
5
6
7
8
9
10
11
12
13
Time, h
pJQ855 +ACC
pJQ855 -ACC
pQF70 +ACC
B
Fluor units
1000
800
600
400
200
0
1000
10^4
10^5
10^6
10^7
10^8
Bacterial cell number/mL
pJQ855
pQF70

Fig. 19. Luminescence of the nine deletion plasmids of pJQ855 in *E. coli* DH5α, and of *E. coli* DH5α/pJQ855 (as a positive control) and *E. coli* DH5α/pQF70 (as a negative control). The cells from each construct were incubated with and without ACC induction. The error bars represent the standard error at each point (n=3).


- ACC
+ACC
800
700
600
500
400
300
200
100
0
Fluor units
pJQ70
pJQ46
pJQ149
pJQ195
pJQ220
pJQ292
pJQ404
pJQ510
pJQ722
pJQ741
pJQ855
Different deletion plasmids

(fluorescence counts) per $1x10^8$ cfu using the standard cfu curve verse the absorbance of bacterial growth (OD_{600nm}) (Appendix III and IV).

Under the conditions mentioned above, the luminescence of LuxAB from the nine upstream-deleted clones of *E. coli* DH5α/pJQ855 as well as both *E. coli* DH5α/pJQ855 (as positive control) and *E. coli* DH5α/pQF70 (as negative control) was detected (Fig. 19). Each deletion clone containing a different upstream fragment was incubated in M9-ammonia minimal medium and treated either with or without ACC. Without ACC induction, in all cases there was only a low level of luminescence; however, with ACC induction it was found with plasmids that contained an upstream region beyond -722 bp (pJQ741 and pJQ721) the luminescence was maintained at a level similar to the positive control pJQ855 (750 fluor units). Once the upstream region became shorter than -722 bp, the luminescence decreased dramatically to below 100 fluor units. These results indicate that the *acdS* promoter is not only induced by ACC, but also needs a -722 bp region to initiate transcription. On the other hand, when the upstream region was shortened up to -510 bp, a lower luminescence (50 to 100 flour units) was detected and the same level was found from -510 bp to -149 bp, suggesting that weak transcription was possible with just this upstream region. Although the luminescence was lower in this region, a similar luminescence between ACC induction and no ACC induction was observed, suggesting the weak transcription by this region was not be affected by ACC. However, no luminescence could be detected when the upstream region was shorter than -46 bp. This indicated that the -46 bp region is not long enough to harbor a promoter region.

3.2.5. Analyzing the regulation of the promoter

A search of the databases using sequence information from the region of the UW4 DNA upstream of *acdS* showed that there was an open-reading frame of 510 nucleotides in length that encoded 169 amino acids plus a stop codon (Grichko and Glick, in preparation). This small protein starts from position -166 bp and ends at -673 bp on the opposite strand. The deduced amino acid sequence from the DNA sequence is shown in Fig. 20. Based on the results of the deletion experiments, this ORF appears to be a necessary component of the ACC-dependent transcription of *acdS*. Thus, based on the assumption that this small protein may be a regulator controlling the expression of the ACC deaminase structural gene (*acdS*), its gene was named *acdR*, previously called this protein Bacca, although it is referred to as AcdR in this thesis (Grichko and Glick, in preparation).

To determine whether or not this small protein in the upstream region is a member of the promoter-regulater, the amino acid sequence of AcdR, as deduced from the upstream DNA sequence, was analyzed for sequence similarity (Altschul et al., 1997) by BLAST sequence searches in GenBank database. The results showed that this small protein, i.e. AcdR, has a 36.6% amino acid identity to the leucine-responsive regulatory protein (Lrp) of *E. coli* (Willins et al., 1991). The Lrp-like proteins have recently been reported to be major promoter-regulators in many bacteria (Friedberg et al., 1995).

Alignment of the amino acid sequence of AcdR with other reported Lrp proteins from different bacterial strains (Fig. 21) showed that the

Fig. 20. The deduced amino acid sequence of the ORF upstream of the UW4 *acdS* gene. The ORF starts from position -166 and ends at -673. Both putative -10 and -35 regions and Shine-Dalgarno sequence (SD) are bolded. Several direct and inverted repeats are underlined as in Fig. 9.

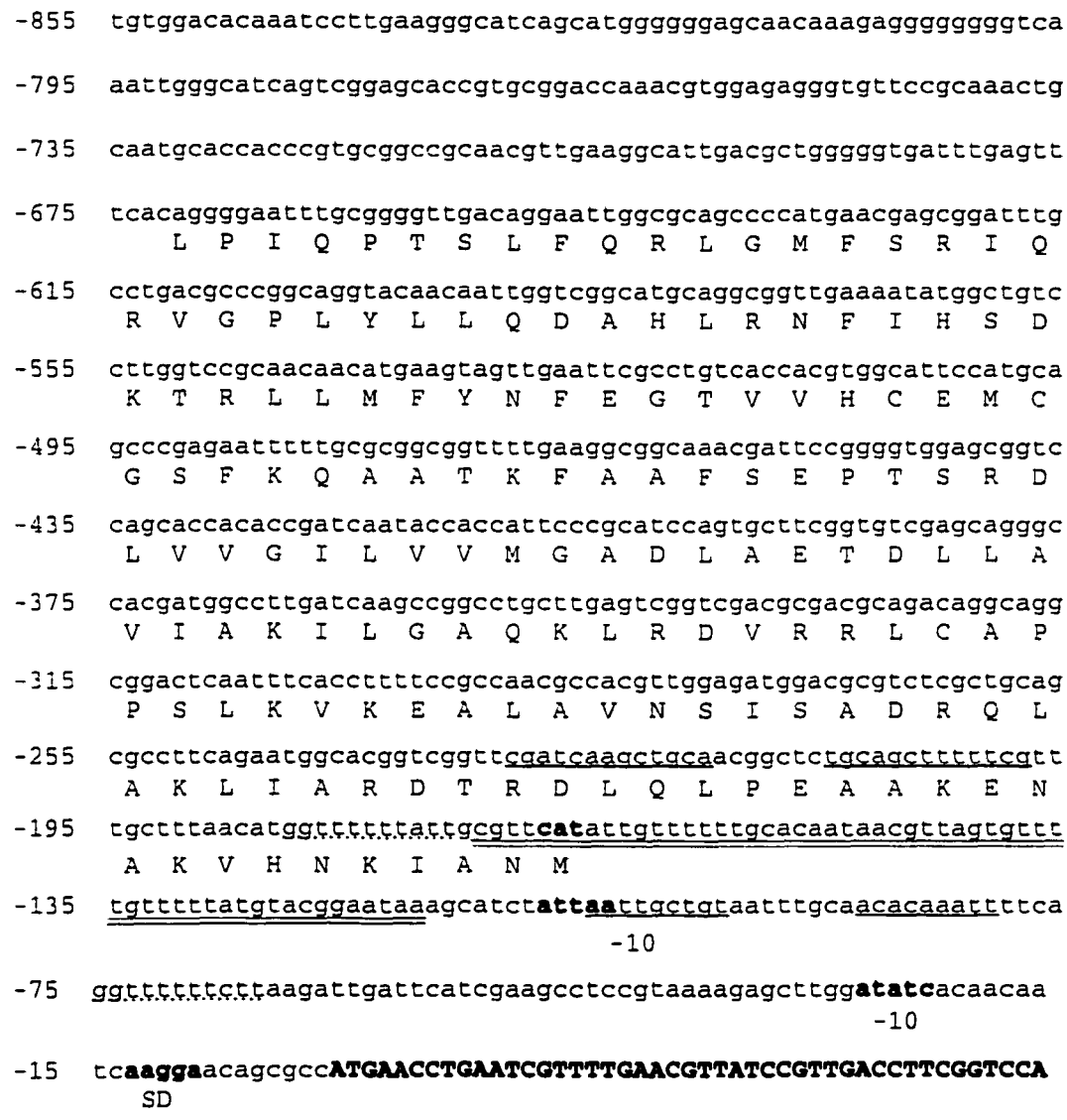

-855 tgtggacacaaatccttgaagggcatcagcatgggggggagcaacaaagagggggggtca
-795 aattgggcatcagtcggagcaccgtgcggaccaaacgtggagagggtgttccgcaaactg
-735 caatgcaccacccgtgcggccgcaacgttgaaggcattgacgctgggggtgatttgagtt
-675 tcacaggggaatttgcggggttgacaggaattggcgcagccccatgaacgagcggatttg
L P I Q P T S L F Q R L G M F S R I Q
-615 cctgacgcccggcaggtacaacaattggtcggcatgcaggcggttgaaaatatggctgtc
R V G P L Y L L Q D A H L R N F I H S D
-555 cttggtccgcaacaacatgaagtagttgaattcgcctgtcaccacgtggcattccatgca
K T R L L M F Y N F E G T V V H C E M C
-495 gcccgagaatttttgcgcggcggttttgaaggcggcaaacgattccggggtggagcggtc
G S F K Q A A T K F A A F S E P T S R D
-435 cagcaccacaccgatcaataccaccattcccgcatccagtgcttcggtgtcgagcagggc
L V V G I L V V M G A D L A E T D L L A
-375 cacgatggccttgatcaagccggcctgcttgagtcggtcgacgcgacgcagacaggcagg
V I A K I L G A Q K L R D V R R L C A P
-315 cggactcaatttcacctttccgccaacgccacgttggagatggacgcgtctcgctgcag
P S L K V K E A L A V N S I S A D R Q L
-255 cgccttcagaatggcacggtcggttcgatcaagctgcaacggctctgcagcttttttcgtt
A K L I A R D T R D L Q L P E A A K E N
-195 tgctttaacatggtttttttattgcgttcattgtttttgcacaataacgttagtgttt
A K V H N K I A N M
-135 tgtttttatgtacggaataaagcatctattaattgctgtaatttgcaacacaaatttttca
-10
-75 ggtttttttcttaagattgattcatcgaagcctccgtaaaagagcttggatatcacaacaa
-10
-15 tcaaggaacagcgccATGAACCTGAATCGTTTTGAACGTTATCCGTTGACCTTCGGTCCA
SD

Fig. 21. Alignment of the deduced amino acid sequence of AcdR with the reported sequences of Leucine-responsive regulatory proteins (Lrp) from different strains. The bold letters indicate residues in the protein AcdR that are identical with at least one other aligned protein and are labeled above with asterisks. A bold asterisk represents residues that are common to all the aligned proteins. The presumed helix-turn-helix motif is underlined. The numbers beside sequences represent the numbers of nucleotides in each sequence.

AcdR: Lrp-like protein found in the upstream region of the ACC deaminase gene.

Eco: Leucine-responsive regulatory protein (Lrp) from *Escherichia coli*, accession: JH0412.

Hin: Leucine-responsive regulatory protein from *Haemophilus infuenzae Rd*, accession: U32833.

Bja: Leucine-responsive regulatory protein from *Bradyrhizobium japonicum*, accession U85623.

Bsu: Leucine-responsive regulatory protein from *Bacillus subtilis*, accession: U93876.

BkdR: Bkd operon transcriptional regulator from *Pseudomonas putida*, accession: P42179

Ppu: Lrp-family transcriptional regulator protein from *Pseudomonas putida*, accession: D89015.

```
          *    **  **     **  *    *********  **********
AcdR      MNA IKNHVK ANEKAAEPLQ LDRTDRAILK ALQRDASISN   39
Eco       M       VD SKKRPGK  D LDRIDRNILN ELQKDGRISN   31
Hin       MSKEIKKMEK KRNKA      LDAIDIKILN ELQRNGKISN   35
Bja       M                  E LDRLDRRILS ILQEDGRIAN   22
Bsu       MG                IM LDETDKAILR DLQEDASISN   24
BkdR      MR                 K LDRTDIGILN SLQENARITN   23
Ppu       M                PSA IDRTDRALLA ALQDNARLTV   24

          * ***** ** * ****** * *  ** **   ***** ****
AcdR      VALAEKVKLS PPACLRRVDR LKQAGLIKAI VALLDTEALD   79
Eco       VELSKRVGLS PTPCLERVRR LERQGFIQGY TALLNPHYLD   71
Hin       IDLSKKVGLS PTPCLERVKR LEKQGVIMGY RALLNPELLD   75
Bja       VELAERIGLS PTSIGERLKR LQREGFVEGY GARLNPHRLG   72
Bsu       LNLSKKIGLS PSACLARTKN LVEAGIIKKF TTIVDEKKLG   74
BkdR      AELARSVNLS PTPCFNRVRA MEELGVIRQQ VTLLSPEALG   73
Ppu       AELADSVALT TSPCWRRVKL LEESGYITGY QAILSPKALG   74

          **  *** *  *** *****  ** ** ***    **** ***
AcdR      AGMVVLIGVV LDRSTPESFA AFKTAAQKFS GCMECHVVTG  119
Eco       ASLLVFVEIT LNRGAPDVFE QFNTAVQKLE EIQECHLVSG  111
Hin       APLLVIVEIT LVRGKPDVFE EFNAAIQALE EIQECHLVSG  115
Bja       LGLLVFVEVL LDKTTPDNFE RFARAVKLAP EVLECHMVAG  112
Bsu       IEVTALALIN LSPLNRETIH SFLEDINKFP QVQECYTLTG  114
BkdR      LDVNVFIHVS LEKQVEQSLH RFEEEIAERP EVMECYLMTG  113
Ppu       FGVTAFVSIM MDSHSKEMAR AFEQRLMDIP EIVACHNISG  114

           * *    *  **   * **   * ** **** * ***   *
AcdR      EFNYFMLLRT KDSHIFNRLH ADQLLYLPGV RQIRSFMGLR  159
Eco       DFDYLLKTRV PDMSAYRKLL GETLLRLPGV NDTRTYVVME  151
Hin       DFDYLLKTRV ADMAEYRKLL GTTLLRLPGV NDTRTYVVME  155
Bja       GFDYLVKARL ADMTAYRRFL GETLLSMPGV RETRTYAVME  152
Bsu       SHDYMLKIVA KDMESYRNFI IDSLMQNPAI SGVDTSIVMS  154
BkdR      DPDYLLRVLL PSIQALERF  LDYLTRLPGV ANIRSSFALK  152
Ppu       RYDFLLEILA RDLESFGEFT REVLQRLPGV KEIYSSFSFK  154

          **  *  ***
AcdR      QVLSTPQIPL              169
Eco       EVKQSNRLVI KTR          164
Hin       EVKQTNFLVL K            166
Bja       EIKRDGPLPV G            163
Bsu       TEKRTVSVPI DEM          167
BkdR      QVRYKTALPL PANGMTLRE    171
Ppu       AVKEKRVIPV AQKHI        169
```

Table 7. Amino acid composition of the protein AcdR from *E. cloacae* UW4.

AcdR-*Ecl*: Lrp-like protein found from the upstream region of ACC deaminase gene from *Enterobacter cloacae.*

BkdR-*Ppu*: Bkd operon transcriptional regulator from *Pseudomonas putida.*

Lrp-*Eco*: Leucine-responsive regulatory protein (Lrp) from *Escherichia coli.*

Lrp-*Bsu*: Leucine-responsive regulatory protein from *Bacillus subtilis.*

Lrp-*Ppu*: Lrp-family transcriptional regulator protein from *Pseudomonas putida.*

Lrp-*Bja*: Leucine-responsive regulatory protein from *Bradyrhizobium japonicum.*

Lrp-*Hin*: Leucine-responsive regulatory protein from *Haemophilus infuenzae Rd.*

Amino acid	**AcdR-** *Ecl*	**BkdR-** *Ppu*	**Lrp-** *Eco*	**Lrp-** *Bsu*	**Lsp-** *Ppu*	**Lrp-** *Bja*	**Lrp-** *Hin*
Alanine	20	11	5	9	17	11	9
Cysteine	3	2	2	2	2	1	2
Aspartic acid	9	6	11	11	8	8	9
Glutamic acid	7	15	12	11	12	15	15
Phenylalanine	7	5	5	4	8	6	4
Glycine	7	6	9	6	7	14	8
Histidine	4	2	2	2	3	2	1
Isoleucine	9	8	7	17	11	6	11
Lysine	11	4	10	12	8	5	17
Leucine	**23**	**25**	**23**	**17**	**17**	**23**	**25**
Methionine	5	5	3	7	5	5	5
Asparagine	6	8	8	8	2	3	8
Proline	7	9	7	5	6	7	6
Glutamine	8	7	7	4	5	2	5
Arginine	12	15	16	5	12	18	10
Serine	9	9	8	14	13	4	5
Threonine	7	8	9	11	7	7	8
Valine	13	12	15	9	11	12	14
Tryptophan	-	-	-	-	1	-	-
Tyrosine	2	4	5	3	4	4	4
Leucine (mol%)	**13.6**	**15.5**	**14.0**	**10.8**	**10.7**	**15.0**	**15.1**

sequence of AcdR was similar to other reported Lrp sequences. For example, AcdR had 38.6% identity to the Lrp of *Haemophilus influenzae* (Tatusov et al., 1996), 36.0% identity to the Lrp of *Klebsiella pneumoniae* (Friedberg et al., 1995), 32.4% identity to the Lrp of *Bradyrhizobium japonicum* (King and O'Brian, 1997), 29.1% identity to the Lrp of *Bacillus subtilis* (Sorokin et al., 1997), and 27.6% to 32.0% identity to the Lrp of *Pseudomonas putida* (Madhusudhan et al., 1993). Although the identity with each Lrp protein was not especially high, the total identity of AcdR in which an amino acid from AcdR was identical with at least one amino acid from all of the aligned proteins was found to be 65.7% (Fig. 21).

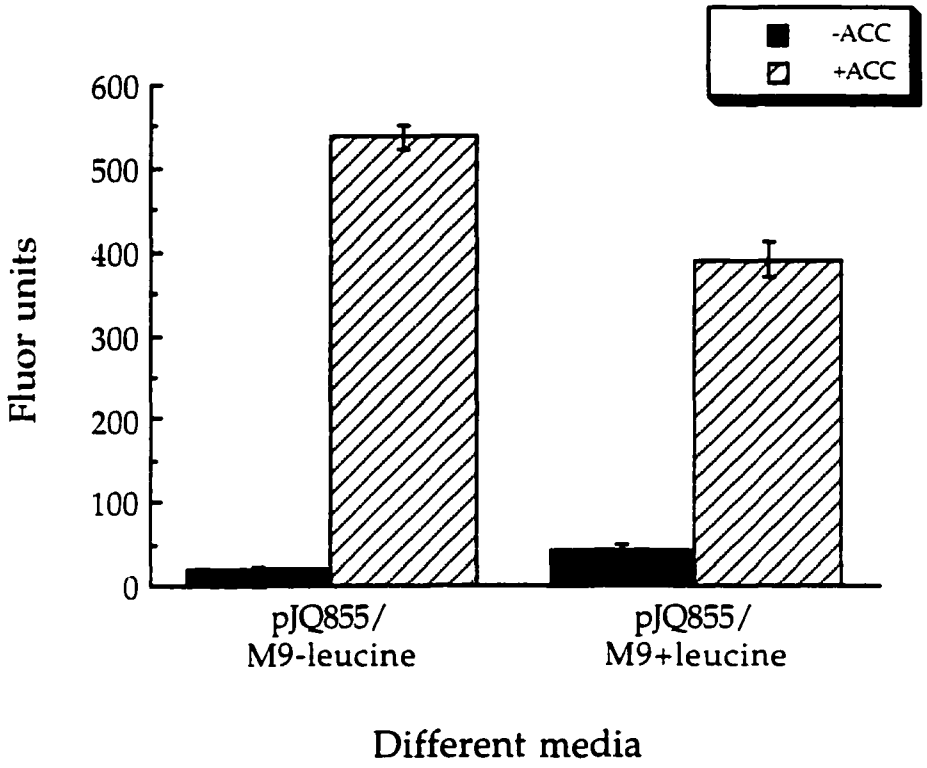
An interesting feature of all of the reported Lrp proteins is that they are unusually high in leucine content. For example, the Lrp from *E. coli* contains 23 leucine residues (14 mol%) (Willins et al., 1991). Similarly, AcdR also has 23 leucine residues with 13.6 mol% leucine in the protein (Table 7). Furthermore, from an alignment of the peptide chains, a conserved helix-turn-helix motif located in the N-terminal part of the Lrp family that allows the Lrp to bind to DNA was also identified in AcdR (Fig. 21). The designated conserved region of AcdR fits the criteria for a helix-turn-helix (Dodd and Egan, 1987; Brennan and Mathews, 1989) and is shown as underlined in Figure 21. Of the 22 amino acid residues within the conserved region, there are 18 residues that corresponded to residues in other Lrp proteins. Although the turn within the conserved region, position 47 in AcdR, is normally a glycine residue in a helix-turn-helix motif in most Lrps, it is a lysine residue in AcdR. However, an asparagine in this position is found in BkdR, a Lrp-like protein from *Pseudomonas putida* (Madhusudhan

et al., 1993). To meet the criteria for a helix-turn-helix, amino acid positions, 46 and 48, which flank the turn, should be hydrophobic, and positions 42 and 53 should be uncharged (either hydrophobic or polar), which is the case in AcdR. There are no proline residues from 41 to 46 and 53 to 58 in AcdR and the Lrps, and the two prolines at positions 51 and 52 are probably at the start of the second helix. A comparison of AcdR with the frequency matrix of amino acids in the conserved region (Dodd and Egan, 1987), positions 42 to 46 (LAEKV), positions 48 and 49 (LS), positions 51 to 52 (PA), and position 55 (R) have the highest frequency of occurrence. These data analyses suggested that *acdR* encodes a protein related to the Lrp family of proteins and probably acts as a regulator of expression of *acdS* in *E. cloacae*. However, a search of the databases using either NBCI or DDBJ failed to find a Lrp-like protein from the upstream region of *E. cloacae* CAL2.

