Cloning of 1-Aminocyclopropane-1-carboxylate Deaminase Gene from Soil Microorganism

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Abstract

From collected soil samples, 55 isolates of bacteria including 8 species were capable of growing on 1-aminocyclopropane-1-carboxylate (ACC) as a nitrogen source owing to induction of the enzyme ACC deaminase and the subsequent conversion of ACC to α-ketobutyrate and ammonia. Among these bacterial isolates *Pseudomonas fluorescens* strain J2 had the highest growth rate. The chromosomal DNA of *Pseudomonas fluorescens* strain J2 was purified, cut with *Sau*3AI, inserted into *Bam*HI site of pGEM-3Zf(+) plasmid and cloned in *Escherichia coli* strain XL1-Blue. Six clones with high growth rate on the medium containing ACC and ampicillin were selected for further investigation. ACC deaminase assay, restriction endonuclease digestion patterns, and hybridization results indicated that all plasmid clones contained an overlapping region of ACC deaminase gene from the J2 genome. However, ACC deaminase activity of *Escherichia coli* containing smaller plasmid clones were less active than those with larger plasmid clones and all of the transformed *Escherichia coli* had lower ACC deaminase activity than that of *Pseudomonas fluorescens* strain J2. This might have been due to the incompletion of ACC deaminase gene or some factors from host cell or plasmid vector used.

1. Introduction

1-Aminocyclopropane-1-carboxylate deaminase (ACC deaminase) is an enzyme catalyzing the degradation of ACC into α-keto-butyrate and ammonia [1],[2]. This enzyme was found in some microorganisms [3], thus enabling them to grow on minimal media containing ACC as a sole nitrogen source. ACC is a precursor of ethylene in the pathway of ethylene biosynthesis [4]. Ethylene is synthesized from ACC by using enzyme ACC oxidase and ACC is derived from S-adenosylmethionine by using enzyme ACC synthase [5]. Ethylene is a plant hormone that affects many processes of plant growth and development such as shoot and root growth, leaf abscission, plant senescence and fruit ripening [6],[7].

Ethylene plays an important role in the ripening of climacteric fruits such as tomato and mango. Control of tomato fruit ripening has been achieved by many researchers by means of controlling ethylene production. Antisense RNA to ACC synthase [8] or ACC oxidase [9] have been used efficiently to repress ethylene biosynthesis and fruit ripening in transgenic tomato. ACC deaminase gene from soil microorganism was also transformed to tomato to control fruit ripening [10]. In this study we have cloned an ACC deaminase gene from soil bacteria which is a basic step for further experimentation

2. Materials and Methods

2.1 Soil samples and bacterial strains isolation

Soil samples from 35 places were collected and screened for organisms that are capable of growing on applied Dworkin and Foster (ADF) medium [10,11] containing ACC as a sole nitrogen source. Bacterial isolates were then classified by the method described by Kreig and Holt [12]. The isolates, grown well on this medium, were chosen and reconfirmed by examining the growth curve in liquid culture [13] and the most efficient strain was selected for further use as a DNA source of cloning.

2.2 DNA extraction and cloning

Chromosomal DNA of the *Pseudomonas fluorescens* strain J2 was extracted as described by Sambrook *et al.* [14], partially digested with *Sau*3AI, ligated into the *Bam*HI site of pGEM-3Zf(+) and transferred to *E. coli* strain XL1-Blue. The transformed white colonies were transferred into ADF medium with ACC to select for the ACC degrading clones. The plasmids from the positive clones were then purified and digested with seven restriction enzymes to make a restriction map to determine the sizes of the inserts.

2.3 Measuring enzyme activity

ACC deaminase catalyzes the degradation of ACC into α-ketobutyric acid and ammonia. For comparison, the ACC deaminase enzyme was extracted from both bacterial isolates and *E. coli* clones by passing the crude extract through the G-25 sephadex column [15] and the activity of the enzyme was assayed by measuring the amount of α-ketobutyric acid, an outcome of the enzyme-substrate reaction, with 2,4-dinitrophenylhydrazine [16].