In addition to the high leucine content of the Lrp protein itself, the addition of exogenous leucine, *in vitro*, reducing the extent of binding of Lrp is another feature of Lrp. In most regulation of Lrp, leucine is considered to be a repressor for Lrp binding to DNA (Willins et al., 1991). To look at whether or not AcdR behaves similarly to other Lrp-like proteins in the presence of leucine, cells containing pJQ855 were incubated in M9 minimal medium with or without leucine (20 μg/mL). The results showed that the presence of leucine decreased luminescence by 27.3% (Fig. 22).

The G+C contents of *acdR* and *acdS* were 56.4 and 58.3 mol%, respectively, which is similar to the genomic G+C content of *Enterobacter* (52-60%). However, the intergenic region between *acdR* and *acdS* (165 bp) is

Fig. 22. Luminescence of *E. coli*/pJQ855 incubated with or without exogenous leucine with *E. coli*/pQF70 as a negative control. Each incubation was also treated with or without ACC induction. The experiments were carried out in triplicate. The error bars represent the standard error at each point.


-ACC
+ACC
600
500
400
300
200
100
0
Fluor units
pJQ855/
M9-leucine
pJQ855/
M9+leucine
Different media

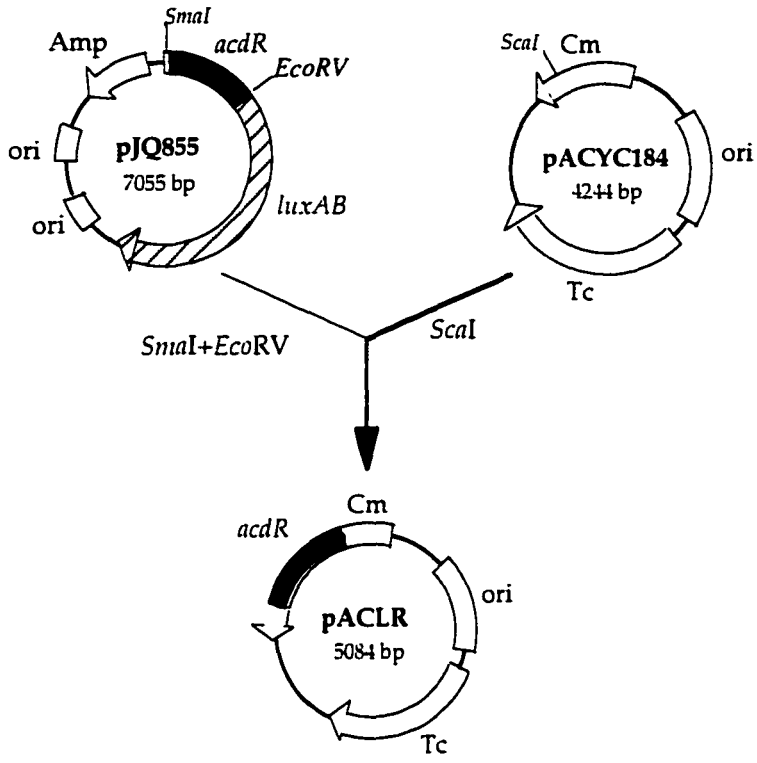
only 33.3 mol% G+C. This unusually low G+C content may play a role in transcriptional regulation by facilitating DNA bending (Inoue et al., 1997).

3.2.6. *trans* complementation of *acdR* mutants by AcdR

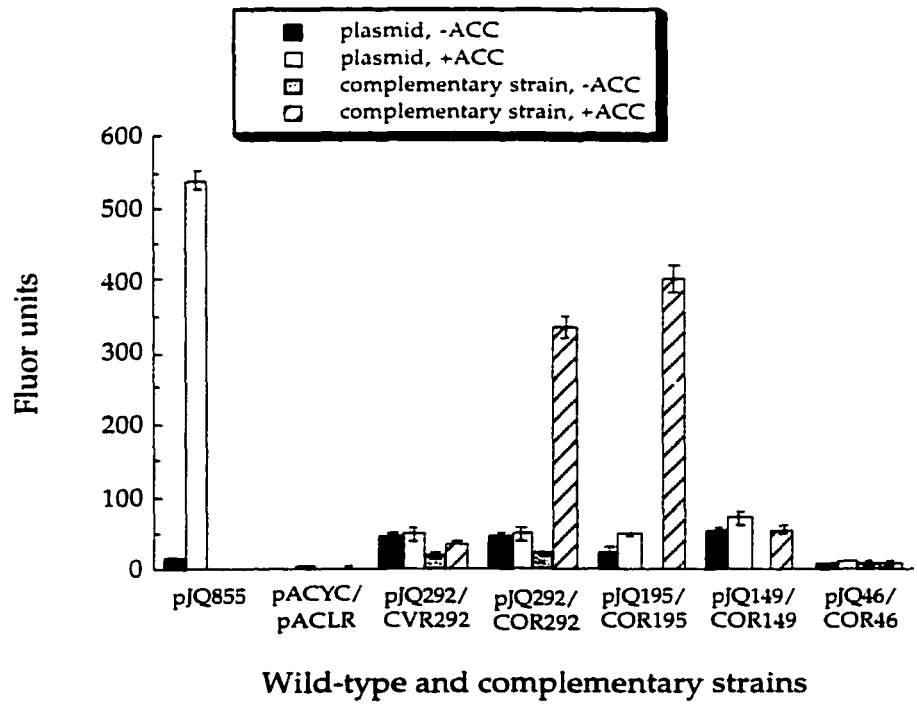
In order to confirm the hypothesis that AcdR regulates the expression of *acdS*, the intact *acdR* gene was subcloned from pJQ855 into vector pACYC184 (Fig 23A). To do this, pJQ855 was digested with *SmaI* and *Eco*RV to obtain a intact *acdR* gene, and then the gene was inserted into the unique *Sca*I site on pACYC184, disrupting the chloramphenicol resistance gene on the vector. The constructed plasmid, named pACLR, was used to *trans* complement pJQ292, pJQ195, pJQ149 and pJQ46. Each of the four plasmids that contain *luxAB* genes fused to different deleted upstream regions of the *acdS* gene (Fig. 16) and had produced low levels of luminescence. Since the *acdR* gene is located between -165 to -672, pJQ292 and pJQ195 contain 25% and 6%, respectively, of the *acdR* gene, whereas pJQ149 and pJQ46 completely lack the gene. The four plasmids present in *E. coli* DH5α cells were complemented separately with pACLR within the same cell. The four complemented strains were named *E. coli* COR292, COR195, COR149 and COR46 respectively. A cell containing pJQ292 was also complemented with vector pACYC184 as a control and named *E. coli* CVR292. Luminescence from both cells before and after complementation with pACLR from different deleted plasmids was detected with and without ACC induction, and pJQ855, carrying the *acdR* gene on the same strand (in *cis*-regulation), was used as a positive control; pACYC184, pACLR only, and *E coli* CVR292 were used as negative controls. As with previous deletion

Fig. 23. Construction of pACLR and luminescence of various strains complemented with this construct. **A**: Construction of pACLR from pJQ855 and pACYC184. Key: *luxAB*, luciferase reporter genes; *acdR*, putative regulator protein of ACC deaminase gene; ori, origin of replication; Tc, tetracycline resistance gene; Cm, Chloramphenicol resistance gene. **B**: Luminescence of various bacterial strains before (first two columns) and after complementation (the next two columns) and incubated with or without ACC induction. CVR292: *E. coli* DH5α contains both pJQ292 and vector pACYC184. COR292: *E. coli* DH5α contains both pJQ292 and pACLR. COR195: *E. coli* DH5α contains both pJQ195 and pACLR. COR149: *E. coli* DH5α contains both pJQ149 and pACLR. COR46: *E. coli* DH5α contains both pJQ46 and pACLR. All experiments were carried out in triplicate. The error bars indicate standard error.

A


SmaI
Amp
acdR
EcoRV
ori
pJQ855
7055 bp
ori
luxAB
ScaI
Cm
pACYC184
4244 bp
ori
Tc
SmaI+EcoRV
ScaI
Cm
acdR
pACLR
5084 bp
ori
Tc

B


plasmid, -ACC
plasmid, +ACC
complementary strain, -ACC
complementary strain, +ACC
Fluor units
600
500
400
300
200
100
0
pJQ855
pACYC/ pACLR
pJQ292/ CVR292
pJQ292/ COR292
pJQ195/ COR195
pJQ149/ COR149
pJQ46/ COR46
Wild-type and complementary strains

experiments (section 3.2.4), no luminescence could be detected from the four deleted plasmids with or without ACC induction. However, among the four strains tested for complemention, two strains, *E. coli* COR292 and COR195, regained the ability to produce luminescence when induced with ACC (Fig. 23B). The level of the luminescence from *E. coli* COR292 and COR195 (333.2 and 401.3 fluor units) were 62.0% and 74.7% of that of pJQ855 (537.4 fluor units), respectively. The lower luminescence may be due to the lower production of AcdR since it was cloned into pACYC184 which is lower copy number plasmid. A much lower level of luminescence (55.5 fluor units) was obtained from strain COR149, which seemed to have a basal level of transcription without regulation by AcdR. In addition, no luminescence was detected from strains COR46 and CVR292. *Trans* complementation by AcdR showed that the *acdR* gene encodes a protein that affects the expression of the gene located on the opposite strand and that the regulation requires ACC as an inducer. Furthermore, the upstream region between -195 and -149 may harbor the binding site for AcdR, since no complementary functions were obtained when the region was deleted shorter than -149, but luminescence was detected when the upstream region was extended to -195. These results suggested that AcdR not only has the main features of Lrp proteins but also, as with other Lrp proteins, act as positive regulators for the expression of the *acdS*. It was also of interest to note that AcdR could not be replaced by the indigenous Lrp present in the *E. coli* host strain since no detectable luminescence was obtained from *E. coli* CVR292.

3.2.7 Characterization of the induction of the promoter

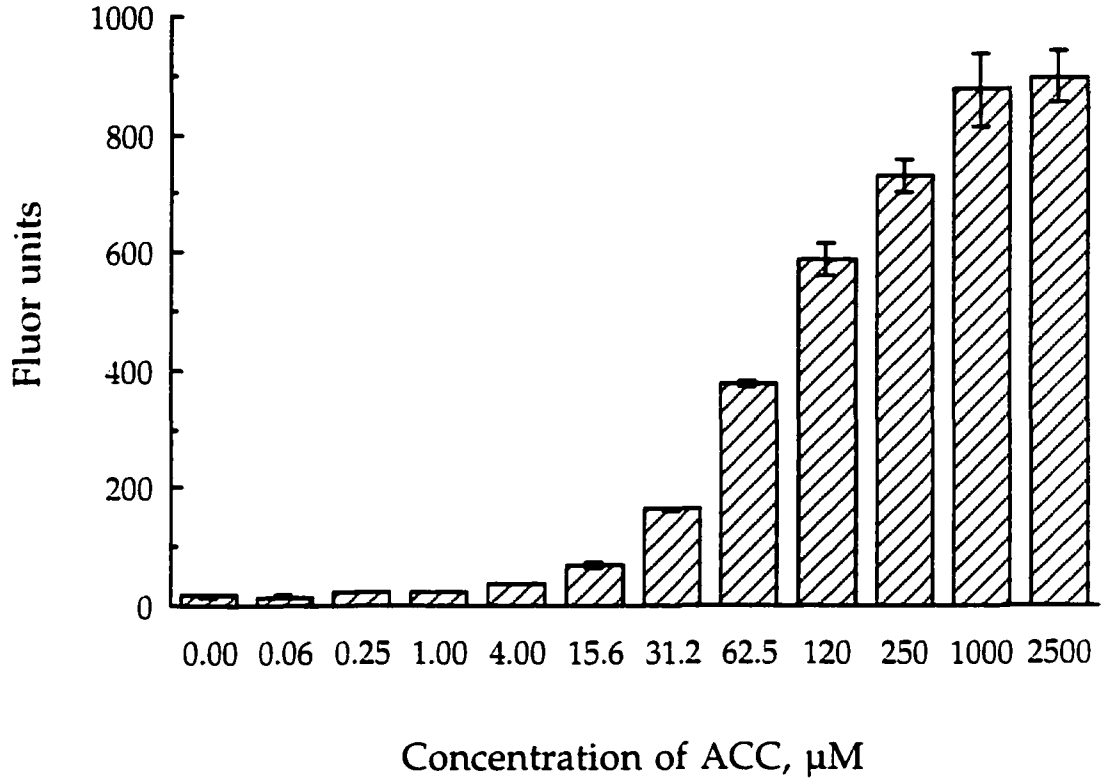
Since ACC deaminase hydrolyzes ACC through a unique regiospecific ring-opening reaction, only a few (1S, 2S) isomers of ACC analogs can function as weak substrates (Walsh et al., 1981; Honma, 1993c). Induction of the *acdS* gene by ACC has been found to be necessary for ACC deaminase expression. In order to understand the mechanism of gene induction, different amino acids including ACC analogs, such as DL-α-amino-n-butyric acid, DL-β-amino-n-butyric acid and N-methyl-DL-alanine, were tested as inducers. *E. coli* DH5α/pJQ855, which contains the 855 bp upstream region conjugated with *luxAB* genes, was used to quantify the luminescence produced using different substances as inducers. In these experiments, the cells were incubated in M9-ammonia medium and different substances (shown in Table 8) were added separately as inducers (1%) with ACC as a positive control. The results showed that, by far, the highest luminescence was detected with ACC as the inducer. The next highest luminescence, although much lower than ACC, was obtained from L-alanine induction. The ACC analog α-aminoisobutyric acid, and the amino acid L-valine were also weak inducers (Table 8). Unlike L-alanine, D-alanine, did not act as an inducer.

E. coli DH5α/pJQ855 was further used as a probe in this study to monitor the sensitivity of the promoter to ACC induction with the CytoFluor Reader. In this experiment, the cells were incubated in M9-ammonia medium with different ACC concentrations, ranging from 62.5 nM to 10 mM, each concentration was tested in triplicate and the entire experiment was repeated. The conditions for induction followed from

Table 8. Luminescence of *E. coli* DH5α/pJQ855 induced with various substances. Each substance was added at the final concentration of 1%. Each experiment was performed with eight samples for statistics analysis. * The difference between the substance and the blank control is considered extremely significant at $P<0.0001$ by ANOVA test. Notes: the reason for using mixed DL-leucine was that only this mix was available.

Substances	Fluor units	Standard Error (n=8)
None	12.85	± 0.30
ACC	771.09*	± 24.71
α-aminoisobutyric acid	40.95*	± 3.47
DL-α-amino-n-butyric acid	5.08	±0.48
DL-β-amino-n-butyric acid	13.27	± 0.99
N-methyl-DL-alanine	18.78*	± 0.53
L-alanine	136.44*	± 3.94
D-alanine	12.75	± 0.84
L-serine	13.07	± 1.24
L-valine	29.79*	±1.90
DL-leucine	20.47*	± 2.14

Fig. 24. Induction of pJQ855 at different ACC concentrations. The experiments were performed in triplicate. The error bars indicate standard error.


1000
800
600
400
200
0
Fluor units
0.00 0.06 0.25 1.00 4.00 15.6 31.2 62.5 120 250 1000 2500
Concentration of ACC, µM

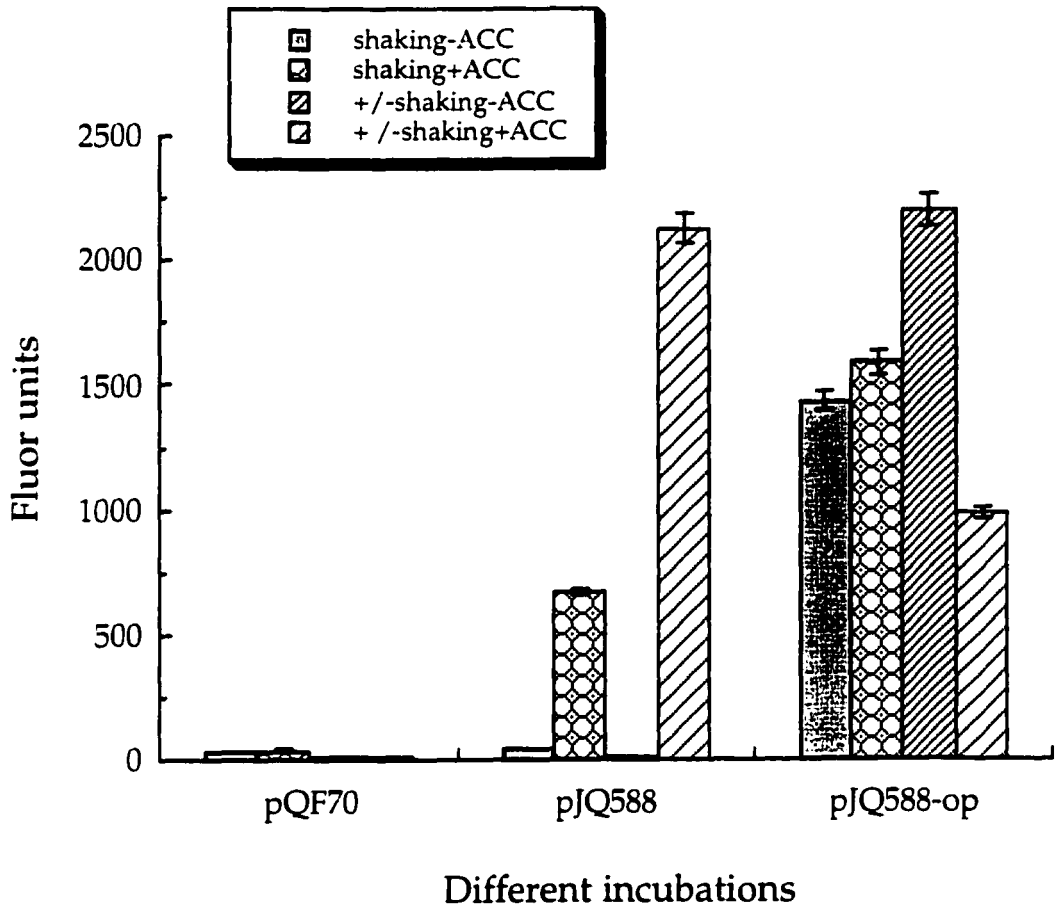
previous experiments; ACC was added at 4-5 h incubation and detection took place at 1.5 h after induction, where the cells were concentrated to 1 x 10^8 in 50 µL in microwell plates. The highest concentration of ACC that resulted in induction was at 1 mM. The concentrations higher than 1 mM had little effect on luminescence, that is, the luminescence began to plateau (data is not shown). Concentrations of ACC below 0.2 mM showed a linear relation with the level of luminescence (Fig. 24). However, luminescence was undetectable when the ACC concentration was below 250 nM.

3.2.8. Expression level of the *acdS* promoter

The expression of the *acdS* gene in *E. cloacae* UW4 was studied and it was found that the expression not only requires ACC as inducer but it is also regulated by AcdR, a Lrp-like protein. Further studies were undertaken to compare gene expression under this promoter-regulator system with other promoters and within different strains. A plasmid, pJQ588-op, constructed in this study (section 3.2.4.) contains the 588 bp *Sau*3A fragment subcloned from p4U2 into pQF70 with insert in an antisense orientation with respect to the *luxAB* reporter genes. Since this insert, when tested, produced luminescence, it was believed that it contained a promoter located on the opposite strand of *acdS*, and that it was the promoter for the *acdR* gene.

A comparison of luminescence between *E coli*/pJQ855 and *E. coli*/pJQ588-op was performed and the cells were grown either with or without ACC (1 mM) induction. Two different measurements of luminescence were used. The first detection of luminescence was after 1.5 h shaking of the culture after ACC induction, and second determination

Fig. 25. Luminescence of pQF70, pJQ588 and pJQ588-op, incubated either with or without ACC, and either with 1.5 h shaking after ACC induction or 2 h without shaking after ACC induction (labeled as +/- shaking). The experiments were carried out in triplicate. The error bars represent the standard error at each point.


shaking-ACC
shaking+ACC
+/-shaking-ACC
+ /-shaking+ACC
2500
2000
1500
1000
500
0
Fluor units
pQF70
pJQ588
pJQ588-op
Different incubations

occurred after 2 h of further incubation without shaking after the first determination. The vector pQF70 was used as a negative control. The results (Fig. 25) from these experiments demonstrated that *E. coli*/pJQ855 produced luminescence only under ACC induction, that is, without the addition of ACC, no luminescence could be detected. However, *E. coli*/pJQ588-op could produce luminescence with or without ACC, suggesting that the promoter in the antisense orientation is a constitutive promoter that does not need ACC to be induced, and the level of luminescence was 2.4-fold higher than the ACC deaminase promoter that was regulated by AcdR.

Additional experiments which involved leaving the bacterial culture on the bench without shaking for 2 h, showed dramatically increased levels of luminescence for *E. coli*/pJQ855 when the cells were incubated with ACC; the level was 3.1-fold higher than usual. In *E. coli*/pJQ588-op strain, a small increase (1.5-fold) was found when cells were incubated without ACC, however, when the cells were incubated with ACC the level of luminescence decreased to 62% of the shaking culture. This finding suggests that the expression of the *acdS* gene in *E. cloacae* UW4 has a bi-regulation system: AcdR positive regulation and an FNR (fumarate and nitrate reduction)-like regulation. A consensus sequence for an FNR-binding site, i.e., A-A-TTGAT----ATCAAT has been identified in the upstream region with the sequence AA-ATTGAT--ATCGA located at position -60 to -49 (Grichko and Glick, in preparation). The expression of luminescence from *acdS* promoter was only one-third of the level of that from the promoter on the opposite strand, suggesting that the AcdR-regulated promoter is weak. However, the

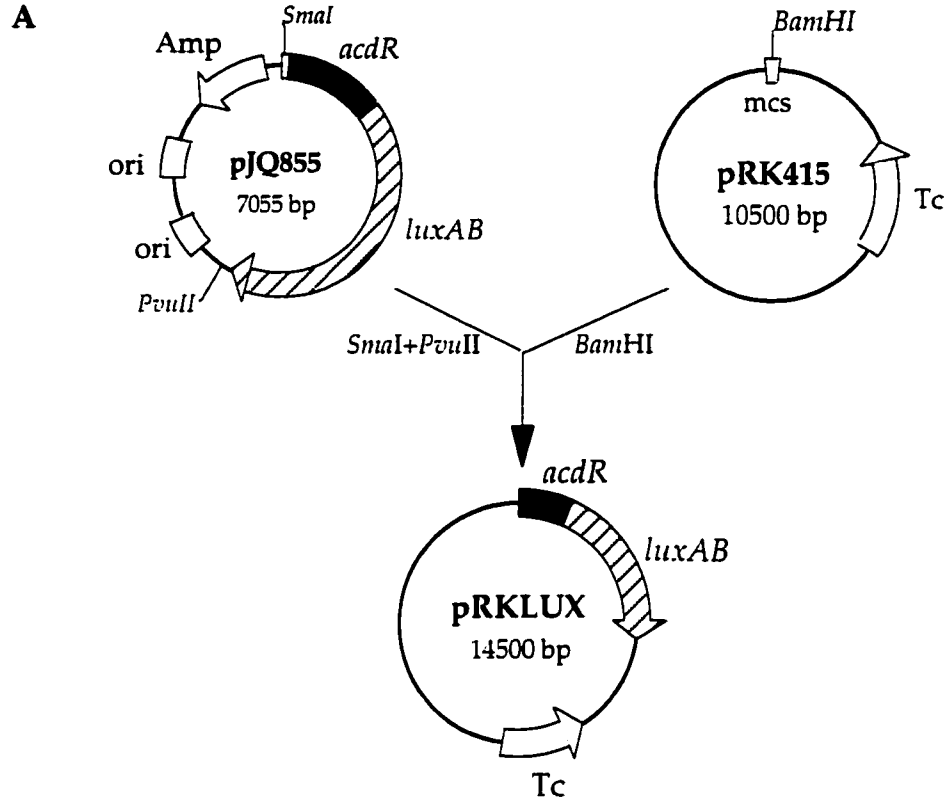
expression of both promoters under anaerobic conditions are at the same level.

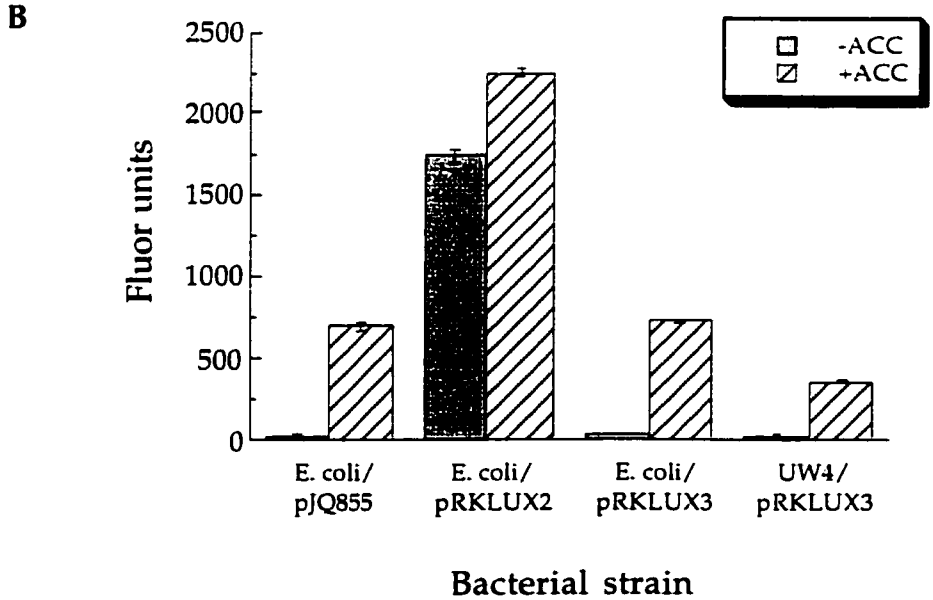
3.2.9. Expressions of the *acdS* promoter in different cells

To ascertain whether or not the expression levels of the gene driven by the AcdR-regulated *acdS* promoter are same in both *E. coli* and in *E. cloacae*, the AcdR-promoter conjugated *luxAB* genes cassette was excised from pJQ855 and cloned into pRK415 (Fig. 26). To construct the new plasmid, pJQ855 was digested with *Sma*I and *Pvu*II and pRK415 was digested with *Bam*HI. The 5.5 kb fragment from pJQ855 and linear pRK415 were treated with Klenow polymerase to generate blunt ends and the two fragments were ligated together. The ligation resulted with inserts in two orientations, pRKLUX2 (with a sense orientation) and pRKLUX3 (with an antisense orientation (Fig. 26A). Since the expression of pRKLUX3 will be driven by the AcdR-regulated promoter, pRKLUX3 was transformed into both *E. coli* DH5α and *E. cloacae* UW4.

Luminescence as detected from both pRKLUX2 and pRKLUX3 in *E. coli*, and pRKLUX3 in either *E. coli* or *E. cloacae*. All the tested cells were incubated in M9 minimal medium with or without ACC and pJQ855 was used as a control. The results showed that *E. coli*/pRKLUX3 had the same level of luminescence as *E. coli*/pJQ855, suggesting both plasmids pJQ and pRK may have a similar copy number in *E. coli*. However, pRKLUX3 in *E. cloacae* UW4 had a lower level of luminescence when detected under the same conditions (Fig. 26B). Increasing the ACC concentration used for the induction of *E. cloacae* UW4/pRKLUX3 had no effect on luminescence (data

Fig. 26. Construction of pRKLUX and luminescence of various strains carrying this construct. **A**: Plasmid pRKLUX was constructed by cloning the *acdR::luxAB* gene from pJQ855 into pRK415. Key: *acdR*, regulator protein gene for expression of ACC deaminase gene; *luxAB* , luciferase reporter gene; ori, origin of replication, Amp, ampicillin resistance gene; Tc, tetracycline resistance gene. **B**: Luminescence of different strains carrying different pRKLUX construct incubated with or without ACC induction. The experiments were carried out in triplicate. The error bars represent the standard error at each point.


A
SmaI
Amp
acdR
ori
pJQ855
7055 bp
ori
luxAB
PvuII
BamHI
mcs
pRK415
10500 bp
Tc
SmaI+PvuII
BamHI
acdR
luxAB
pRKLUX
14500 bp
Tc


B
2500
2000
1500
1000
500
0
Fluor units
-ACC
+ACC
E. coli/ pJQ855
E. coli/ pRKLUX2
E. coli/ pRKLUX3
UW4/ pRKLUX3
Bacterial strain

not shown). The lower expression in wild-type strain UW4 may be due to slower growth than *E. coli* or that perhaps the copy number of the plasmid was lower in UW4 than in *E. coli*. The pQF plasmid was reported to display a copy number of 13 in *Pseudomonas* spp. strain but 39 in *E. coli* (Farinha and Kropinski, 1989).

A comparison of the luminescence from both pRKLUX2 and pRKLUX3 showed that both produced light. Unlike pRKLUX3, which produces luminescence only in the presence of ACC, pRKLUX2 produces luminescence with or without ACC induction. The construct pRKLUX2 contains the *acdR*-promoter conjugated *luxAB* gene cassette inserted behind the P_{lac} promoter on vector pRK415 and had a higher light output (2.2-fold) without ACC induction than that of pRKLUX3 with ACC induction. When ACC was added, the luminescence from pRKLUX2 was increased another 1.3-fold (Fig. 26B). The results suggested that the light output in ρRKLUX2 without ACC induction may due to a transcription from the P_{lac} promoter on pRK415 which does not need ACC as an inducer. After adding ACC the higher luminescence produced may be due to an combination of transcription by both the P_{lac} promoter and the ACC-induced promoter. A comparison of the luminescence from pRKLUX2- and pRKLUX3-transformed *E. coli* indicated that the ACC-induced promoter is not a strong promoter even with regulation by AcdR.

3.3. Assessment of the model with different soil bacteria

In order to assess whether or not the *acdS* gene in different strains of PGPR contributes to lowering the level of ACC within plants, resulting in

decreased ethylene production, a simple system studying the growth of young canola seedlings under gnotobiotic conditions (Lifshitz et al., 1987) with different bacterial inoculants was used in this study. In such a system, the high levels of ethylene production during seed germination and root development have been shown to inhibit root elongation (Jackson, 1991; Hall et al., 1996; Glick et al., 1997). If the high level of ethylene in the root could be decreased by inoculating the seeds with bacteria that contain AcdS, the inhibition of root elongation by high levels of ethylene will be relieved, therefore root elongation will occur.

3.3.1. Construction of different AcdS strains

A 3.8 kb fragment carrying the *acdS* and *acdR* genes was subcloned from p4U2 (a clone from the *E. cloacae* UW4 library) into plasmid pRK415 to generate pRKACC which was then transformed into *E. coli* DH5α (Shah et al., 1998). Plasmid pRK415 is a broad-host-range (bhr) vector conferring tetracycline resistance, that can be introduced to other bacteria by conjugation when used in conjunction with the helper plasmid pRK2013 (Figurski and Helinski, 1979). Since *E. coli* strains are not normally found in the rhizosphere, it may behave differently when in association with seeds. Further experiments were aimed at conjugating both plasmids pRK415 and pRKACC into soil bacteria that do not have an expressible *acdS*. Two strains, *Pseudomonas putida* ATCC 17399 and *Pseudomonas fluorescens* ATCC 17400 (both psychrophiles), were selected as they cannot grow on minimal medium with ACC as the nitrogen source and neither have ACC deaminase activity nor promote root elongation (Table 9). Both pRK415 and pRKACC,

present in separate *E. coli* cells, were conjugated via triparental mating into these two *Pseudomonas* strains and selected on DF-tetracycline selective agar. The transconjugants were further confirmed by streaking all three strains, that of the donor cells, cells containing the helper plasmid and transconjugants, on the same selective agar on which only the transconjugant can grow. To distinguish the transconjugants from donor cells, *P. putida* ATCC 17399 cells were grown on selective medium at 4°C for 3 days. The transconjugants from *P. fluorescens* ATCC 17400 were further grown on Pseudomonad F agar or King's B agar to examine growth and fluorescence. Plasmids were extracted from some of the transconjugants and analyzed by restriction endonuclease digestion. Following these steps, transconjugants from each strain were obtained (data not shown).

Two other constructs were also made by conjugating both pRK415 and pRKACC from *E. coli* into *P. putida* GR12-2 and *E. cloacae* CAL3. Although these two strains have ACC deaminase activity (Table 5), it was found that they do not contain the same ACC deaminase gene as strains UW4 and CAL2 (Shah et al., 1998). After conjugation, the transconjugants were selected on DF-tetracycline agar, and the *E. cloacae* CAL3 transconjugants were further confirmed on Citrate agar and the transconjugants from *P. putida* GR12-2 were confirmed on Pseudomonad F agar. The plasmids were isolated from both of the strains to confirm that the cells were transconjugants.

3.3.2. Expression of AcdS within different bacterial strains

Before ACC deaminase assays were performed, all of the constructs were examined for their ability to grow on minimal medium with ACC as a sole source of nitrogen. The wild-type strains CAL2, UW4, GR12-2 and CAL3 served as positive controls, and in the experiment, two different ACC induction methods and protein extraction techniques (section 2.15.2) were compared. In the first method, the cells were inoculated in minimal medium at 1% (v/v) with 3 mM ACC as a sole source of nitrogen and grown for two days, after which the proteins were extracted following sonication. The second method involved growing the cells in minimal medium with ammonia for one day and the cells were collected and concentrated ten-fold in a second culture of minimal medium with 5 mM ACC followed by 6 hours of incubation. The concentrated cell resuspension was then treated with toluene and directly used for ACC deaminase assays. It was obvious from the results (Table 9) that a five- to seven-fold greater activity can be obtained from the second induction method than can be from the first, and the results for different bacteria from both methods were consistent. The data summarized in Table 9 clearly shows that all of strains, *E. coli*, *P. putida*, *P. fluorescens*, and *E. cloacae*, can express the cloned *acdS* genes from the plasmid pRKACC. After pRKACC was introduced, *P. putida* ATCC 17399 and *P. fluorescens* ATCC 17400, which normally cannot grow on minimal medium with ACC as nitrogen source, acquired the ability to grow on ACC-agar. The activity of ACC deaminase from both transformed pseudomonads displayed the same activity as that of *E. coli* transformed with pRKACC. On the other hand, when pRKACC was introduced into both *P. putida* GR12-2

Table 9. ACC deaminase activity of the different *acdS* transformants.

* The assay was performed with the first ACC induction method, as described in text.

** The assay was determined with the second ACC induction method as described in text

The activity of ACC deaminase was expressed in nmoles/mg protein/ h.

+: bacteria grew well

-: no bacterial growth

nd: not determined

Strains	Growth on minimal medium with ACC	ACC deaminase activity*	ACC deaminase activity**
E. cloacae CAL2	+	476	2451 ± 19
E. coli DH5α/pUC18	–	14	67 ± 6
E. coli DH5α/p4U2	+	285	1996 ± 28
E. coli DH5α/pRKACC	+	454	2641 ± 39
P. putida ATCC 17399/pRK415	–	0	58 ± 9
P. putida ATCC 17399/pRKACC	+	507	2855 ± 84
P. fluorescens ATCC 17400/pRK415	–	0	56 ± 11
P. fluorescens ATCC 17400/pRKACC	+	490	2842 ± 78
P. putida GR12-2	+	232	2440 ± 24
P. putida GR12-2/pRK415	–	189	2013 ± 22
P. putida GR12-2/pRKACC	+	531	2291 ± 41
E. cloacae CAL3	+	151	226 ± 11
E. cloacae CAL3/pRK415	–	169	252 ± 13
E. cloacae CAL3/pRKACC	+	678	1617 ± 60

Fig. 27. The appearance of canola roots following treatment of the seeds with various bacterial strains. (A) $MgSO_4$ only; (B) *E. coli* DH5α/pUC18; (C) *E. coli* DH5α/p4U2; (D) *P. putida* ATCC 17399/pRK415; (E) *P. putida* ATCC 17399/pRKACC; (F) *E. cloacae* CAL2.

A
B
C
D
E
F

and *E. cloacae* CAL3, increases in the levels of ACC deaminase activity was observed, especially in *E. cloacae* CAL3.

3.3.3. Testing the different constructs for promotion of root elongation

To investigate the effect of the pRKACC transconjugants on root elongation, both pRK415 and pRKACC were introduced into *E. coli* DH5α, *P. putida* ATCC 17399 and *P. fluorescens* ATCC 17400. In the experiment, pRK415 (the vector for pRKACC) served as a negative control for comparison to the pRKACC constructs. All of the constructs, as well as strain CAL2 which served as a positive control, were incubated with canola seeds and the inoculated seeds were grown under gnotobiotic conditions for five days. The appearance of the young seedlings treated with the indicated bacterial strains are depicted in Fig. 27 and the root length of each seedlings was measured and summarized in Table 10. The canola roots are visibly longer in panels C, E, and F of Fig. 27 (every instance in which the bacterium contains the *acdS* gene) than in panels A, B, and D (when the *acdS* gene is not present). Promotion of root elongation was found to be more effective with pRKACC than with pRK415 in both pseudomonad strains. In *E. coli*, the roots of seedlings treated with p4U2 (a clone contains *acdS* gene) were on the average 23% longer than those treated with pUC19. For *P. putida* ATCC 17399 there was a 62% increase in root length when the *acdS* gene was present; with *P. fluorescens* ATCC 17400 there was a 31% increase in root length under similar conditions. The extent of growth promotion from both pseudomonads carrying plasmid pRKACC was similar to that observed with wild-type *E. cloacae* CAL2, which had a 36% promotion of root

Table 10. Effect of the different bacteria containing either or not containing ACC deaminase on growth of canola roots in a gnotobiotic condition and the survival of the treated bacteria in the rhizosphere.

nd: not determined

*: Data are given as means ± standard error.

**: the seedling numbers of each treatment were the same as the root numbers in the next column.

a: indicates that a strain containing ACC deaminase treatment is significantly different from the respective wild-type strain at the 0.01% probability level as determined by the t-test.

b: indicates that a strain treatment is significantly different from the respective control $MgSO_4$ at the 0.01% probability level as determined by the t-test.

Strains	Root length* (mm) ±SE**	Colonies/root (root number)
E. coli/pUC19	47.4 ± 2.0	359 (75)
E. coli/p4U2	58.2 ± 2.4[a]	142 (82)
P. putida ATCC 17399/pRK415	45.7 ± 1.7	300 (94)
P. putida ATCC 17399/pRKACC	74.1 ± 2.2[a]	690 (87)
P. fluorescens ATCC 17400/pRK415	50.9 ± 1.9	250 (92)
P. fluorescens ATCC 17400/pRKACC	66.6 ± 1.9[a]	6300 (95)
E. cloacae CAL2	61.5 ± 2.0[b]	3400 (88)
$MgSO_4$	45.2 ± 2.2	2.5 (81)

elongation compared with blank control roots (no bacteria). Bacteria were recovered from the roots of seedlings after root measurement and it was shown (Table 10) that both *Pseudomonas* spp and *E. cloacae* CAL2 were able to bind canola seeds to a 10-fold greater extent than *E. coli*. An interesting pattern observed was that the number of cells containing pRKACC with the *acdS* gene and wild-type strain CAL2 recovered were much higher than that of the constructs containing pRK415. These results suggest that bacteria containing the *acdS* gene may be able to use the exuded ACC from the root as a nitrogen source and mutilply and better colonize the root surface.

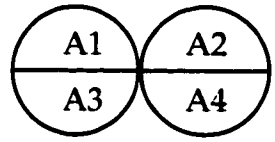
3.3.4. Detection of ACC exudation using pJQ855 as a probe

The construct JQ855, which contains the *acdR* gene and the promoter of the *acdS* gene conjugated with reporter *luxAB* genes, can be used as a probe to detect whether or not ACC is exuded from roots and taken up by soil bacteria. Since the expression of *luxAB* genes in pJQ855 is inducible by ACC, and the concentration of ACC used for induction showed a linear relationship with luminescence produced by pJQ855 when the ACC concentration was less than 1 mM, the levels of luminescence can reflect the ACC concentration in a test culture. Previous experiments with this construct have shown that the lowest ACC concentration that produced detectable luminescence was 250 nM (Fig. 24). In this experiment, canola seeds were inoculated with *E. coli* DH5α, *E. coli* DH5α/pQF70 (the vector of pJQ855), *E. coli* DH5α/pJQ855 and water, and incubated for different times. The seeds were then plated on minimal-ammonia medium to recover the cells. After adding n-decyl aldehyde, the substrate for *luxAB*, the agar plates

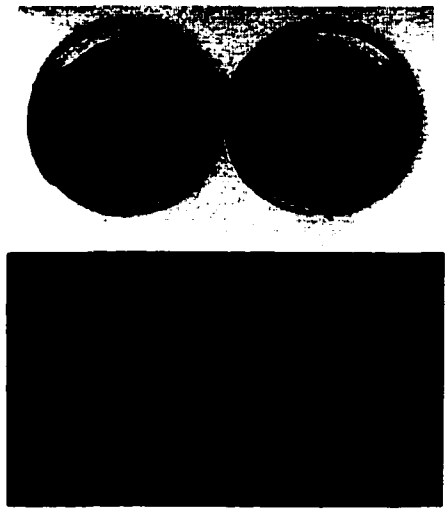
Fig. 28. Luminescence image of individual canola seeds or roots after inoculation of the seeds with or without bacteria (either *E. coli*/pQF70 or *E. coli*/pJQ855). Each upper panel is a photograph of the seeds or roots and each lower panel is their luminescence image on X-ray film, the plates were exposed to X-ray film for 5 min. **A**: Seeds were incubated with or without bacteria for 1 h and placed in a growth pouch for 24 h after which the seeds were placed on M9-ammonia agar overnight. The seeds were incubated with H_2O (A1), *E. coli* DH5α (A3), *E. coli*/pQF70 (A2), and *E. coli*/pJQ855 (A4). **B**: Seeds were incubated with or without bacteria for 1 h and placed in growth pouch for 48 h, after which the seedlings were transferred to M9-ammonia agar and exposed to X-ray film for 5 min. The seeds were treated with H_2O (B1), *E. coli*/pQF70 (B2), and *E. coli*/pJQ855 (B3).

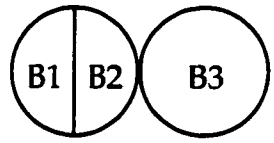
A





B





with the seeds were exposed to X-ray film for 5 min. Both pJQ855 and pQF70 treatments had detectable luminescence and no signals were observed from the *E. coli* DH5α and water treatments (Fig. 28). The luminescence from both pJQ855 and pQF70 treatments seemed extremely strong for an exposure period of only 5 min, however, there was no detectable difference in the intensity of luminescence between the treatments. Further efforts to assess the difference between pJQ855 and pQF70 treatments involved the use of CytoFluor Reader. A thousand canola seeds were germinated in water in small petri dishes overnight. The seed exudates were collected and added separately to bacterial cultures containing either pJQ855 or pQF70, and luminescence was detected after 1.5 h induction. However, even after several repeats, the readings from both cells were not significantly different. Among several detections only one reading showed a detectable small difference between pJQ855 and pQF70. The difference suggested that germination of 1,000 seeds during overnight could exude 8.6 ng of ACC.