2.4 Southern hybridization

Southern hybridization of the selected recombinant plasmids was performed by employing one of these plasmids as a probe using an ECL® labelling and detection kit from Amersham company. The recombinant plasmids were cut with *Eco*RI and *Hin*dIII, electro-phoresed on 1% agarose gel, transferred to nylon membrane and hybridized with the insert from one plasmid.

3. Result

3.1 Isolation of bacteria from soil samples

From 35 soil samples; 55 isolates of bacteria, capable of growing on ADF medium, were selected and classified into 8 species i.e., *Alcaligenes xyloxosidans* subsp. *denitrificans*, *Enterobacter* sp., *Klebsiella pneumoniae*, *Pseudomonas cephacia*, *Pseudomonas fluorescens*, *Pseudomonas mendocina*, *Pseudomonas putida* and *Xanthomonas maltophilia* (Table 1). Among these; the first six strains, showing the highest efficiency in utilization of ACC as a nitrogen source, are shown on the growth curve in Figure 1. They were *Klebsiella pneumoniae*, *Pseudomonas putida*, *Pseudomonas fluorescens* strain B1, *Pseudomonas fluorescens* strain J2, *Pseudomonas fluorescens* strain E1 and *Xanthomonas maltophilia*. Among these, the *Pseudomonas fluorescens* strain J2 possessed the highest growth rate and was thence used for the chromosomal DNA isolation.

3.2 Cloning of ACC deaminase gene

The library was constructed in *E. coli* strain XL1-Blue using pGEM-3Zf(+) as a vector and plated on a blue-white selective medium. From this medium 1,327 white colonies were transferred to ADF medium containing ACC on which 110 colonies appeared after 3 days of incubation. The growth curve of all clones grown on ACC containing medium were studied and 6 of them were chosen on their highest growth rate basis. These were assigned C103, C39, C105, C107

Table 1. Species of selected bacteria capable of growing on the medium containing ACC as a sole nitrogen source

Species	No. of isolates found
Alcaligenes xylosoxidans subsp. *denitrificans*	8
Enterobacter sp.	6
Klebsiella pneumoniae	7
Pseudomonas cepacia	7
Pseudomonas fluorescens	7
Pseudomonas mendocina	8
Pseudomonas putida	5
Xanthomonas maltophilia	7

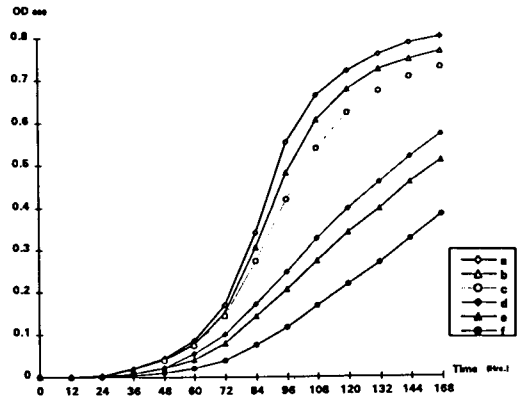


Figure 1. Growth curve of bacterial isolates in ADF medium containing ACC
(a) *Pseudomonas fluorescens* strain J2
(b) *Pseudomonas fluorescens* strain E1
(c) *Pseudomonas fluorescens* strain B1
(d) *Pseudomonas putida*
(e) *Xanthomonas maltophilia*
(f) *Klebsiella pneumoniae*

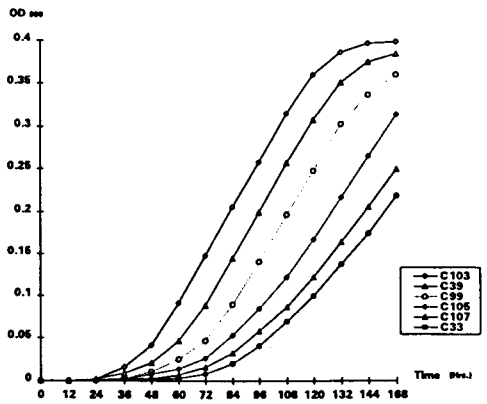


Figure 2. Growth curve of *E. coli* clones in ADF medium contraining ACC

and C33 clones respectively (Figure 2).