3.4. Creation of an ACC deaminase defective mutant

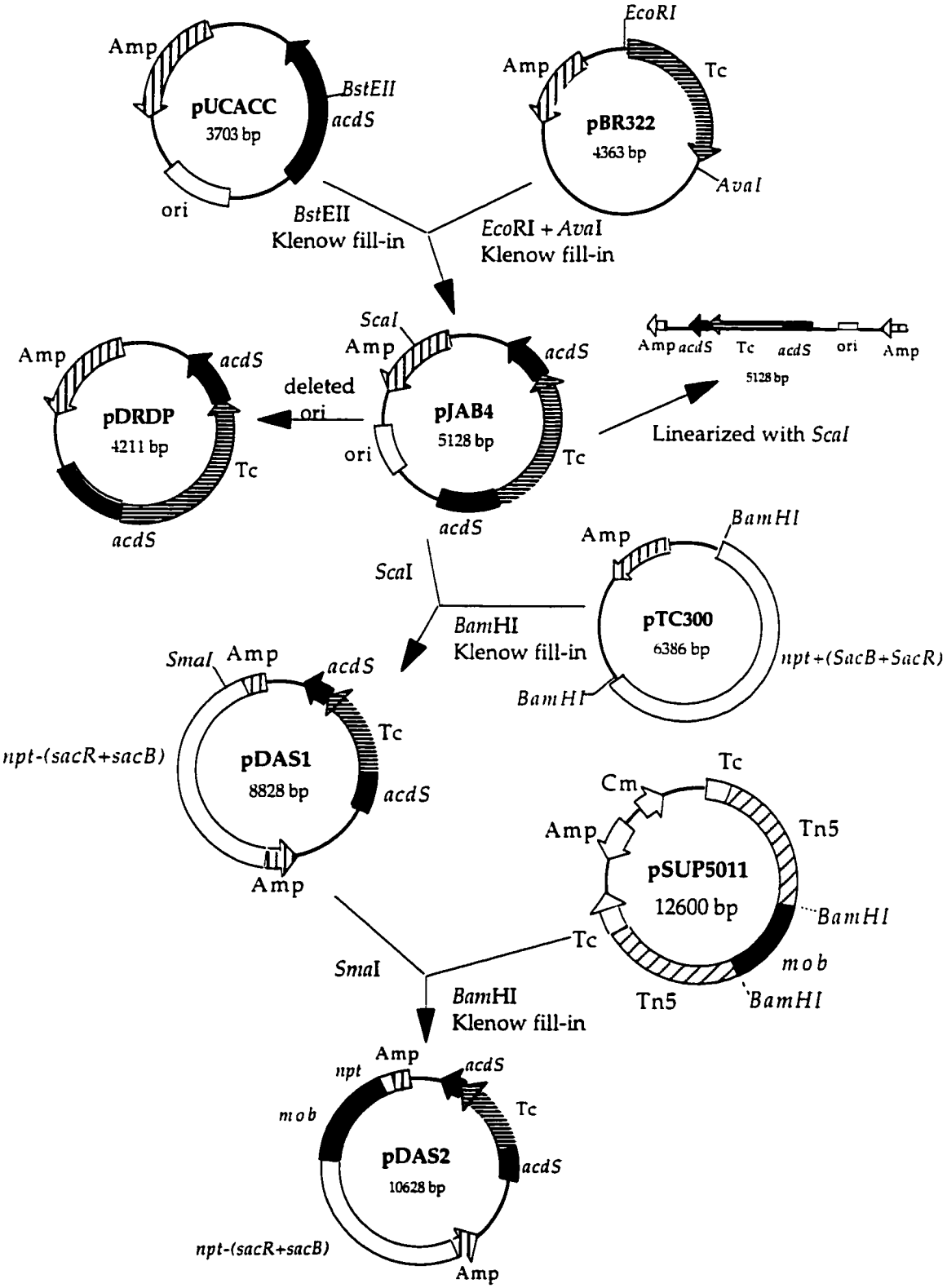
In an effort to fully understand the role of ACC deaminase in the stimulation of root elongation, strains of *E. cloacae* UW4 and CAL2 were constructed, by gene replacement, that lack a functional ACC deaminase gene. The procedure to create an ACC deaminase minus mutant followed a series of steps: A construct that contained the disrupted ACC deaminase gene was created and maintained in *E. coli* cells; the construct was transformed or conjugated into the wild-type strains of UW4 and CAL2.

Clones in which homologous double-crossover between the wild-type *acdS* gene in the bacterial genome and a disrupted *acdS* gene on the constructed plasmid occurred were selected and the selected *E. cloacae* UW4 or CAL2 were then tested for the presence of the ACC deaminase mutation (AcdS$^-$) using PCR and Southern hybridization. Finally the ACC deaminase activity as well as the ability to promote root elongation was determined in both wild-type and AcdS$^-$ mutant strains.

3.4.1. Construction of *acdS*$^-$ in *E. coli*

The starting plasmid, pUCACC, which derived from pUC18 contains a cloned copy of *acdS* (Shah et al., 1998). To create the *acdS*$^-$, the *acdS* gene on pUCACC was interrupted with a tetracycline resistance (Tc^r) cassette. In addition to generating the mutation in the gene, the Tc^r gene once incorporated into the vector can serve as a marker for selecting cells where homologous recombination has occurred. Plasmid pUCACC was digested with *Bst*EII which cuts pUCACC at a unique site located in the middle of *acdS* gene, and the Tc resistance gene was excised from pBR322 by digesting it with *Eco*RI and *Ava*I. The restriction overhangs were completed using Klenow fill-in and the new blunt ended fragment was ligated into the unique *Bst*EII in pUCACC, interrupting the *acdS* gene. The resulting plasmid, pJAB4 (Fig. 29), carrying *acdS*::Tc^r was transformed into both UW4 and CAL2 .

Fig. 29. Schematic representation of the different constructs that were used to generate an *acdS* deficient mutant by gene replacement. Key: *acdS*, ACC deaminase gene; ori, origin of replication; Amp, ampicillin resistance gene; Cm, Chloramphenicol resistance gene; Tc, tetracycline resistance gene; *mob*, mobility site; *npt*, neomycin phosphotransferase/kanamycin resistance gene.

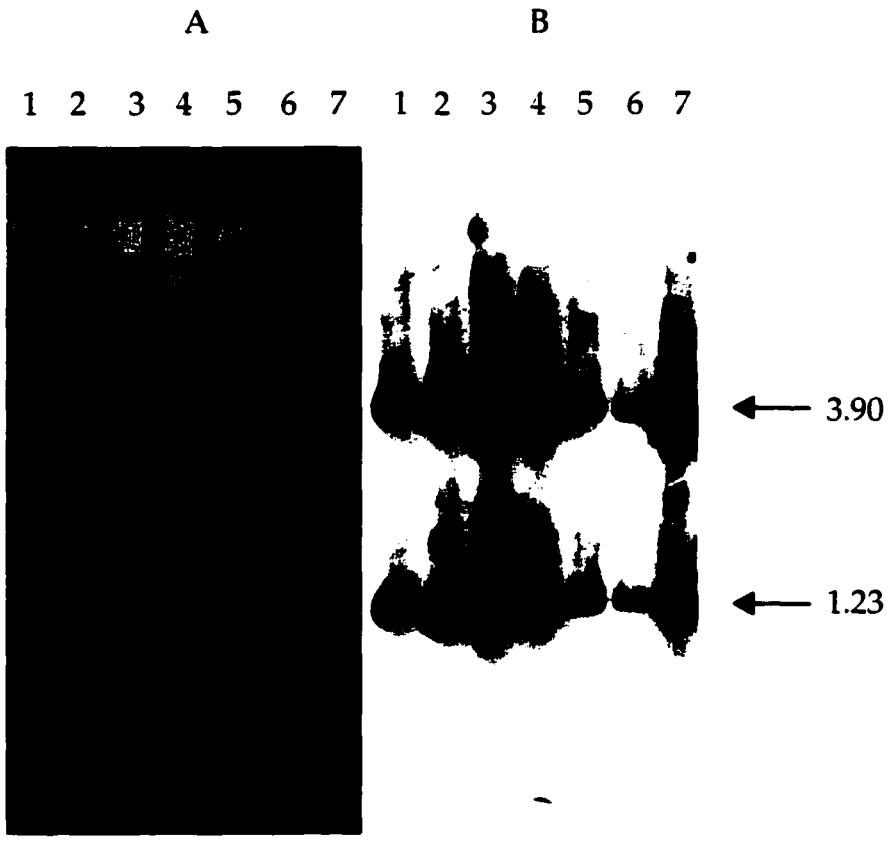

Amp
pUCACC
3703 bp
BstEII
acdS
ori
BstEII
Klenow fill-in
EcoRI
Amp
Tc
pBR322
4363 bp
AvaI
EcoRI + AvaI
Klenow fill-in
ScaI
Amp
acdS
pJAB4
5128 bp
ori
Tc
acdS
deleted
ori
Amp
acdS
pDRDP
4211 bp
Tc
acdS
Amp acdS Tc acdS ori Amp
5128 bp
Linearized with ScaI
ScaI
BamHI
Klenow fill-in
Amp
BamHI
pTC300
6386 bp
npt+(SacB+SacR)
BamHI
SmaI
Amp
acdS
pDAS1
8828 bp
Tc
acdS
Amp
npt-(sacR+sacB)
Tc
Cm
Amp
Tn5
pSUP5011
12600 bp
BamHI
mob
Tc
Tn5
BamHI
SmaI
BamHI
Klenow fill-in
Amp
npt
acdS
mob
Tc
pDAS2
10628 bp
acdS
npt-(sacR+sacB)
Amp

3.4.2. Selection of an *acdS*⁻ mutant by transformation with pJAB4

Plasmid pJAB4 was successfully transformed into *E. cloacae* CAL2, and the transformants were selected using tetracycline. To verify that the transformants were strains of *Enterobacter*, and not other bacteria, such as *E. coli*, citrate agar was used. Citrate agar contains bromothymol blue, which changes from green to blue as carbon dioxide released from citrate degradation recombines with water and sodium in the medium to produce sodium carbonate. Species of *Enterobacter* are able to use citrate as a sole carbon source and will therefore change the colour of the medium from green to blue. Once the species identity of the transformant was confirmed, the genomic DNA was isolated and digested with *Sal*I. Since one *Sal*I site is within the Tc resistance gene and the *acdS* gene in strain UW4 is known to be located in a 2180 bp *Sal*I-*Sal*I fragment, a double crossover recombination event can be identified using Southern hybridization with the Tc resistance gene as a probe. If a double crossover had occurred, the *Sal*I digestion pattern of the mutant should produce two hybridization bands, 2.05 kb and 1.57 kb. Two possible single crossover events, at the different flanks of the Tc gene, will give hybridization bands of either 1.57 kb and 1.23 kb or 4.47 kb and 2.05 kb. However, with no recombination event, the *acdS*::Tc cassette would still be on pJAB4, giving two fragments of 3.90 kb and 1.23 kb.

In this experiment, the *Sal*I digested DNA from the pJAB4-transformants was hybridized with the Tc resistance gene, two bands appeared after colour development from DIG-labeled hybridization, they were at the positions the 3.90 kb and 1.23 kb digestion fragment (Fig. 30), indicating that the bands were from the plasmid. In other words, pJAB4

Fig. 30. Southern hybridization of the pJAB4 transformants with tetracycline resistance gene (*tet*) as a probe. **A**: Agarose gel electrophoresis of genomic DNA of the transformants that were digested with *Sal*I. **B**: Autoradiogram of the nylon membrane-blotted DNA from the gel shown in panel A after hybridization with ^{32}P-labeled *tet* gene. Lane 1-6, different transformants; lane 7, plasmid pJAB4 as a positive control.


A
B
1 2 3 4 5 6 7
1 2 3 4 5 6 7
3.90
1.23

was stably maintained in *E. cloacae* CAL2, which is possible because both *Enterobacter* and *Escherichia* belong to the same Family, *Enterobacteriaceae*.

3.4.3. Transformation with linear pJAB4 and a replication-deficient pJAB4

Previous attempts at creating the mutant suggested that a construct would have to be created that allowed for selection of double crossover events as well as the selection of cells that lost the replacement vector. To meet that end, a linearized version of pJAB4 (Fig. 29) was the next construct that was tried. The construct pJAB4 was cut at the unique *Sca*I site of the vector, generating a blunt-ended linear plasmid. The linearized plasmid was transformed into *E. cloacae* UW4 and CAL2 and transformants were grown on selective medium containing tetracycline. The transformants obtained were tested for the double crossover recombination using colony-PCR. With primers P1 and P2 (Shah et al., 1998) the wild-type *acdS* gene could be amplified as a 0.8 kb fragment and the interrupted *acdS* gene would result in a 2.2 kb fragment. Only a single fragment of 2.2 kb is expected once double crossover recombination occurs. If two bands, 0.8 kb and 2.2 kb, are obtained together after PCR, it would indicate that either the plasmid had recyclized in the transformant or that a single crossover occurred. To further distinguish between the various cases, Southern hybridization is needed. In this experiment, after transforming linearized pJAB4 into strains UW4 and CAL2, two putative mutant colonies were obtained. PCR on both colonies indicated the absence of a 2.2 kb band. One colony produced the 0.8 kb PCR band that represented the *acdS* gene in the wild-type strain.

Another approach to force the loss of pJAB4 from transformants was to excise the origin of replication from the plasmid. After a scaled-up plasmid preparation, pJAB4 was modified by digesting it with both *Sca*I and *Sap*I. After separation on an agarose gel, the fragment without the origin of replication was extracted from the agarose gel and self-ligated to generate the replication deficient plasmid, pDRDP (Fig. 29). Following transformation the cells were placed on selective medium, with the idea that only those cells that have integrated the mutant gene would survive, without the plasmid remaining in the cell. Several attempts to introduce pDRDP into strain UW4 and CAL2 were made. Most of the attempts resulted in no transformed colonies, however, one transformation produced two colonies, which were tested using citrate agar and PCR. As with the colonies obtained above, both tests came up negative.

Of importance in this experiment is the frequency of the recombination and that of transformation itself. Although *Enterobacter* is closely related to *Escherichia*, efficient transformation of *Enterobacter* was found to be very difficult. Thus, without a high frequency of transformation, it is hard to obtain the desired mutant.

3.4.4. Transformation with an inducible suicide plasmid

In this construct, the *sacB-sacR* genes of *Bacillus subtilis* (Gay et al., 1983) were inserted into pJAB4. The *sacR* gene is a regulatory locus believed to function as an attenuator for the *sacB* gene (Steinmetz and Aymerich, 1986); the *sacB* gene codes for the enzyme levansucrase and confers sucrose sensitivity on Gram-negative bacteria. The expression of *sacB* gene has been

reported to be lethal in a wide range of Gram-negative bacteria in the presence of 5% sucrose (Gay et al., 1985; Ried and Collmer, 1987). Thus the *sacB* gene, allowing positive selection of gene replacement, has been used as a conditional (sucrose-inducible) lethal gene for Gram-negative bacteria to discriminate between either maintenance or integration of the entire vector, and double recombination events. Successful selection using the *sacB-sacR* gene cassette for gene replacement in Gram-negative bacteria such as *Rhizobium* has been obtained by Hynes et al. (1989) and Quandt and Hynes (1993).

The *nptI-sacB-sacR* cassette constructed by Ried and Collmer (1987) offered an easy means of cloning the *sac* genes into another plasmid, the cassette was obtained from pTC300, constructed by Dr. T. Charles, as a 3.7 kb *Bam*HI fragment. After pTC300 was digested with *Bam*HI, the overhangs were completed by Klenow fill-in, and the blunt-ended fragment was ligated into *Sca*I digested pJAB4 (Fig. 29). The ligated product, named pDAS1, was transformed into *E. coli* DH5α and the clones containing pDAS1 were selected on LB agar containing both tetracycline and kanamycin. Furthermore, the plasmids from positive clones were isolated and digested with *Hind*III, which has four cut sites in pDAS1, while pJAB4 has only two *Hind*III sites (data not shown). The concentration of sucrose necessary to induce *sacB* and result in the death of the bacteria was tested at 5% and 7.5%, and the threshold for inhibition of growth of *E. coli* DH5α/pDAS1 was found to be 7.5% sucrose.

Once pDAS1 was constructed, it was introduced into both strains UW4 and CAL2 by both transformation and electroporation. Despite the

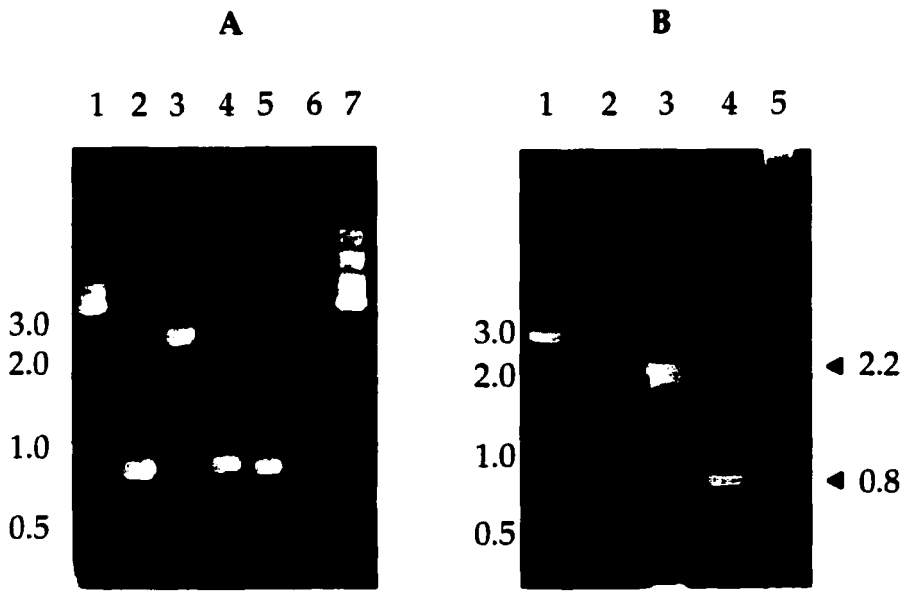
fact that a wide range of conditions were attempted for both transformation and electroporation no transformants were obtained.

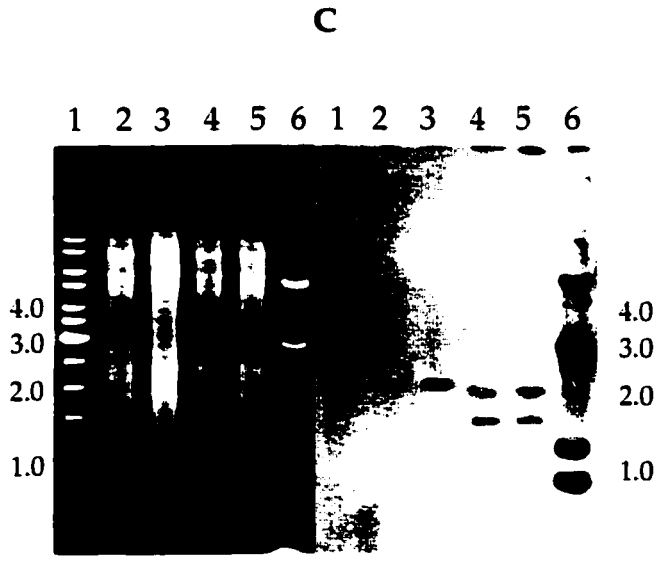
3.4.5. Conjugation of pDAS1

To overcome the transformation barrier, pDAS1 was reconstructed by inserting the mobilization (*mob*) site of RP4 (a promiscuous P1-type R-factor) that allows the carrier vector to be efficiently mobilized from *E. coli* into other Gram-negative bacteria using RP4 in *trans* as a helper plasmid (Simon et al., 1983). A *mob* site in a 1.8 kb *Bam*HI fragment was excised from pSUP5011 (provided by Dr. T. Charles) and the overhangs were completed using Klenow fill-in. The fragment was then inserted into the *Sma*I site in the *npt* gene on pDAS1 (Fig. 29). The new construct, pDAS2, was transformed into *E. coli* S17-1 and grown on medium with tetracycline. Since the *mob* insert came without a selectable marker, it was inserted into the kanamycin resistance gene on the *npt-SacR-SacB* cassette. Thus, resulting transformants were tested for the insert by replica plating, and clones containing pDAS2 were selected based on resistance to tetracycline and sensitivity to kanamycin. *E. coli* S17-1 was used because it can function as both a helper and a donor in plasmid mobilization since it has RP4 integrated into its chromosomal DNA. Therefore, it can directly mobilize the plasmid within its cell into the recipient cell.

When *E. coli*S17-1/pDAS2 (*acdS*::Tc) was mated with either *E. cloacae* UW4 (*acdS*) or CAL2 (*acdS*) and grown on selective agar (TSB/Amp/Tc), hundreds of transconjugants were obtained for *E. cloacae* UW4, but none from *E. cloacae* CAL2. Species identification on citrate agar

Fig. 31. Identification of the *acdS* mutant by PCR and Southern hybridization. **A**: PCR products amplified from strain UW4 (lane 2), pDAS1 (lane 3), and two transconjugants (lane 4 and 5). **B**: PCR products amplified from two transconjugants after an extended culture in 7.5% sucrose medium (lane 3 and 4), and strain UW4 (lane 2). **C**: Southern hybridization of the *Sal*I digested genomic DNA of transconjugants using the UW4 *acdS* gene as a probe. Lane 2-3, wild-type strain UW4, lane 4-5, one of the transconjugants that produced a 2.2 kb PCR product; lane 6, plasmid pDAS1 digested with *Sal*I. Lane 1 in all of panels is DNA marker.


A
1 2 3 4 5 6 7
3.0
2.0
1.0
0.5
B
1 2 3 4 5
3.0
2.0
1.0
0.5
2.2
0.8


C
1 2 3 4 5 6 1 2 3 4 5 6
4.0
3.0
2.0
1.0
4.0
3.0
2.0
1.0

showed that roughly 90% of the transconjugants were *E. cloacae* and not *E. coli*. Furthermore, several transconjugants were tested by colony PCR, and two amplified PCR fragments, 0.8 kb and 2.2 kb, were obtained from one cell, indicating that pDAS2 was present within strain UW4. The 0.8 kb fragment was amplified from the wild-type strain *acdS* as a control, while the 2.2 kb fragment was from the *acdS*::Tc present on the replacement vector (Fig. 31A). However, at this stage the homologous recombination event was not obtained, since none of the tested colonies showed a single 2.2 kb fragment. To select for the mutant, one transconjugant was cultured in TSB broth containing tetracycline for three days with transfer into fresh medium each day. The purpose of the extended culture was to allow for double recombination event to occur putting the tetracycline resistance gene (Tc) onto the chromosome. Furthermore, the *sacB* gene could be used to select for cells that have integrated the mutant gene (*acdS*::Tc) into the chromosome and lost pDAS2. In the replacement construct, plasmid pDAS2 is easily lost, since once recombination occurred the Tc resistance gene is transferred onto the chromosomal DNA, and because the pUC-derived vector is not stable in species of *Enterobacter*. Thus, cells from the extended culture that were able to grow on TSB-tetracycline agar supplemented with 7.5% sucrose were likely to be mutants UW4 *acdS*::Tc.