3. 3 Restriction mapping of the insert

Plasmids from the selected clones were purified and the sizes of the inserts were determined. Restriction mapping showed no *Bam*HI, *Eco*RI, *Hin*dIII, *Kpn*I and *Pst*I sites except for the sites of *Eco*RV and *Xho*I in individual plasmid inserts (Figure 3). The sizes of the inserts varied from about 400 bp in clone C33 to 1,100 bp in clone C39.

3.4 Measurement of ACC deaminase activity

The expression of ACC deaminase gene was induced by growing the selected clones and the original six bacterial isolates on minimal media containing ACC as the only nitrogen source. Determination of the activity of ACC deaminase was as described earlier in the materials and methods. Among the six bacterial isolates, *Pseudomonas fluorescens* strain J2, used for the chromosomal DNA isolation and cloning, gave the highest enzyme activity. It is noted that the activity of this original strain was two times as strong as that of clone C103 which was the most active among the transformants (Table 2).

3.5 Southern hybridization of the recombinant plasmids

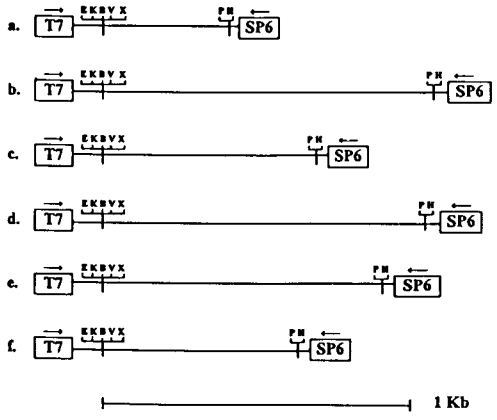
The insert in the recombinant plasmids was excised by double digestion with *Eco*RI. and *Hin*dIII. All Southern hybridization of these plasmids using the insert from the C33 clone showed a similar result (data not shown). In other words, no significant differences were noted in all the 6 recombinant plasmids.

4. Discussion

The majority of soil microorganisms capable of growing on ACC medium isolated

Table 2. ACC deaminase activity in bacterial isolates and in *E. coli* clones

Bacterial isolates and *E. coli* clone	ACC deaminase activity (unit/l)
Klebsiella pneumoniae	12.2
Pseudomonas fluorescens strain B1	43.7
Pseudomonas fluorescens strain E1	48.1
Pseudomonas fluorescens strain J2	52.0
Pseudomonas putida	21.6
Xanthomonas maltophilia	15.9
Escherichia coli clone C33	5.8
Escherichia coli clone C39	23.5
Escherichia coli clone C99	11.8
Escherichia coli clone C103	25.3
Escherichia coli clone C105	8.4
Escherichia coli clone C107	7.9



E=*Eco*RI, K=*Kpn*I, B=*Bam*HI, V=*Eco*RV, X=*Xho*I, P=*Pst*I, H=*Hind*III

Figure 3. Restriction map of recombinant plasmid from *E. coli* clones
(a) C33 (b) C39 (c) C99
(d) C103 (e) C105 (f) C107

in this experiment were *Pseudomonas* sp. with *Pseudomonas fluorescens* strain J2 as the best one. *Pseudomonas* sp. has been previously found to carry ACC deaminase gene [3,10]. However, the ACC deaminase activity was noted in all 6 bacterial isolates but *Pseudomonas fluorescens* strain J2 gave the highest activity. The *E. coli* transformants screened from the library produced by strain J2 chromosomal DNA also showed the activity of ACC deaminase enzyme. The α-ketobutyrate produced by the degradation of ACC by this enzyme was detected in all samples although *E. coli* clones were less active than those of the soil bacterial isolates. It is worth noting that the longer the inserts, the higher the enzyme activity. This might be due to the incompleteness of the gene cloned. Southern hybridization results also confirmed that all clones have the same gene with different length. Restriction mapping with some restriction enzymes coresponded well with ACC deami-nase gene reported by Klee *et al.* [10] and Sheehy *et al.* [3] in which there are *Eco*RV and *Xho*I sites at the 5' end of the gene. However, the longest insert of about 1.1 kb may not be completed yet. This problem has to be clarified by determining the nucleotide sequences of the genes and comparing with those of the previously reported ones.

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6. References

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