In this way, four putative mutant colonies were isolated and tested by PCR. Two of them had only one band as expected that was 2.2 kb (Fig. 31B), suggesting that the two transconjugants may be exconjugants having a *acdS*::Tc cassette replacing the wild-type gene on the chromosome, and the strain was designated UW4-AcdS$^-$.

3.4.6. Identification of *acdS* mutant

Further confirmation of the presence of the mutation in strain UW4-AcdS$^-$ was provided by Southern hybridization as described in section 3.3.3. The different digestion pattern between the wild-type and mutant strain can be recognized from the hybridization with either an *acdS* probe or a Tc probe. The expected sizes of the hybridization bands from *Sal*I digested genomic DNA were 2.05 kb and 1.57 kb for the mutant where a double crossover recombination had occurred. Strain UW4/pDAS1 would give two bands, 7.60 kb and 1.23 kb in size, when hybridized with the tetracycline resistance gene probe; and three bands, 7.60, 2.18 and 1.23 kb with the *acdS* probe. The genomic DNA from the strain UW4-AcdS$^-$ was digested with *Sal*I and hybridized with *acdS* probe, two hybridization bands resulted with an *acdS* probe and as expected the bands were 2.05 kb and 1.57 kb in size (Fig. 31C). The result from Southern hybridization further confirmed that a double crossover had occurred in the transconjugant UW4-AcdS$^-$ and that it harbors the disrupted *acdS* gene (*acdS*::Tc) on its chromosome.

Whether or not strain UW4-AcdS$^-$ is an ACC deaminase deficient mutant in its phenotype was examined by testing UW4-AcdS$^-$ for ACC deaminase activity. The ACC deaminase activity in both strains UW4 and UW4-AcdS$^-$ were compared. The result showed that the ACC deaminase activity from wild-type strain UW4 was 20-fold higher than the mutant strain UW4-AcdS$^-$ (Table 11), and that no activity was obtained from UW4-AcdS$^-$, indicating that strain UW4-AcdS$^-$ is in fact an ACC deaminase deficient mutant.

Table 11. ACC deaminase activity of wild-type strain UW4 and its mutant UW4-AcdS$^-$ and the ability of these strains to promote the elongation of canola roots under gnotobiotic conditions. The experiments for the root promotion were repeated three times and showed as separate tests.

* The assay was performed with the second ACC induction method, and the activity of ACC deaminase was expressed in nmoles/mg protein/h.

** Data are given as means ± standard error.

a: indicates that strain UW4 treatment is significantly different from the respective control $MgSO_4$ at the 0.01% probability level as determined by the t-test.

nd: not determined.

Strains	ACC deaminase activity*	root length ± SE (mm)** / (sample size)		
		1st test	2nd test	3rd test
$MgSO_4$	nd	21.2 ± 1.0 (73)	31.5 ± 1.5 (49)	31.9 ± 1.5 (62)
E. cloacae UW4-AcdS$^-$	2.1	25.0 ± 1.0 (103)	34.8 ± 1.9 (57)	35.6 ± 2.1 (66)
E. cloacae UW4	1139	31.1 ± 1.3 (76)[a]	40.3 ± 2.0 (59)[a]	41.9 ± 2.0 (59)[a]

3.4.7. Testing the UW4-AcdS$^-$ mutant for promotion of root elongation

The ACC deaminase mutant that was created in this study was further tested for the ability to promote the elongation of canola seedling roots. Canola seeds were inoculated with both mutant and wild-type strain UW4 cells with 0.1 M $MgSO_4$ used as a blank control, and the experiment performed on three separate occasions. The root lengths were measured on the fifth day after inoculation with the exception of the first test in which the measurement was done on the fourth day (Table 11). The results from the three experiments were consistent and showed that the roots of canola seeds treated with wild-type strain UW4 were significantly longer ($P<0.0001$) than the roots from the blank control and mutant UW4, while no significant difference was found between the mutant strain UW4-AcdS$^-$ and the control, although some promotion was found in all experiments.

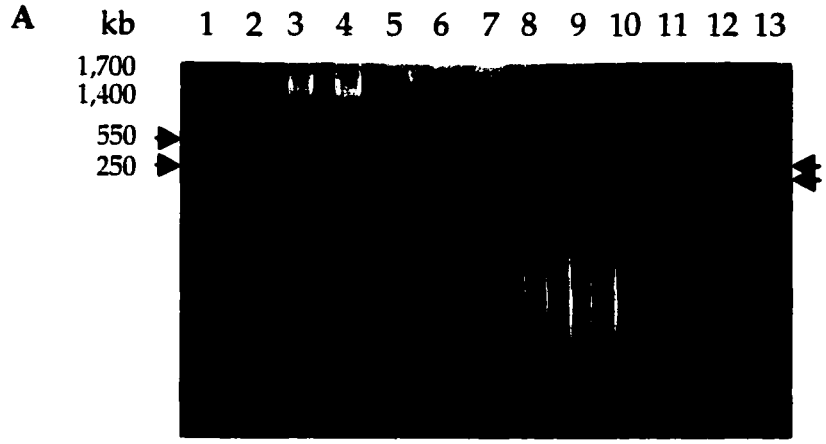
3.5. A study on the evolution of ACC deaminase

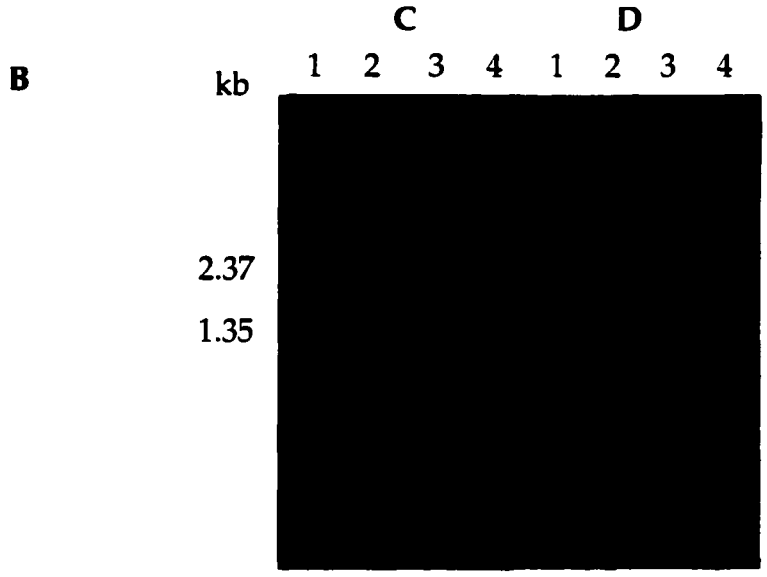
The diversity the *acdS* genes in different soil organisms suggests that the gene may be horizontally transferred among different organisms. Determining the localization of the *acdS* gene and its arrangement in the genome will provide understanding as to the genetic background of the gene.

3.5.1. Localization of the *acdS* gene

Several soil bacteria have been reported to carry megaplasmids and many of them have been well characterized. For example, the metabolic plasmids TOL and NAH/SAL from *Pseudomonas* have been well-studied

Fig. 32. **A**: Detection of the megaplasmids of strains UW4 and CAL2. Lanes 1-2, the megaplasmids of strain C58 that are 500 kb and 250 kb in size; lane 3-4, the megaplasmids of strain 2011 that are 1,700 and 1,400 kb in sizes; lane 5-7, strain UW4; lane 8-10, strain CAL2; lane 11-12, strain GR12-2. The arrows are used to label the bands and estimate the size. **B**: Northern hybridization of total RNAs that were isolated from strain UW4 and the clone p4U2 by Qiagen column. **C**: Formaldehyde agarose gel electrophoresis of total RNAs. **D**: Autoradiogram of the nylon membrane-blotted RNA from the gel shown in panel C after hybridization with a ^{32}P-labeled *acdS* DNA probe. Lane 1, total RNA isolated from strain UW4 grown in DF-minimal medium containing 3 mM ACC; lane 2, total RNA isolated from *E. coli*/p4U2 grown in M9-ammonia medium; lane 3 and lane 4, total RNA isolated from *E. coli*/p4U2 grown in M9 minimal medium with 5 mM and 10 mM ACC, respectively. Numbers indicated on the side are the size (in kb) of RNA marker.


A
kb
1 2 3 4 5 6 7 8 9 10 11 12 13
1,700
1,400
550
250


B
C
D
kb
1 2 3 4 1 2 3 4
2.37
1.35

and large plasmids, such as these, are usually involved in the degradation of different hydrocarbons. To initiate this experiment, attempts were made to isolate the plasmid component from both strains UW4 and CAL2 by conventional large plasmid isolation techniques (section 2.3.1). One large, clear band was obtained after separating the preparation on an agarose gel. However, the band was later identified as contaminating chromosomal DNA since it showed a smear pattern after restriction digestion.

A modified in-well lysis technique (section 2.3.2) was also used to determine the plasmid profiles of each strain on horizontal agarose gels and two known megaplasmid-containing bacteria, namely *Agrobacterium tumefaciens* C58 with two plasmids (500 kb and 250 kb), and *Rhizobium meliloti* 2011 with two megaplasmids (1,700 kb and 1,400 kb), were used as size standards (Jumas-Bilak et al., 1998). In addition to the two size markers, *P. putida* GR12-2 was also used as a control, although the sizes of the large plasmids from this strain were difficult to identify, it showed two bands on the gel, indicating that strain GR12-2 may contain two large plasmids. It was known that the cell does contain a large plasmid (Y. Hong, Ph.D. thesis). Results of the in-well lysis gel are shown in Fig. 32A, and it can be seen that no clear bands were obtained from either strain UW4 or CAL2. However, the standard size plasmids from both *Agrobacterium tumefaciens* and *Rhizobium meliloti* 2011 could be clearly seen on the gel and two large plasmids from *P. putida* GR12-2 were observed and the sizes were estimated to be between 250 kb and 500 kb.

3.5.2. Arrangement of *acdS* gene in the genomic DNA

In order to understand the genomic background of *acdS* in strain UW4, total RNA from wild-type *E. cloacae* UW4 and *E coli* DH5α/p4U2, the clone containing *acdS* gene, was analyzed by Northern hybridization. The *acdS* clone p4U2 was incubated in M9 minimal medium without ACC, and M9 medium with ACC at 5 mM and 10 mM, respectively. The RNA samples were first extracted with phenol (section 2.13.1.1), a method that results in a higher quantity of RNA, some plasmid DNA was found in the sample. To eliminate competing DNA, the RNA samples were separated from DNA with the Qiagen RNeasy Mini Kit (section 2.13.1.2), after which, the RNA sample was relatively pure although it was still contaminated with a small amount DNA. After treating the RNA samples with DNase, no DNA bands could be seen in the gel. However, the results from the hybridization showed a degraded hybridized region (Fig. 32B). Although the results were not perfect, it was clear that no hybridization signal was observed when p4U2 was incubated without ACC. When the clone p4U2 was incubated with 5 mM and 10 mM ACC, a weak signal with incubated 5 mM ACC was obtained (data not shown), while a strong signal from 10 mM incubation was similar to that from wild-type strain UW4. The signals were at an area below 1.35 kb. Furthermore, isolation of RNA using CsCl ultracentrifugation (section 2.13.1.3), produced the same results obtained with the Qiagen kit.

3.5.3. Isolation of clones from ACC agar

From previous experiments, it seemed that two different mechanisms were involved in permitting bacteria to grow on medium containing ACC as a sole source of nitrogen. One mechanism is related to the presence of the *acdS* gene, confirmed at both the gene and enzyme activity levels, the second involves growth on ACC-agar through ACC deaminase activity by way of different ACC deaminase genes. For example, the ACC deaminase-containing strains *Pseudomonas putida* GR12-2, *Pseudomonas fluorescens* CAL1 and *Enterobacter cloacae* CAL3, did not produce any positive signals during PCR analysis and Southern hybridization using the UW4 *acdS* gene as a probe (Shah et al., 1998). Thus, there probably are enzymes, in addition to the one encoded by *acdS*, that cleave ACC.

A genomic library of strain UW4 was constructed in vector pUC19 in *E. coli* (sections 2.6) and screened for the ability to grow on solid minimal medium containing ACC. Of the 3142 clones in the genomic library 7 clones were able to survive on the ACC-agar. However, many attempts to isolate plasmids from five of these seven clones failed. Plasmids were, however, isolated from clones pWA1 and pWA2 using the PEG method (section 2.2.2). Both plasmids, pWA1 and pWA2, were digested with *Hind*III and *Sma*I and the sizes of inserts were 4 kb in pWA1 and 0.8 kb in pWA2. The ability of each clone to grow using ACC as the sole source of nitrogen was investigated using either M9-ACC agar or M9-ACC broth. The growth of the clones on M9-ACC agar was shown to be very slow, and not at all comparable to the growth of wild-type strain UW4. However, in M9-ACC broth, both clones reached an OD_{600nm} of 1.0 after three days incubation

Table 12. ACC deaminase activity of *E. coli* clones carrying pWA1 and pWA2 and the ability of those clones to use ACC as a sole source of nitrogen. ACC deaminase activity was expressed in nanomoles per milligram of protein per hour.

-: no bacterial growth.

nd: not detectable.

Strains	Growth in minimal medium with ACC (OD_{600})	ACC deaminase activity of cells grown in nutrient medium	ACC deaminase activity of cells grown in minimal medium
E. coli/pUC18	–	16.8	nd
E. coli/pWA1	1.04	11.6 ± 2.8	85.6 ± 15.4
E. coli/pWA2	1.27	13.4 ± 2.4	69.8 ± 14.7
E. cloacae UW4	2.17	57.6 ± 7.1	1890 ± 18

Fig. 33. Nucleotide sequence and the deduced amino acid sequence of clone pWA2 that was isolated from an *E. cloacae* UW4 library together with its flanking regions. An inverted repeat sequence is underlined.

```
agccgccccggcaccgccgaactgATGCGCGAAGCCATGGCCCTGGGCGTG  51
                          M  R  E  A  M  A  L  G  V
GAGAACGTCGGTGGCCTGGACCCGTGCGGCATCGACAACGACCCGATCGCC  102
 E  N  V  G  G  L  D  P  C  G  I  D  N  D  P  I  A
CAACTGGATTTCGTCTTCAAGCTGGCCAGCGAATTTGACCGTGGCGTCGAC  153
 Q  L  D  F  V  F  K  L  A  S  E  F  D  R  G  V  D
ATTCACCTGCACGACAAGGGTGAGCTGGGTCTGTGGCAGATCGCGCTCATC  204
 I  H  L  H  D  K  G  E  L  G  L  W  Q  I  A  L  I
GCCGACTACACCGAGCGCTTCGGCCGCCAAGGCCAGGTGATGATCAGTCAT  255
 A  D  Y  T  E  R  F  G  R  Q  G  Q  V  M  I  S  H
GCCTACTGCCTCGGCATGCTGCCATGGAGCCAGgTCAAACCGGTGGCCGAG  306
 A  Y  C  L  G  M  L  P  W  S  Q  V  K  P  V  A  E
CGCCTGGCGACAcTGGGTATTTCGCTGATGAGTTCGGCCCCGGCCGATTGC  357
 R  L  A  T  L  G  I  S  L  M  S  S  A  P  A  D  C
GCGGTGCCGCCGTTCCTGGCCCTGCGCGAAGTCGGCGTGAATCTGTGCCTG  408
 A  V  P  P  F  L  A  L  R  E  V  G  V  N  L  C  L
GGCTCGGACGGCATTCGTGATGCATGGTCGCCGATGGGCAACGGCGACATG  459
 G  S  D  G  I  R  D  A  W  S  P  M  G  N  G  D  M
CTGGAGCGCGCGATGCTGCTGGCGTTCCGTTTCGATTTGAACAAGGACGAA  510
 L  E  R  A  M  L  L  A  F  R  E  D  L  N  K  D  E
GAACTGGCGGCGGCGTTCGAGGCGGCGACCgTCAACGGCGCCCAGGCACTG  561
 E  L  A  A  A  F  E  A  A  T  V  N  G  A  Q  A  L
GGTTGCGCAGCCAATCGCCTGCAGGTCGGCCAGCCGGCGGACTTCCTGCTG  612
 G  C  A  A  N  R  L  Q  V  G  Q  P  A  D  F  L  L
ATGCCCGTGCAAACGTTGGGTGAAGCGGTGGTTTCGCGACCGCTGCGTCAA  663
 M  P  V  Q  T  L  G  E  A  V  V  S  R  P  L  R  Q
GTCTATCGCGCCGGTCGTTTGATCGCTGCGGGTAGTCGTTTGCTGGAAAGC  714
 V  Y  R  A  G  R  L  I  A  A  G  S  R  L  L  E  S
CGCCTGTGAagcgcatcggtttgcgggccagttgcgtggtcggcttcgacg  765
 R  L  *
gcacgcagcatgtgctgtggcgcgacggcgaagtggtgttcgaaggctcgc  816

gcatcgagtttgtcgggcgcggttatccgggcccggtagatcagtggaccg  867

attacggcaacgcgctgatcggccccggcttcatcgatctggatgcccttg  918

gcgatcaccgccgacgaaggcattcgtgccggcaagaagatccccggtacc  969

gagctcgaattc                                         981
```

with normal shaking (Table 12). The slow growth of the clones on ACC agar is consistent with the results which showed that both clones have a very low level of ACC deaminase activity.

Both clones were further incubated in M9-minimal medium with different amino acids as substitutes for ACC as a sole source of nitrogen, the substitutes included L-alanine, L-serine, L-threonine, L-aspartic acid, L-arginine, L-asparagine, L-histidine, L-glutamic acid, L-tryptophan and L-proline. None of the amino acid substitutes were able to serve as a sole nitrogen source for bacteria growth. Also, protein extracts from both clones were analyzed with these amino acids as possible substrates for an amino acid deamination, and no activity was obtained.

3.5.4. Analysis of the gene from pWA2

The inserts from pWA1 and pWA2 were analyzed using PCR and Southern hybridization for similarity to each other and the known ACC deaminase gene from strain UW4. The results showed that neither insert was homologous with the UW4 *acdS* gene, and the two clones contain different inserts (data not shown). The 0.8 kb fragment cloned in pWA2 was sequenced in both directions (performed by Dr. S. Shah). The primers used for the sequencing, in addition to universal and reverse primers, were AB7986 and AB7955. The resultant DNA sequence contained an open-reading frame that consisted of 696 nucleotides predicted to encode a protein of 232 amino acids (Fig. 33). The sequence CGGGCGCGGTT followed by several T residues was found in the 3′ terminal non-encoding region as an inverted repeat that was 107 nucleotides downstream from the

Fig. 34. Alignment of the deduced amino acid sequence from pWA2 with the other gene sequences obtained from GenBank. The dashes represent gaps in the sequence. The number beside the sequences indicate the nucleotide numbers in each sequence.

CodA: cytosine deaminase from *E. coli*, P25524. (Danielsen et al., 1992)

AtzC: N-isopropylammelide isopropyl amidohydrolase from *Pseudomonas* sp. ADP, AF17572. (Sadowsky et al., 1998)

Ctd: creatinine deaminase from *Bacillus* sp., D38505.

```
CodA:   CNNALQTII- NARLPGEEGL WQIHLQDGKI SAIDAQS-GV MPITENSLDA  48
AtzC    MSKDFDLIIR NAYLSEKDSV YDIGIVGDRI IKIEAKI--- EGTVKDEIDA  47
Ctd     MR------IT NAQVKNYAEL VDITIEGERI STITPSALRP EDRRADDYDA  44
WA2     MR-------- ---------- ---------- ---------- ----------   2

CodA:   EQGLVIPPFV EPHIHLDTTQ TAGQPN---- WNQSGTLFEG IERWAERKA-  93
AtzC    KGNLVSPGFV DAHTHMDKSF TSTGERLPKF WSRPYTRDAA IEDGLKYYK-  96
Ctd     AGRLVSPQFA EAHIHLDYAN TAGVPR---- ENSSGTLFEA IEIWADRKTQ  90
WA2     ---------- ---------- ---------- ---------- ----------   2

CodA:   -LLTHDDVKQ RAWQTLKWQI ANGIQHVRTH VDVSDATLT- ALKAMLEVKQ  141
AtzC    -NATHEEIKR HVIEHAHMQV LHGTLYTRTH VDVDSVAKTK AVEAVLEAKE  145
Ctd     GFHIKEDIKA KALQAARRAA EHGVGFIRTH VDVTDPTFA- GFEAIADVRD  139
WA2     ---------- ---------- ---------- ---------- ----------   2

CodA:   EVAPWIDLQI VAFPQEGILS YPNGEALLEE ALRLGADVVG AIPHFEFTRE  191
AtzC    ELKDLIDIQV VAFAQSGFFV DLESESLIRK SLDMGCDLVG G--VDPATRE  193
Ctd     ENSSGTLFEA VAFPQNGIYA YEGGQKLISD AMSAGADVVG GIPHLEPTRD  189
                                          * ** **  ** *  *  *
WA2     ---------- ---------- ---------E AMALGVENVG GLD--PCGID   21

CodA:   YGVE-SLHKT FALAQKYDRL IDVHCDEIDD EQSRFVETVA ALAHHEGMGA  240
AtzC    NNVEGSLDLC FKLAKEYDVD IDYHIHDIGT VGVYSINRLA QKTIENGYKG  243
Ctd     DGVE-SVKWL FDLAEKHSAP IDIHTDEIDD PHSRFVEVLA AEAAKRDMGA  238
        *    **   **** * **   *** ** *   *  *  *   *  *  *
WA2     NDPIAQLDFV FKLASEFDRG VDIHLHDKGE LGLWQIALIA DYTERFGRQG   71

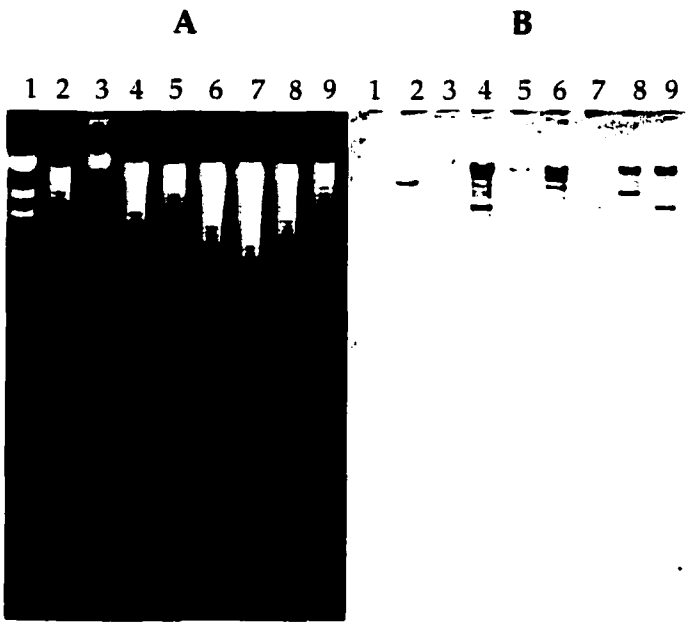
CodA:   RVTASHTTAM HSYNGAYTSR LFRLLKMSGI NFVANPLVNI HLQGR-FDTY  289
AtzC    RVTTSHAWCF ADAPSEWLDE AIPLYKDSGM KFVTC----- ------FSST  282
Ctd     QTVVSHSVAM AYYSPGYMAR LLPKLAASKV RFAVCPNENL HLQGLGFQGP  288
        **  *** *     *  * *   *                          **
WA2     QVMISHAYCL GMLP--W-SQ VKPVAERLAT LGISL----- ------MSSA  107

CodA    PKRRGITRVK EMLESGINVC FGHDDVFDPW YPLGTANMLQ VLHMGLHVCQ  339
AtzC    PP--TMPVIK -LLEAGINLG CASDNIRDFW VPFGNGDMVQ GALIETQRLE  329
Ctd     VPRGVAPVKQ -LTEWGIPVS FCQDSLNDPF YPMGDGDLLR ILDSGLHVSH  337
        *     *     * * * *** *** *** * ********   *     *
WA2     PADCAVPPFL ALREVGVNLC LGSDGIRDAW SPMGNGDMLE RAMLLAFRFD  157

CodA    LMGYGQINDG LNLITHHSAR TLNLQ-DYGI AAGNSANLII LPAENGFDAL  388
AtzC    LKTNRDLGLI WKMITSEGAR VLGIEKNYGI EVGKKADLVV LNSLSPQWAI  379
Ctd     MLTASHLKNA LSFITTNPAG NLGLD-NYDI AENSPANLLV LDASSEKEAV  386
        *     *  *     * ***   **   *     ** *** *   *     ***
WA2     LNKDEELAAA FEAATVNGAQ ALGCAANR-L QVGQPADFLL MPVQTLGEAV  206

CodA    RRQVPVRYSV RGGKVIASTQ PAQTTVYLEQ PEAIDYKR  426
AtzC    IDQAKRLCVI KNGRIIV--- ---------K DEVI--VA  403
Ctd     QRKAS----- ---------- ---------- ------VL  393
            *  *  * ** **                       *
WA2     VSRPLRQ-VY RAGRLIAAGS ---------- -RLLESRL  232
```

Fig. 35. Southern hybridization analysis of the seven PGPR strains with pWA2 gene as a probe. **A**: Agarose gel of the genomic DNAs from the seven strains digested with *Hind*III. **B**: Hybridization signals of the nylon membrane-blotted DNA from the gel shown in panel A after hybridization with a DIG-labeled pWA2 gene. Lane 1, DNA 1 kb marker; lane 2, UW1; lane 3, UW2; lane 4, UW4; lane 5, CAL1; lane 6, CAL2; lane 7, CAL3; lane 8, GR12-2; lane 9, a diluted DNA sample from strain UW4.


A
B
1 2 3 4 5 6 7 8 9 1 2 3 4 5 6 7 8 9

TGA stop codon. This sequence is potentially capable of forming a hairpin-like loop with 8 GC pairs and may represent a transcriptional termination site.

The amino acid sequence was deduced from the putative ORF sequence and compared with the ACC deaminase gene sequence, and only 17.5% identity was found between them. The sequence from pWA2 was further analyzed with those of other proteins in the SwissPort and PIR databases and those derived from translation of genes in the GenBank and EMBL databases (Altschul et al., 1997). The sequence of pWA2 had less than 35% overall sequence identity with any known protein. The highest identities were shown with N-isopropylammelide isopropyl amidohyfrolase (*atzC*) from *Pseudomonas* sp. ADP (32.1%) (Sadowsky et al., 1998) and creatinine deaminase from *bacillus* sp. (25.4%) (accession number D38505 in GenBank database). Moreover, the sequence of pWA2 had a 20.5% identity with cytosine deaminase (*codA*) from *Escherichia coli* (Danielsen et al., 1992). After alignment of the pWA2 sequence with the sequences of the three genes mentioned above, it was found that identity between pWA2 and at least one of three genes was 42.2%, and the sequence of pWA2 is shorter than these three genes (Fig. 34).

A search for conserved sequence motifs, however, was more informative. Several regions proposed to be involved in active-site metal coordination in both amino acid sequences of AtzC and CodA are bolded in Fig. 34 (Sadowsky et al., 1998). The five metal-liganding amino acid residues of CodA were reported to be four histidine residues (at position of 61, 63, 214, 246) and one aspartic acid (at position 313). Two histidine

residues (214 and 246) and one aspartic acid residue (313) of CodA were found to match with corresponding positions in the pWA2 sequence.

The genomic DNA from all of the seven strains UW1, UW2, UW4, CAL1, CAL2, CAL3 and GR12-2 was digested with *Hind*III and hybridized with a probe that was generated by PCR using primers AB7955 and AB7986 designed from the pWA2 gene. The results are shown in Fig. 35. The *Hind*III digested DNA from strain UW1 yielded a single weak band (lane 2) approximately 10.0 kb in size; three very weak bands from strain CAL2 (lane 6), all larger than 10.0 kb; one or two bands from strain GR12-2 with one band around 9.5 kb (lane 8). In strain UW4, three bands were hybridized with the probe. Two of which were extremely large, around 20 kb, and one band is clear and the size was around 8.0 kb (lane 4). The resolution of the three bands from strain UW4 became clearer when the amount of the DNA loaded in the lane was decreased (lane 9). However, no hybridization signals were obtained from strains UW2, CAL1 and CAL3. The results suggested that the putative ACC deaminase gene on pWA2 may also be present in other strains such as strains UW1, CAL2 and GR12-2, and may have low homology with other genes in the same strain UW4.

4. DISCUSSION

Soil contains a large variety of different microorganisms that vary in their ability to use unusual carbon or nitrogen sources. The ability of a certain microorganism to survive on unusual substrates such as opine, or naphthalene may provide a competitive advantage to the microorganism in the some soil environments. The enzyme ACC deaminases has been found in a number of soil microorganisms that can hydrolyze ACC into α-ketobutyrate and ammonia, however, its exact role in microorganisms is still unclear (Glick et al., 1999). Since ACC in nature is produced mainly by plants as a precursor to ethylene (Adams and Yang, 1979), it was proposed (Glick et al., 1995; 1997; 1998) that one potential function of ACC deaminase may be within plant-microbe interactions as one of the mechanisms that soil bacteria use to stimulate the growth of plants (see model in section 1.4).

Many studies on ACC deaminase have been directed toward elucidating its biochemical properties (Honma and Shimomura, 1978; Honma, 1985, 1986, 1993b). These studies 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. ACC deaminase genes were first isolated from pseudomonads (Klee et al., 1991; Sheehy et al., 1991), and subsequently, the gene was introduced into tomato plants (Klee et al., 1991; Sheehy et al., 1993; Robison et al., unpublished), tobacco and *Arabidopsis* (Romano et al., 1993), and the level of ethylene production in the

transgenic plants was dramatically decreased due to diminished levels of ACC.

The success of introducing bacterial ACC deaminase genes into plants emphasized its importance in nature, stimulated further study of microorganisms that contain ACC deaminase and encouraged investigations into its role in plant-growth-promoting rhizobacteria.

The focus of this study was to investigate the role of ACC deaminase in PGPR. As a first step, ACC deaminase genes from two PGPR were isolated and transformed into different soil bacteria. Following this, an ACC deaminase-deficient mutant was generated, and all strains were tested for their effects on the growth promotion of canola seeds. The study also focused on the regulation of the ACC deaminase gene in PGPR and its evolution.

4.1. Isolation and characterization of ACC deaminase genes

In this study, ACC deaminase genes (*acdS*) were isolated from two PGPR strains, *E. cloacae* UW4 and CAL2. This is the first report of ACC deaminase genes being isolated from strains of *Enterobacter cloacae*. Even though all seven PGPR strains initially studied were able to grow on ACC-agar, and five of them had ACC deaminase activity, observations based on Southern hybridization analysis using PCR-generated ACC deaminase gene probes (Shah et al., 1998) showed that only two of them contain genes for the known ACC deaminase. This suggested that more than one type of ACC deaminase gene may exist (Shah et al., 1998).

The deduced amino acid sequence from the DNA sequences of the isolated ACC deaminase genes were highly homologous (95-99% identity) with the reported pseudomonad ACC deaminase genes, with the exception of *P.* sp. strain ACP (81-82% identity) (Shah et al., 1998). The *acdS* genes from both *Enterobacter* strains can be expressed in *E. coli* cells with their native promoters. The Km for ACC of the crude cell-free extracts from strain UW4 is 10.7 ± 1.8 mM, indicating that the ACC deaminase gene from *Enterobacter* is similar to those in pseudomonads in that it binds the substrate ACC relatively poorly. The ACC deaminase gene isolated from the yeast *Hansenula saturnus* showed a 60-64% identity at the amino acid level with the reported ACC deaminase genes. Other reported putative ACC deaminase genes, from *E. coli* and *Pyrococcus horikoshii*, had a low identity (30-34%), however, neither were tested for ACC deaminase activity. And since we have never detected ACC deaminase activity in *E. coli*, there is a real question as to whether this is a bona fide ACC deaminase.

ACC deaminase is a member of the PLP-dependent protein family (Honma and Shimonmura, 1978). The catalytic mechanisms of PLP-dependent enzymes have been proposed to reside in three main portions of the active site: namely, the ε-amino group of a lysine residue, a glycine-rich region and a S-X-H-K sequence (section 1.2.3.). The lysine residues in PLP-dependent enzymes are known to bind the carbonyl group of PLP through an internal aldimine. Lys-51 of ACC deaminase was identified as an active site residue that is responsible for PLP-binding, and was shown to have similar functions with active site lysine residues in other PLP-dependent enzymes, such as Lys-87 in the β-subunit of tryptophan synthase (Honma,

1993); Lys-145 in D-amino acid transaminase (Manning, 1992); Lys-258 in aspartate aminotransferase (Malcolm and Kirsch, 1985) and Lys-74 in alanine dehydrogenase (Chowdhury et al., 1998).

In addition to the lysine residue, which forms a Schiff base (C=N) with PLP through its ε-amino group, a glycine-rich region (Gly-232, Gly-233, Gly-234, Ser-235 and Ala-237) in the β-subunit of tryptophan synthase is proposed to bind the phosphate group of PLP through hydrogen bonds (Hyde et al., 1988). This glycine-rich region was also found to be highly conserved in several threonine dehydratases, members of the PLP-dependent protein family, that contain the sequence Gly-Gly-Gly-X-Ser-Ala at positions 232-237 (Datta et al., 1987). The phosphate of PLP in glycogen phophorylase b was also reported to link two glycine residues, findings that suggest that the phosphate of PLP may bind to a glycine-rich region in other PLP-dependent enzymes. A search for glycine-rich regions in ACC deaminase revealed that there is such a region (GGLG) at positions 168-171 and it is flanked by a highly conserved sequence of 9 amino acids. The flanking region has been found in all ACC deaminase gene sequences and is located next to Cys-162 (Fig. 35). Cys-162 was previously shown to provide a reactive thiol group for the enzymatic reaction and is in close proximity to the active sites of the enzyme (Honma, 1993b). A comparison of glycine-rich regions between the ACC deaminase gene and the β-subunit of tryptophan synthase gene at the secondary protein structure has shown that the position of the GGLG region in ACC deaminase is located before the seventh of eleven helices, while the GGGSN region in the β-subunit of tryptophan synthase is situated before the ninth, out of a total of 13 helices (data not

shown). A further computer search for Prosite patterns using the MOSAIC program revealed that ACC deaminase contains many double glycine (GG) groups, such as GGKVE (positions 29-32), GGNKT (48-52), GGIQS (74-78), GGKPF (152-156), GGLGF (168-172), GGAPA (323-327). The double glycine groups all share the myristyl pattern (N-myristoylation site), and some of them may be involved in the enzymatic reaction. The sequence of ACC deaminase was also analyzed for the presence of the Ser-X-His-Lys sequence, however, no such sequence was found. A vaguely similar sequence Ser-Try-Glu-Lys was found at positions 141-144. Thus, the Lys-51 residue and the GGLG (168-172) region may be the catalytically important residue and ACC deaminase may in fact have active sites different from other PLP-dependent enzymes.

4.2. Regulation of the *acdS* gene expression

The upstream sequence of the ACC deaminase gene was obtained from the UW4 genomic library clone p4U2. The clone contains the 855 bp upstream region of *acdS*, and once promoter activity was confirmed with an ACC deaminase assay, the insert was sequenced in both directions. When 528 bp and the 229 bp upstream fragments from p4U2 were subcloned separately into the promoter-probe vectors, pKO-1 and pQF70, the results showed that the expression of both reporter genes was very low, though detectable. However, for those subclones that contained the same upstream fragments in the reverse orientation, the expression from both reporter genes was detected at high levels, indicating that a promoter is located on the opposite strand.

When the intact 855 bp upstream fragment was cloned into pQF70, a high level of luminescence was detected by both X-ray film and the CytoFluor Reader. Based on these results, deletion experiments were performed on the 855 bp fragment where the upstream region was shortened from position -855 toward to the ATG start codon. The resultant nine constructs contain deletions of different portions of the upstream region. These constructs were all sequenced and tested for expression, and it was found that the maximum level of luminescence was produced as long as the upstream region was longer than 722 bp, but that the luminescence immediately decreased once the deletion was beyond the -510 position, indicating that more than 510 bp and less than 722 bp of the upstream region of *acdS* is required for downstream gene expression. A small protein encoded within the upstream nucleotide sequence, named AcdR (for a protein that regulates transcription of the ACC deaminase structure gene, *acdS*), was found to be located on the opposite strand from *acdS* gene starting at -166 and ending at -672. Analysis of AcdR for sequence similarity to other known protein in GenBank suggested that it had 36.6% identity at the amino acid level to the Lrp (leucine-responsive regulatory protein) family.

It is known that during DNA replication and recombination, large nucleoprotein complexes involving multiple protein-DNA and protein-protein interactions are formed, and the DNA within these complexes is bent. Several proteins such as cAMP receptor protein (ACP) (Wu and Crothers, 1984), integration host factor (IHF) (Robertson and Nash, 1988), λ cI repressor (Kim et al., 1989), λ Cro (Kim et al., 1989), and λ replication

protein O (Zahn and Blattner, 1985) are known to facilitate the bending of DNA in replication complexes. In addition to these more thoroughly characterized proteins, Lrp, a DNA-binding protein, has recently been recognized to be a global regulatory protein in *E. coli*, and controls the expression of many operons through the induction of DNA bending (Lin et al., 1991; Wang and Calvo, 1993; Janes and Bender, 1999; Stauffer and Stauffer, 1998). Lrp can act as a positive regulator of transcription, although in some cases it may repress transcription. So far, Lrp is known to regulate more than 40 genes and operons in *E. coli* and the target genes are involved in cellular processes as diverse as amino acid biosynthesis, transport, and degradation, fimbrial synthesis, tRNA synthesis, maltose transport, outer membrane structure, osmoregulation, and others (Napoli et al., 1999). Some of the operons regulated by Lrp in *E. coli* include *glnA* (Ernsting et al., 1992), *gltBD* (Ernsting et al., 1992), *ilvIH* (Plakto et al., 1990), *livJ* and *livK* (Haney et al., 1992), *lysU* (Gazeau et al., 1992), *oppABCDF* (Austin et al., 1990), *sdaA* (Lin et al., 1990), *serA* (Lin et al., 1990) *ompC* and *ompF* (Ernsting et al., 1992) and *tdh* (Lin et al., 1990).

Lrp is a dimer consisting of two identical 18.8 kDa subunits. Lrp was mapped at 20 min in the *E. coli* genome (Anderson et al., 1976) and approximately 3000 molecules of the monomer are present in an *E. coli* cell (Willins et al., 1991). The N-terminal portion of Lrp contains a helix-turn-helix motif (Willins et al., 1991) that is proposed to be responsible for DNA binding, the middle third of Lrp mediates transcriptional activation and the C-terminal third determines the response of the protein to leucine (Plakto and Calvo, 1993). Binding sites for Lrp are usually located within several

hundred base pairs upstream of the promoter. The binding of Lrp to DNA facilitates the formation of a high-order nucleoprotein structure in the promoter-regulatory region. One study showed that Lrp could induce a DNA bend of ~52° upon binding to a single binding site, and the angle of bending is increased to at least 135° when Lrp binds to two adjacent sites (Wang and Calvo, 1993).

More recently some regulatory proteins resembling Lrp have been found in other bacteria. Some of them are highly homologous to the *E. coli* Lrp, such as, the Lrp from *Enterobacter aerogenes, Klebsiella pneumoniae, Salmonella typhimurium* (Friedberg et al., 1995; Tatusov et al., 1996) and *Bacillus subtilis* (Sorokin, 1997). However, some of them have low sequence identity (around 30%) with the Lrp from *E. coli*, such as MdeR (Inoue et al., 1997) and BkdR (Madhusudhan et al., 1993) from *Pseudomonas putida*, and PutR (Keuntje et al., 1995) from *Rhodobacter capsulants*. Although these proteins have low identity, it was found that all of the proteins contained the same conserved helix-turn-helix sequence for DNA-binding, and were located on the opposite strand upstream of the operon or gene that they control with similar regulatory functions. Thus, all of these proteins are classified as members of the Lrp family (Inoue et al., 1997).

One important feature of Lrp and Lrp-like proteins is that many operons under the control of Lrp are also subject to control by leucine, positively or negatively, however, some are not. For operons regulated by Lrp, leucine has an antagonistic effect (Haney et al., 1992; Newman et al., 1992), and, *in vitro*, leucine was found to reduce the extent of binding of Lrp to the DNA (Willins, 1991).

The results from this study suggest that *acdS* gene expression is regulated by a system not unlike those involving Lrp and Lrp-like proteins. During this study it was shown that: (1) AcdR, the regulator of the *acdS* promoter, is an Lrp-like protein. A conserved helix-turn-helix motif located in the N-terminal part of *AcdR* was recognized according to Dodd and Egan's systematic method (1987). The conserved motif is a typical DNA binding sequence that allows the Lrp-like protein to bind to DNA molecules. AcdR also has a high leucine content and its activation for the expression of *acdS* is repressed by addition of leucine to the growth medium.

(2) The binding site for AcdR appears to be located in the region between -195 and -149. Evidence for the position of the binding site comes from the observation that when upstream region was deleted shorter than 149 bp, the product of *acdR* carried by pACLR failed to *trans* complement the *acdS* promoter. However, when *trans* completed with the deletion constructs that contained upstream regions greater than 195 bp, expression of the reporter genes was restored. These results suggested that AcdR controls *acdS* expression by binding to DNA between -195 and -149.

In *E. coli*, it was reported that Lrp can bind to several specific sites that were located within a several hundred base pair region upstream of the promoter (Wang and Calvo, 1993). In *Pseudomonas putida*, xylR, which regulates the expression of *xylS*, was shown to bind to two UAS (upstream activator sequence) regions, -136 to -154 and -169 to -184. Comparison of the upstream sequence of *acdS* with the UAS reported from *P. putida* revealed that there is a sequence in UW4 that is similar to the UAS consensus sequence at position -80 to -96, located within one of the inverted repeats (-

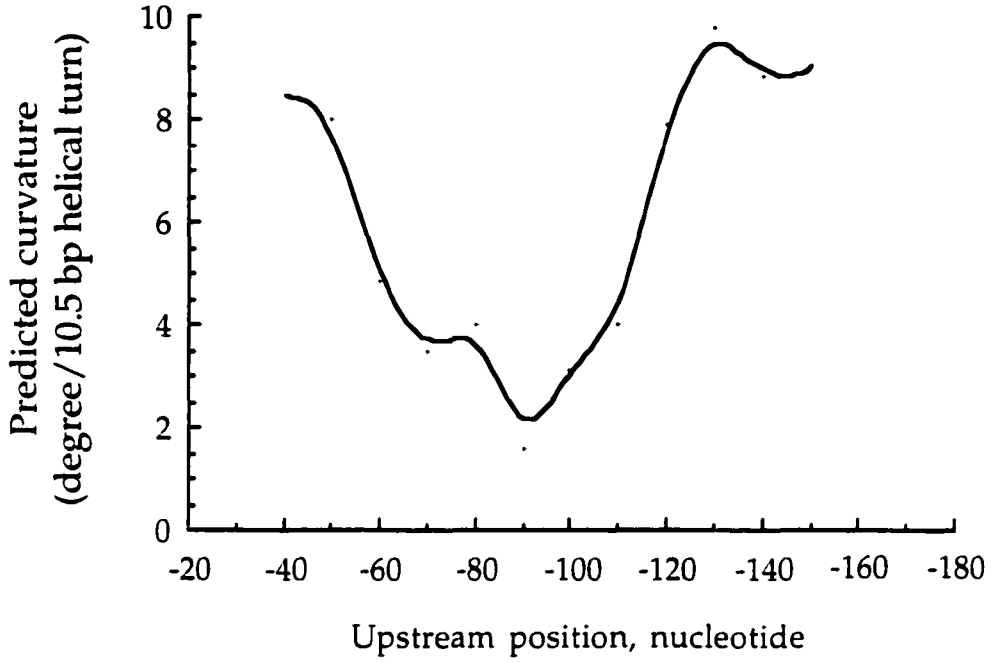
80 to -105) (Fig. 36B). The UAS-like sequence and the inverted repeat are both palindromic sequences, and either of them could be one of the binding sites for AcdR.

In addition to xylR, several regulatory proteins, such as pseudomonad BkdR and PutR (Keuntje et al., 1995; Madhusudhan et al., 1995) have also been identified as positive transcriptional activators, whose DNA-binding sites were found within regulatory regions. The sequences for Lrp binding sites from *E. coli* and BkdR (Madhusudhan et al., 1995) contain a consensus sequence with a number of consecutive thymine residues A/GTTTTNTNT (Fig. 36C). Two distinct features of the upstream sequence of *acdS* are an AT-rich region (generally known as a UP element) (Rao et al., 1994) between -195 and -105 and a large direct repeat (-116 to -174) (Fig. 20). Within the large direct repeat is a large run of consecutive thymine residues TTGTTTTTT, which is itself repeated, and a small sequence GGTTTTTTATT located at position -176 to -184 that contains six thymine residues in succession (Fig. 20 and Fig. 36C). Either of the thymine-rich sequences could be an AcdR binding site.

(3) The 165 bp intergenic region between *acdS* and *acdR* has a relatively low G+C content (33.3% mol) compared to the genomic G+C content of *Enterobacter* (52-60% mol). The intergenic G+C content is also lower than the intergenic G+C content reported in BkdR (50% mol). Low G+C content is often found within regulatory regions, and was proposed to play a role in transcriptional regulation by facilitating DNA bending and/or strand separation (Madhusudhan et al., 1993). The bending of the promoter DNA would then facilitate the activation of RNA polymerase and promote

Fig. 36. Computer analysis of the upstream region between *acdS* and *acdR*. **A**: Potential DNA bending between *acdS* and *acdR*. **B**: The bend center was estimated by the the lowest point of curve, and the nucleotide sequence around the lowest point contains a UAS-like binding site that is located within an inverted repeat, both are palindromic sequences. **C**: Comparison of the DNA binding sites for Lrp among putative AcdR and Lrp as well as BkdR. The direct repeats of the sequence from one of BkdR and AcdR are underlined.

A



B

-96 -80

UAS-like sequence: tgtAATTTGCAACACAAATTttc

-105 -80

Additional inverted repeats: attAATTGCTGTAATTTGCAACACAAATTttc

C

Sequence	Organisms	Strain	gene
AGAATTTTATTCT	*Escherichia*	*coli*	Lrp
TTTATTCTNAAT	*Escherichia*	*coli*	Lrp
GAGTTTGCGCATGAGAC	*Pseudomonas*	*putida*	BkdR
TGATTTTGTCTCAT	*Pseudomonas*	*putida*	BkdR
GTTTATGCGGAATGTTTAT	*Pseudomonas*	*putida*	BkdR
AGAATTTTTCTCTCT	*Pseudomonas*	*putida*	BkdR
CGTTCATATTGTTTTTTGCACAA	*Enterobacter*	*cloacae*	AcdR
CGTTAGTGTTTTGTTTTTATGTACGGAATAA	*Enterobacter*	*cloacae*	AcdR

functional protein-protein interactions when these DNA-binding proteins are relatively close together (Madhusudhan et al., 1993). Computer analysis of a portion of the region of DNA upstream of *acdS* using Bend.it software (www2.icgeb,trieste.it/~dna/cgi-bin/Bendit2) predicts the presence of a bend at -60 to -120 of the upstream region of *acdS* (Fig. 36A and B), and the bending center (the lowest point) was estimated to be within -85 to -90. Interestingly, the bending center is located in the UAS-like sequence (-80 to -96), consistent with the possibility that the UAS-like sequence may be the one of the binding sites for AcdR.

(4) AcdR is an essential regulator for the promoter of *acdS* and acts to activate the expression of *acdS*. Expression from deleted AcdR constructs was just barely detectable, but expression could be regained when the deleted construct was *trans* complemented by an intact AcdR construct. Furthermore, the complementation only occurred with cloned AcdR, not with Lrp from host *E. coli* cells, indicating that the regulation of *acdS* by AcdR is specific, and is consistent with the notion that ACC must react with AcdR before transcription of *acdS* gene is activated.

(5) The regulation of AcdR and expression of *acdS* is inducible by ACC. In the absence of ACC, the expression of the promoter could not be detected in either pJQ855 or the complemented strains. On the other hand, without AcdR, ACC cannot induce the expression of *acdS*, which is shown in the experiments using pJP96, pJQ588, and the deleted constructs including pJQ510, pJQ404, and pJQ292. Thus, the expression of *acdS* requires both AcdR and ACC. In this regard, ACC is likely to be either an effector for

AcdR converting it into an activator of *acdS* transcription or an inducer that effectively inactivates a repressor of *acdS* (see discussion 4.2 (12)).

(6) Clone pJQ855 that contained only the *acdS* upstream region was used to test different substances for the ability to induce ACC deaminase, and the results showed that L-alanine, α-ketobutyric acid and L-valine, but not D-alanine or any other amino acid, could induce the promoter, albeit at a very low level compared to ACC. The fact that L-alanine is involved in the induction of the promoter but not D-alanine is interesting in that the latter was reported to be a weak substrate for ACC deaminase (Honma and Shimomura, 1978). These observations may suggest that a similar structure but different stereo-specific conformation is required for alanine to act as either an inducer or substrate. ACC itself can act as both an inducer and a substrate for ACC deaminase, and induction by ACC can occur despite the presence of a pre-existing nitrogen source such as ammonia in the growth medium. It has been proposed that most operons regulated by Lrp are involved in adapting to changes in the nutritional environment (Landgraf et al., 1996). If the activating functions of Lrp *in vivo* requires a small molecule such as an inducer, then that inducer is normally present in cells growing in a minimal medium because the promoter is maximally active under such conditions (Willins et al., 1991).

(7) Expression from the *acdS* promoter appears to be moderately sensitive to the level of AcdR. When the complemented strains, *E. coli* COR292 and *E. coli* COR195 carrying pACLR (in a two plasmid system) were analyzed for expression of the reporter gene *luxAB*, the luminescence levels from these complemented strains were lower (62.0% - 74.7%) than that

of *E. coli*/pJQ855 (which carries both *acdS* and *acdR* genes in a one plasmid system). This result may be due to the fact that the vector, pACYC184, that carries the *acdR* gene is maintained at a low copy number, resulting in low levels of AcdR available for *trans* complementation. The same phenomenon was found during a study of the *E. coli livJ* promoter, in which the expression of the reporter gene was repressed 8-fold when Lrp was expressed from a moderate-copy-number plasmid and increased 30-fold when Lrp was expressed on a high-copy-number plasmid. The *livK* promoter also from *E. coli*, however, was not as sensitive to the concentration of Lrp (Haney et al., 1992). The moderate sensitivity of the *acdS* promoter to the level of AcdR, which is dependent on plasmid-copy-number, might be the reason for the differential expressions of the *acdS* promoter in pRKLUX plasmid when the latter was introduced into different organisms, where pRKLUX might be maintained at different copy numbers in different strains.

(8) The relative strength of different promoters was also addressed in this study, through the use of the reporter gene *luxAB*. In addition to the *acdS* promoter, two other promoters, the *acdR* promoter and the *E. coli* P_{lac} promoter, were conjugated with *luxAB* genes in either vector pQF70 or pRK415. The expression of both promoters demonstrated a 2.2 to 2.4-fold higher level of luminescence than that of *acdS* promoter in the same vector and same conditions, suggesting that *acdS* is not a strong promoter even after activation by AcdR. It was found that the -35 sequence and the -10 sequences in the upstream region of *acdS* were poor matches with typical prokaryotic -35 and -10 promoter sequences. It is generally known that for

most σ^{70} RNA polymerase (RNAP) promoters, the more similar the sequence of the -35 and -10 sites is to the consensus sequence, the higher the affinity of RNAP for the promoter. In the case of *E. cloacae* UW4, the strength of the promoter is also dependent on the distance between the binding site for AcdR and the promoter sequences, and the bending of DNA induced by AcdR. However, alignment of the upstream sequences from all of the isolated ACC deaminase genes (Fig. 12) indicates that the upstream sequence from strain UW4 lacks 35 nucleotides from position -29 to -63 compared to those aligned ACC deaminase upstream sequences, and the gap is located close to the promoter regions, therefore this may be another factor that affects the transcriptional efficiency of the gene.

(9) Strains UW4 and CAL2 have both been identified as species of *Enterobacter* and both contain *acdS*; however, only strain UW4 is fluorescent. Furthermore, strain CAL2 had a 2-fold higher ACC deaminase activity than strain UW4 (Table 2). Thus, despite the fact that CAL2 and UW4 have nearly identical *acdS* genes, the regulation of the *acdS* promoter in these strains is different.

(10) The FNR (fumarate and nitrate reduction) was previously found to be involved in the regulation of the *acdS* gene expression in strain UW4 (Grichko and Glick, in preparation). An FNR-binding consensus sequence was identified in the upstream region, located at positions -49 to -60. FNR is a transcriptional activator for many anaerobically regulated genes (Spiro and Guest, 1990; Khoroshilova et al., 1997; Jordan et al., 1997). It is an iron-dependent, DNA-binding protein, and it is assumed that the redox status of the cell is "sensed" by iron which in its reduced form induces

a conformational change in FNR that promotes DNA binding when cells switch from aerobic to anaerobic growth condition (Spiro and Guest, 1991). Experiments with clone pJQ855 showed that expression from the *acdS* promoter increased 3-fold under ACC induction when the cells were switched from shaking to non-shaking (i.e. aerobic to anaerobic) conditions and, at the same time, the expression from the opposite promoter was decreased to 62% of the shaking culture value that was observed under ACC induction. The results indicate that transcription may switch from the opposite promoter to the *acdS* promoter, when the incubation conditions are changed from aerobic to anaerobic conditions. The promoter switch could be regulated by FNR proteins from the host cells.

(11) In 1991, Lrp was proposed to function as a global regulatory protein in *E. coli* (Willins et al., 1991), and since then, a number of Lrp-like proteins have been isolated from different bacterial strains. Recently, several hypothetical models have been proposed to explain the mechanism of transcriptional regulation by Lrp.

i) In the GcvA-Lrp model for the *gcv* operon (glycine cleavage enzymes) from *E. coli*, in which GcvA (an activator) and Lrp are both necessary for activation of the *gcv* operon (Stauffer and Stauffer, 1998), it was proposed that Lrp induces DNA bending that facilitates the binding of GcvA to the two upstream sites. GcvA is then thought to either activate *gcv* by interacting with RNAP or repress *gcv* by binding to a second site. In this example, Lrp plays a structural role and GcvA is an activator protein. DNA bending mediated by Lrp may also be part of the mechanism of induced super-repression in this case.

ii) Activation by interaction with the C-domain of the α subunit of RNAP. For transcription of the *araBAD* operon (arabinose operon) in *E. coli*, both AraC (an activator) and CAP (catabolite gene activator protein) together stimulate the activity of the promoter of the *araBAD* operon and they likely interact with RNAP via contacts made by the C-terminal domain of the α-subunit of RNAP (α-CTD). The direction of transcription may depend on whether AraC or CAP binds to RNAP (Saviola et al., 1998). Another model for the *catBCA* and *clcABD* operons for the *ortho*-cleavage pathway from *P. putida* suggest that both operons require the transcriptional activators CatR and ClcR, and both regulatory proteins activate RNAP through specific interaction with the α-CTD of RNAP by binding to specific sites (McFall et al., 1998). GalR-RNAP-DNA ternary complexes obtained from the *gal* operon (galactoside operon) also support this model and it is thought that fine regulation depends on the location of the GalR binding site and the sequence of the DNA region between the activator and promoter (Ryu et al., 1998). A more complicated two-hybrid system has been proposed for the AraC (activator)/LexA (repressor) system (Kornacker et al., 1998). A heterodimerization fusion between AraC and LexA homodimers may further regulate either activation or repression in a specific spatial organization.

iii) Activation by integration host factor (IHF) (Bertoni et al., 1998), where the target for the activator (UAS) can be located at over 100 bp from the RNAP binding site. DNA looping induced by IHF binding enhances the recruitment and binding of RNAP to the promoter. IHF-induced bending could bring the two elements into close proximity and the resulting

promoter conformation could stabilize the C-terminal domain of the α-unit of RNAP and the promoter interacts through the UP element (an additional upstream element which is frequently an AT-rich region recognized by RNAP through an interaction with α-CTD) and promoter sites. An interesting finding is that IHF produces a sharp bend of the helix axis resulting in the widening of the major DNA groove, which then remains accessible for interactions with other proteins (Rice et al., 1996). Maximal transcriptional activation by IHF protein in *E. coli* has been investigated and found to be dependent on optimal DNA bending (Engelhorn and Geiselmann, 1998). The role of IHF may be to bend DNA, and bending induced by IHF below 180° is optimal for looping the DNA back towards RNAP. The wild-type protein would thus activate (favour upstream bending) and inhibit (make upstream contacts too tight) transcription initiation.

iv) The binding of Lrp is leucine-dependent (Roesch and Blomfield, 1998; Janes and Bender, 1999). The function of Lrp in both activating and repressing an operon may be regulated by leucine. One study on *fim* (phase variation of type 1 fimbriation) in *E. coli* showed that one of the three binding sites for Lrp is sensitive to leucine-bound Lrp. In the absence of leucine, three Lrp molecules bind to DNA in the regulatory region and activation is inhibited. When leucine is present, one binding site can no longer keep Lrp bound, and activation is stimulated by the remaining two Lrp molecules. Thus, the number of Lrp molecules that bind to DNA is leucine-dependent, and this in turn determines whether Lrp functions as a transcriptional activator or repressor.

v) The binding of Lrp is indirectly dependent on alanine. An interaction similar to that of Lrp and leucine was observed between Lrp and L-alanine (Janes and Bender, 1999). The levels of the enzymes D-amino acid dehydrogenase and alanine racemase were increased under limiting carbon conditions with L-alanine present in the growth medium. The operon *dadAX* in *E. coli* and *dadAB* in *S. typhimurium* and *K. aerogenes* encode enzymes controlled by CAP (catabolite activator protein), Lrp, and/or NAC (nitrogen assimilation control protein). When an Lrp molecule binds alanine it can no longer bind to DNA, and the absence of Lrp at one of the Lrp binding sites results in activation of transcription. In the case of the *dad* operon, the presence of alanine leads to activation of the operon by relieving the Lrp-mediated repression of the promoter. Thus, two possible inducers, leucine and alanine, may play a role in Lrp-mediated activation.

vi) DNA structural transmission model (Sheriden et al., 1998). Several strong σ^{70} promoters contain A+T rich upstream regions that are susceptible to destabilization. When regulatory proteins bind to these A+T rich regions, superhelicity is imposed and the region is destabilized, transferring the susceptibility to the nearby promoter sites. This protein-mediated activation of transcription requires a supercoiled DNA template and occurs in the absence of specific interactions between IHF and RNAP. Recent reports indicate that the protein-mediated, supercoiling-dependent model is also important in eukaryotes (Barton et al., 1997).

(12) Preliminary investigations into the regulation of the *acdS* promoter in strain UW4 were undertaken in this study. The results indicate that this regulation involves a complex series of interactions. To explain or

aid in understanding of the regulation of *acdS* promoter, a few models could be proposed that are conceptually similar to those involving Lrp and Lrp-like protein: the Lrp-effector model and inducer-repressor model. Our experiments suggest that both AcdR and ACC are necessary for the expression of *acdS*. AcdR itself, was found to be an Lrp-like protein and it may function similar to Lrp. AcdR binding to DNA may or may not be induced by ACC. In the first case, ACC could be an effector to stimulate the binding of AcdR to the proper recognition sites of the DNA. This stimulation may be replaced by L-alanine. Once AcdR binds to the DNA strand, it would induce bending of the DNA strand at an angle that allows RNA polymerase to access the *acdS* promoter and initiate transcription. In this case, the binding of RNAP would occur through an interaction of the α-CTD subunit with a specific DNA sequence element located in a distant region, which requires a change in the geometry of the DNA strand. In the second model, AcdR may not need to be induced by ACC, instead there may be a repressor acting on the *acdS* promoter region. ACC could act as an inducer to cause release of the repressor, however, transcription of *acdS* still requires AcdR to bend the DNA for RNAP function. It is also possible that both of the models are active simultaneously, i.e. ACC molecules act either as an effector for AcdR or an inducer for the *acdS* promoter. The fine regulation of the *acdS* gene in strain UW4 is still unclear, and it may involve a complex set of interactions with other other DNA-binding proteins.

4.3. Assessment of the model with different soil bacteria

The role of ACC deaminase in the activity of PGPR is part of a model that describes the potential ability of certain soil microorganisms to hydrolyze ACC (Glick et al., 1998). In this model (Fig. 3), ACC exuded by a plant into rhizosphere is taken up and hydrolyzed by the PGPR, which sets up the conditions for a net outflow of ACC from the plant and a decrease in the level of ethylene production inside the plant. Lowering the level of ethylene results in a relief in the ethylene inhibition of root elongation, and a consequent promotion of root growth.

Evidence that provided strong support for the model of PGPR promotion of plant growth was obtained by introducing the ACC deaminase gene into different soil bacteria that do not normally have ACC deaminase activity. The generated strains, *P. putida* ATCC 17399/pRKACC and *P. fluorescens* ATCC 17400/pRKACC, along with their control strains *P. putida* ATCC 17399/pRK415 and *P. fluorescens* ATCC 17399/pRK415 and wild-type strain CAL2 as a positive control, were used to inoculate canola seeds. The data in support of the model, clearly show that once a bacterium is provided with the enzyme ACC deaminase, it can significantly stimulate root elongation in ethylene sensitive plant seedlings (Table 10 and Fig. 27) and the bacterium becomes better able to survive in the rhizosphere (Table 10). The mechanism for their survival is unclear, though it may be that the bacterium gains the ability to use the nutrients exuded from plants more efficiently. However, the data suggest that any bacteria that can bind to the seeds of ethylene-sensitive plants and actively expresses ACC deaminase can promote the elongation of seedling roots, i.e. it can act as a PGPR.

The construct pJQ855, containing the upstream region of the *acdS* gene, conjugated with *luxAB* genes, can be used as an ACC probe to detect ACC in various conditions. The level of luminescence was dependent on the concentration of ACC and using a CytoFlour Reader luminescence could be detected when the level of ACC was at least 250 nM. When pJQ855 was used to detect ACC in seed exudates, it was difficult to find a significant difference between the probe pJQ855 and control pQF70. Since ACC, in nature, is present at a very low level, the amount of ACC exuded from the seeds may not be sufficient to detect by this method. However, this approach may be useful in estimating somewhat higher levels of ACC such as those found in ripening fruit or flooded plants. Several additional factors may affect the detection of ACC including the strength of the promoter, the sensitivity of the detector and the methods used for seed exudate collection.

4.4. Creation of ACC deaminase defective mutant

To assess the accuracy of the proposed role of ACC deaminase in the model, a mutant was created that lacked a functional ACC deaminase gene. In this study, an AcdS$^-$ mutant of *E. cloacae* UW4 was created by targeted gene replacement, in which an interrupted*acdS* gene present on a plasmid replaced the wild-type version in the genome of UW4. When tested, the mutant displayed little ACC deaminase activity. In addition, as expected, the AcdS$^-$ mutant lost the ability to promote the elongation of canola root (Table 11) compared to wild-type strain UW4, which was shown to significantly promote root elongation in the same experiment. This result, together with the observation that pseudomonads that have been

transformed to produce ACC deaminase acquire the ability to promote root elongation (Table 10 and Fig. 27), provides direct support for the proposed role of ACC deaminase in the functioning of some plant growth-promoting bacteria.

4.5. A possible evolution of deaminase

Some PGPR that have ACC deaminase activity, and are able to both grow on ACC-agar and promote the elongation of canola roots, do not show a hybridization signal when probed with the UW4 ACC deaminase gene. This raises the possibility that other ACC deaminases or enzymes that can cleave ACC in a non-specific way may be present in these other bacteria. A putative gene cloned in pWA2 from strain UW4 allows the host cells to grow in ACC minimal medium, thus, studies on the relationship of this gene to ACC deaminase and other proteins may provide useful information towards our understanding how of ACC deaminase evolved and the physiological importance of ACC deaminase in bacteria.

The putative ACC deaminase from pWA2 displayed low sequence identity with the UW4 ACC deaminase (17.5%) at the amino acid level and no conserved regions were found. Further protein similarity analysis indicated that the putative gene had a 20% - 30% identity with the enzymes cytosine deaminase (CodA), N-isopropylammelide isopropylaminohydrolase (AtzC), and creatinine deaminase (Ctd). An important finding was that the putative ACC deaminase gene shares conserved residues within the active sites with the three enzymes, and the similarity increased up to 65.3% with AtzC, 54.1% with CodA and 55.3%

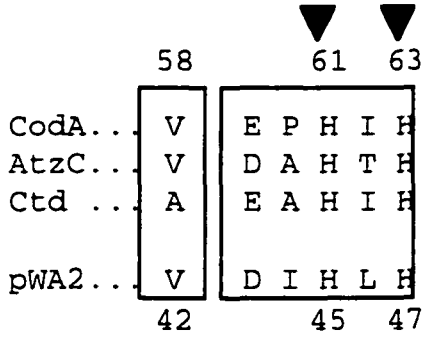
with Ctd. Interestingly, all three of these enzymes are deaminases, but surprisingly, they are not PLP-dependent enzymes. Instead, all three enzymes belong to the amidohydrolases protein superfamily, which share a similar catalytic function in the hydrolysis of cyclic amides (in either 5- and 6- member rings). The key mechanistic feature of the amidohydrolase superfamily is that all of the enzymes contain mononuclear or binuclear metal centers that are essential for catalytic activity. Cytosine deaminase, for example, can bind zinc, manganese, iron, or cobalt (Poter and Austin, 1993). The amidohydrolase protein family is proposed to contain over 70 members, such as dihydro-orotase, urease, adenine deaminase, phosphotriester hydrolase, D-hyhantoinase, allantoinases and dihydropyrimidinase (Holm and Sander, 1997; Kim and Kim, 1998). Based on the comparison of the crystal structure and enzyme architecture of ureases and adenine deaminase, it is thought that these proteins all share a common fold (Holm and Sander, 1997; Jabri et al., 1995).

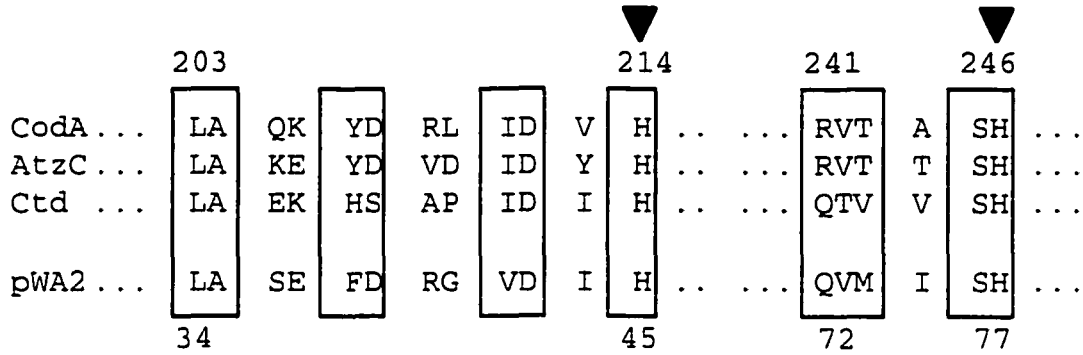
In fact, the sequence identity between each protein in the amidohydrolases superfamily is low, around 10% - 45% (Sadowsky et al., 1998; Kim and Kim, 1998), but all of the enzymes contain an ancient conserved region seen in proteins from eukaryotic, Archaea and prokaryotic organisms, and catalyze similar functions but with different substrates (Holm and Sander, 1997). On the basis of the similar catalytic function and existence of the conserved sequences, it has been proposed that the enzymes that comprise the amidohydrolase superfamily are examples of divergent evolution. From an evolutionary stand point, enzymes possessing the conserved active site region are closely related to each other with respect to

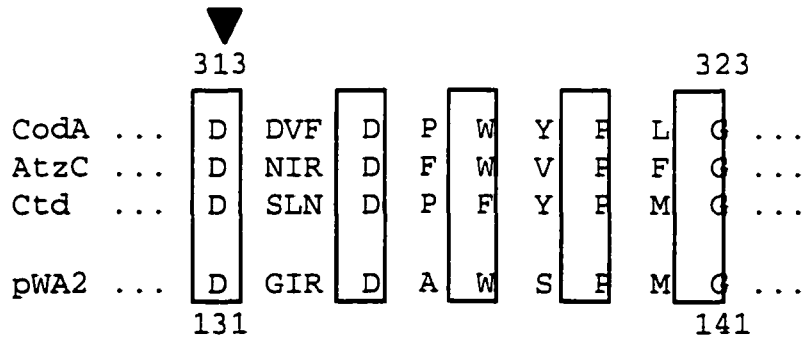
the nature of the substrate on which they act, despite the low similarity (Kim and Kim, 1998).

In the amidohydrolases superfamily, conserved residues have been found and consist of four histidine and one aspartic acid residue (Poter and Austin, 1993), and they are located at different positions but clustered together in the folded polypeptide to bind with metal ions from different directions. For example, the active-site metal coordinating residues in cytosine deaminase are His-61, His-63, His-214, His-246 and Asp-313 (Sadaowsky et al., 1998). The putative ACC deaminase gene cloned in pWA2 was found to match active sites within the amidohydrolases superfamily. Since the sequence of the gene in pWA2 is shorter than the three genes with which it was compared, it was aligned with only the last three residues, i.e. histidine (214, 246) and aspartic acid (313) (the numbering is from the CodA sequence) (Fig. 37). However, the active histidine 61 and histidine 63 together with another aspartic acid, which make up a typical consensus sequence (D-X-H-X-H) found in the N terminal portion of the amidohydrolase superfamily enzymes (Kim and Kim, 1998), was surprisingly found in the sequence of the putative gene in pWA2, although because it was shorter it seemd to lack this portion. The histidine 45 (previously proposed to match histidine 214 in CodA) and histidine 47 that form the H-X-H motif, together with aspartic acid 43 are assumed to be the typical amidohydrolase consensus sequence (D-X-H-X-H) (Fig. 37). The sequence of pWA2 is shorter, but nevertheless contains the consensus D-X-H-X-H sequence which suggests that conserved active residues may

Fig. 37. Pairwise alignment of amino acid sequence from the putative ACC deaminase gene in pWA2 and *E. coli* cytosine deaminase (CodA: P25524), N-isopropylammelide isopropyl amidohydrolase from *P.* sp. ADP (AtzC: AF17572), creatinine deaminase from *Bacillus* sp. (Ctd: D38505) in the regions of CodA indicates to be involved in active-site metal coordination. The five metal-liganding amino acids of CodA are indicated with vertical arrows. Amino acid numbers were determines by counting from the N terminus of the proteins.


58
61
63
CodA...
AtzC...
Ctd ...
pWA2...
V
V
A
V
E P H I H
D A H T H
E A H I H
D I H L H
42
45
47


203
214
241
246
CodA...
AtzC...
Ctd ...
pWA2...
LA QK YD RL ID V H .. ... RVT A SH ...
LA KE YD VD ID Y H .. ... RVT T SH ...
LA EK HS AP ID I H .. ... QTV V SH ...
LA SE FD RG VD I H .. ... QVM I SH ...
34
45
72
77


313
323
CodA ...
AtzC ...
Ctd ...
pWA2 ...
D DVF D P W Y F L G ...
D NIR D F W V F F G ...
D SLN D P F Y F M G ...
D GIR D A W S F M G ...
131
141

coordinate to metal ions, therefore, the putative ACC deaminase gene may have evolved from the amidohydrolase superfamily.

The amidohydrolases have been reported to require metal ions for their activity, and are thus metalloenzymes. In addition, the structure of the substrates on which the enzymes act vary within the large superfamily, i.e. the cyclic amide (5- or 6- member rings), and limited identical residues were found for substrates (Kim and Kim, 1998). Microbial D-hydantoinases were reported to exhibit comparable affinity toward the substrates of other amidohydrolases (Kim and Kim, 1998). Interestingly, *E. coli* cytosine deaminase was reported to act on α-amino-η-butyric acid, although weakly (Katsuragi et al., 1986). From an evolutionary point of view, a minor modification in the specific regions or residues may trigger a significant alternation in substrate specificity. Thus, it is reasonable to suggest that ACC, a 3 member ring amide, may be a non-specific substrate, for the putative ACC deaminase gene product from pWA2. Finally it can be suggested that the mechanism of ACC hydrolysis by the putative enzyme is through the metal-binding catalysis, not PLP-dependent catalysis.

Newly evolved or evolutionary modified proteins may not only arise from point mutations, and insertions or deletions in native proteins, but also by combinations of genes to give chimeric proteins (Chothia, 1992). It is also interesting to find that the same ACC deaminase genes in both strains UW4 and CAL2 have different expression levels and different regulation systems in the upstream region, and the gap in the upstream region of strain UW4 may suggest that one of the genes came from horizontal gene transfer. During transformation, the genes may have

integrated into different chromosomal loci. On the other hand, since ACC deaminase has been found in different microorganisms from different areas, it is not likely to be a recently mutated gene. Furthermore, the G+C content of each of isolated ACC deaminase genes is, in most cases, similar to the chromosomal G+C content, suggesting that ACC deaminase may be a moderately evolved gene.

In this study, attempts to determine whether or not the ACC deaminase gene is present on a megaplasmid were also undertaken. Since after several attempts, such a megaplasmid was not found in either strain of *Enterobacter*, the issue of the physical location of the ACC deaminase gene remains unresolved, although it could conceivably be located on the chromosome.

In summary, ACC deaminase genes were first isolated from PGPR *E. cloacae* UW4 and CAL2. When the gene was introduced into different soil bacteria, they gained the ability to promote root elongation, thus, acting like PGPR. The expression of the *acdS* genes from both strains were found at different levels since the regulation systems involved in both strains were inherently different. Upon characterization, it was found that the regulation requires both AcdR, an Lrp-like protein, and ACC, acting as an inducer. As a means to confirm the role of ACC deaminase in the promotion of plant growth, an ACC deaminase mutant was created by targeted gene replacement. Results with this mutant provide direct proof for the ACC-PGPR function model and provide a first step towards the elaboration of the physiological role of ACC deaminase in bacteria.

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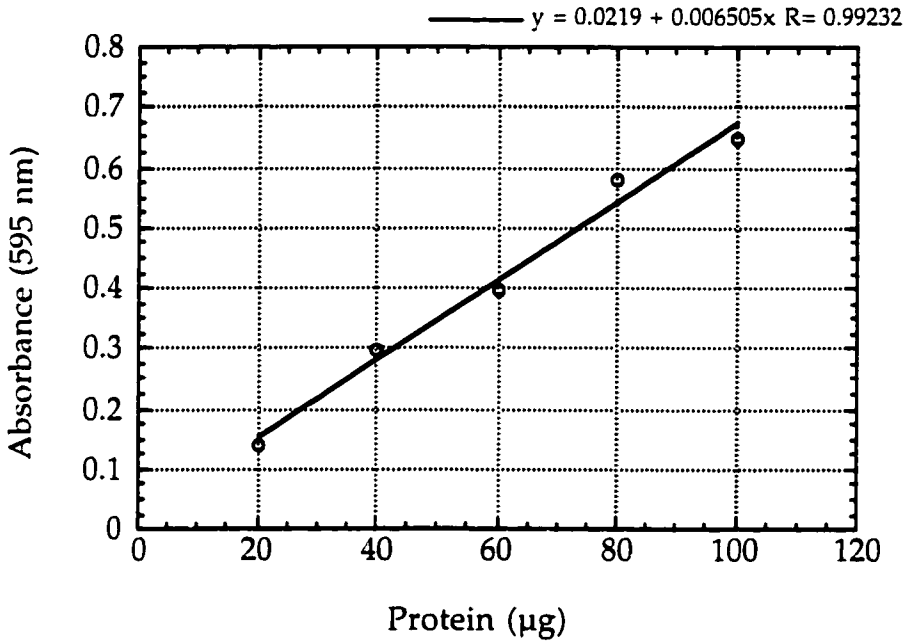
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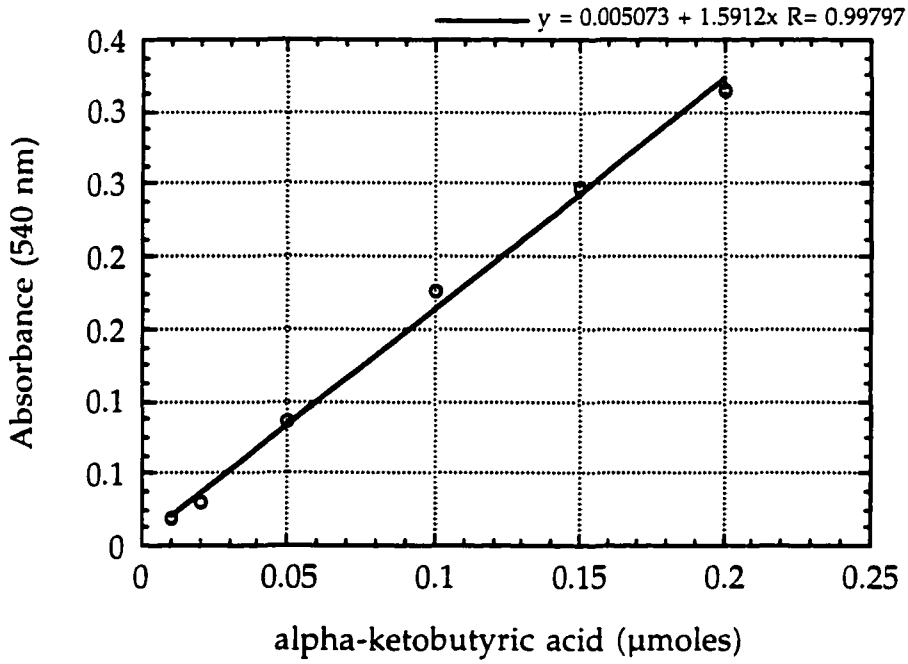
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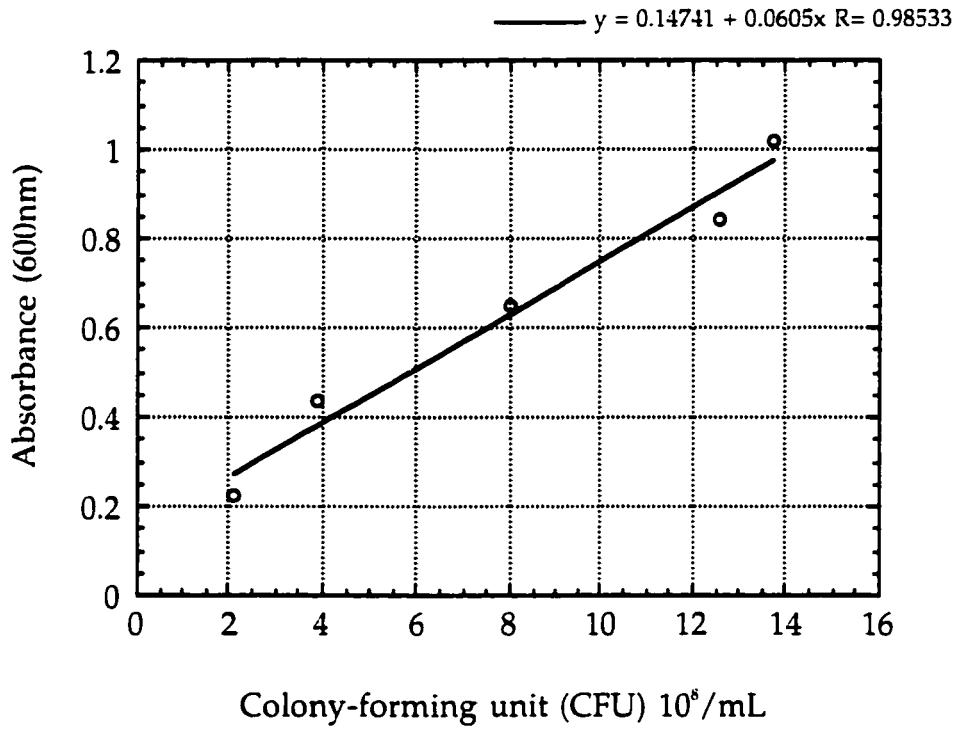
Appendix I. Bradford protein standard curve.


y = 0.0219 + 0.006505x R= 0.99232
Absorbance (595 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
20
40
60
80
100
120
Protein (µg)

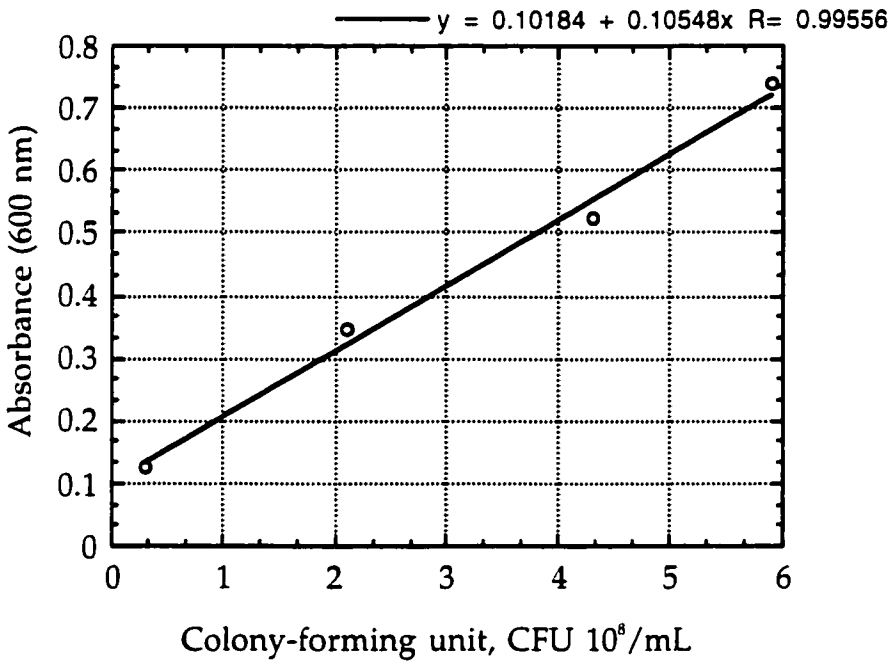
Appendix II. Standard curve for α-ketobutyric acid concentration.


y = 0.005073 + 1.5912x R= 0.99797
Absorbance (540 nm)
0.4
0.3
0.3
0.2
0.2
0.1
0.1
0
0
0.05
0.1
0.15
0.2
0.25
alpha-ketobutyric acid (µmoles)

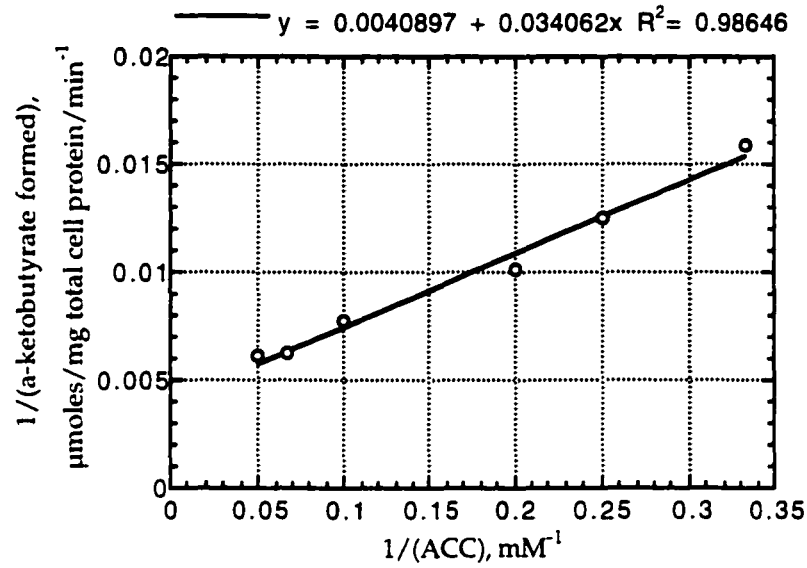
Appendix III. Curve of colony-forming unit (CFU) versus optical density (OD) standard of bacterial growth (*E. coli*) in LB medium.

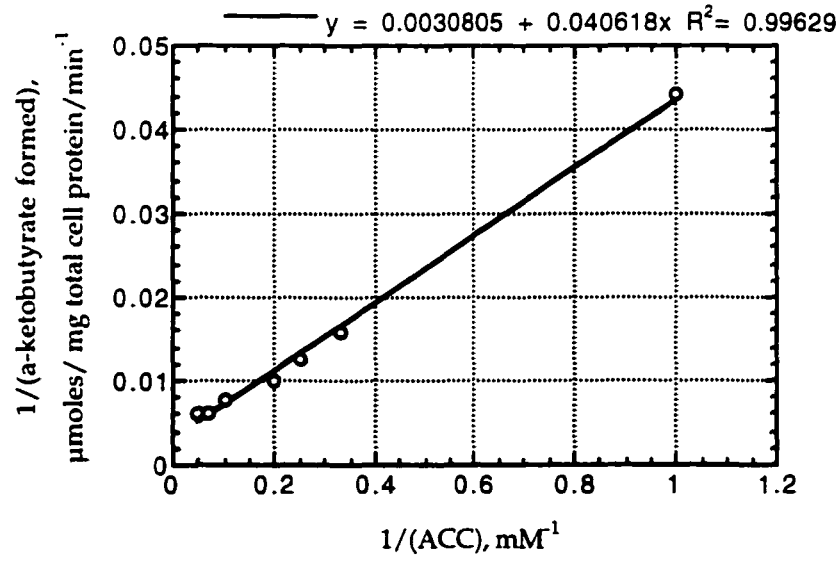

y = 0.14741 + 0.0605x R= 0.98533
Absorbance (600nm)
1.2
1
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
14
16
Colony-forming unit (CFU) 10⁸/mL

Appendix IV. Curve of colony-forming unit (CFU) versus optical density (OD) standard of bacterial growth (*E. coli*) in M9 minimal medium.


y = 0.10184 + 0.10548x R= 0.99556
Absorbance (600 nm)
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
0
1
2
3
4
5
6
Colony-forming unit, CFU 10⁸/mL

Appendix V. Effect of ACC concentration on ACC deaminase reaction.


y = 0.0040897 + 0.034062x R²= 0.98646
1/(a-ketobutyrate formed), µmoles/mg total cell protein/min⁻¹
0.02
0.015
0.01
0.005
0
0 0.05 0.1 0.15 0.2 0.25 0.3 0.35
1/(ACC), mM⁻¹


y = 0.0030805 + 0.040618x R²= 0.99629
1/(a-ketobutyrate formed), µmoles/ mg total cell protein/min⁻¹
0.05
0.04
0.03
0.02
0.01
0
0 0.2 0.4 0.6 0.8 1 1.2
1/(ACC), mM⁻¹